# Genetic characterization and molecular detection of antimicrobial resistance genes and virulence genes present in *Campylobacter* spp. isolated from broiler chickens and human clinical samples in Durban

by

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Submitted in fulfillment of the academic
requirements for the degree of
Master of Science in the
School of Life Sciences,
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December 2016

As the candidate's supervisor I have/have not approved this thesis/dissertation for submission.

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**Abstract**

*Campylobacter jejuni* and *Campylobacter coli* have evolved as the most prevalent *Campylobacter* species which are responsible for gastroenteritis infections in humans. Successful infection and continued fitness of *Campylobacter* species depend on virulence determinants and antimicrobial resistance elements which differ amongst strains of different origin. There is a paucity of information regarding *Campylobacter* virulence genes and the antimicrobial resistance genes in developing countries such as South Africa. Therefore, research is essential to characterize pathogenic markers and to implement strategies for proper control and prevention of infection caused by this pathogen. This study aimed to detect the presence of virulence genes such as: *cadF, hipO, asp, ciaB, dnaJ, pldA, cdtA, cdtB* and *cdtC* as well as the detection of genes associated with antimicrobial resistance which included *gyrA*, $bla_{OXA-61}$and *tetO* present in *C. jejuni* and *C. coli*. Following ethical approval, 100 commercial chicken fecal samples were collected and 100 human clinical isolates were selected from a collection of *Campylobacter* spp. which originated from a private pathology laboratory in South Africa. From the 100 chicken fecal samples 78% were positive for *Campylobacter* growth on mCCDA and from the collection of a 100 human clinical isolates 83% demonstrated positive *Campylobacter* spp. growth following culturing methods. The *cadF* gene was present in 100% of poultry and human clinical isolates. This could indicate that the presence of this gene is needed for successful infection in a host. *C. jejuni* was the main species detected in both poultry and human clinical isolates while *C. coli* were detected at a low incidence (chicken =13%, human=17%) and therefore, not statistically significant in either host ($p>0.05$). The resistance genes *gyrA*(235bp), gyrA(270bp), $bla_{OXA-61}$ and *tetO* were also detected at a higher percentage (51%, 36%, 58% and 68% respectively) in chicken samples compared to human clinical samples (49%, 36%, 53% and 64% respectively). In conclusion, this study demonstrated high prevalence of virulence and antibiotic resistance genes in *Campylobacter* species from South Africa. The high prevalence rates demonstrated the importance of *Campylobacter* spp. as a food borne zoonotic pathogen capable of causing persistent infection due to acquisition of antimicrobial resistance genes and virulence genes via the food chain. The study finally recommended limited and prudent use of antimicrobial agents as a mitigating measure to combat the evolution of multiple drug resistance.

**Keywords**: Antimicrobial resistance; *Campylobacter*; chicken; gastroenteritis; human clinical; pathogenic; virulence

# PREFACE

The experimental work described in this thesis was completed by Samantha Reddy in the Discipline of Genetics, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Westville Campus, under the supervision of Dr Oliver Tendayi Zishiri.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

I certify that the above information is correct

_____

Dr Oliver Tendayi Zishiri (Supervisor)

Date:.....

## PLAGIARISM DECLARATION

I, Samantha Reddy declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

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## DELARATION OF PUBLICATIONS

Chapter 3 and 4 in this thesis each contain manuscripts that are still under peer review

**Publication 1:** Reddy, S. and Zishiri, O.T., 2016. Prevalence of virulence genes related to adherence, invasion, and cytotoxicity in *Campylobacter* spp. isolated from commercial chickens and human clinical cases from South Africa. (Under Review)

**Publication 2:** Reddy, S. and Zishiri, O.T., 2016. Detection and prevalence of antimicrobial resistance genes related to ciprofloxacin, ampicillin and tetracycline resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from chickens and human clinical cases in South Africa. (Under Review)

**Journals submitted to for Review:**
Veterinary Microbiology
Onderstepoort Journal of Veterinary Research
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## LIST OF ABBREVIATIONS

| | |
|---|---|
| °C | Degrees Celsius |
| g/ml | grams per milliliter |
| µl | microliter |
| µm | Micrometers |
| AFLP | Amplified Fragment Length Polymorphism |
| AIDS | Acquired Immune Deficiency Syndrome |
| AME | Aminoglycoside-Modifying Enzyme |
| ATCC | American Type Culture Collection |
| bp | base pair/s |
| BREC | Biomedical Research Ethics Committee |
| CDC | Centers for Disease Control and Prevention |
| CDT | Cytolethal Distending Toxin |
| CFU/g | Colony Forming Units per gram |
| CI | Confidence Interval |
| DNA | Deoxyribonucleic Acid |
| ECDC | European Centre for Disease Prevention and Control |
| EFSA | European Food Safety Authority |
| FDA | Federal Drug Administration |
| flaA-RFLP | flagellin A Restriction Fragment Length Polymorphism |
| FQ | Fluoroquinolones |
| GBS | Guillain-Barré syndrome |
| ITS | Internal Transcribed Spacer |

| | |
|---|---|
| kDa | KiloDaltons |
| MACP | Methyl-Accepting Chemotaxis Protein |
| mCCDA | modified Charcoal Cefoperazone Deoxycholate Agar |
| MDR | Multidrug-Resistance |
| MIC | Minimum Inhibitory Concentration |
| MLEE | multilocus enzyme electrophoresis |
| MLST | multilocus sequence typing |
| MOMP | major outer membrane protein |
| MW | Molecular Weight |
| NARMS | National Antimicrobial Resistance Monitoring System |
| NCBI | National Center for Biotechnology Information |
| OR | Odds Ratio |
| PCR | Polymerase Chain Reaction |
| PFGE | Pulsed-Field Gel Electrophoresis |
| PNS | Peripheral Nervous System |
| QRDR | Quinolone Resistance-Determining Region |
| SE | Standard Error |
| spp. | species |
| STEC | Shiga-toxigenic *Escherichia coli* |
| subsp. | Subspecies |
| Tm | melting temperature |
| WGS | Whole Genome Sequencing |
| VNTR | Variable Numbers of Tandem Repeat |

## Acknowledgements

Firstly, I wish to express my deepest appreciation to my supervisor, Dr Oliver Tendayi Zishiri for giving me the opportunity to pursue my MSc degree with him and for all his support, intellectual guidance, advice and encouragement which made this dissertation possible. I am extremely fortunate to have such a patient, understanding, talented and caring person as my mentor, the experiences and techniques I have learnt here will assist me immensely in my future career.

Special thanks also go to Dr Linda Bester and Christiana Wunmi for their time and guidance through my project from the beginning. I am grateful for all the helpful techniques, ideas and explanations of concepts throughout. The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF, the scholarship has helped me through my research and provided peace of mind during this stressful time.

I am also thankful to everyone at the Department of Genetics for their friendship and kind assistance during the time of my research, Natalie Naidoo, Shirwin Pillay and Shrinav Rai Dawlat thank you for the constant help when I needed it. I would also like to specially thank Mary-Anne Brouckaert for constantly offering help, advice and assistance with everything and making my life so much easier during my studies. Special thanks goes out to Bethany Nathaniel who has been with me since honours, thank you for your encouragement and love through the late and trying nights during my Masters research, I appreciate your help and assistance with everything and especially helping me relax when things became difficult. To Yevette Gounden, thank you for all the hard work and time you put in to help finish labwork towards the end, I really appreciate all of it and I am glad I had you as the student I had to mentor this year, it has been great and I wish you all the best in your future.

I would also like to thank my mother, Sandra, and my sister, Rovaine, for their continuous love and support through the difficult times in my studies; I greatly appreciate everything you both have done for me and love you both dearly. Finally, my heartfelt gratitude goes to the most important person in my life, my significant other, Nick Shiells, for his encouragement, help and motivation with everything, thank you. I am exceptionally fortunate to have such an extraordinary, understanding, patient and thoughtful person in my life since the beginning of my Masters and I wish to express my deepest appreciation to you for always being there and helping me get through the tough, difficult and stressful times during my studies.

# CHAPTER 1
# GENERAL INTRODUCTION

## 1.1. Background

*Campylobacter* has been widely recognized as one of the most causative bacterial food-borne pathogen in diarrheal diseases worldwide (CDC, 2013). The intestinal tract of warm-blooded animals is the region where most of the species belonging to the genus *Campylobacter* reside, majority of campylobacters are pathogenic and infect human and animal gastrointestinal tracts however some are commensal (Kovács, 2014). Initially *Campylobacter* was misidentified and published as a *Vibrio* based on its morphological properties observed by Theodor Escherich who isolated a bacteria, from the colon of a child, this bacteria was observed as non-culturable and spiral-shaped (Epps et al., 2013). Further investigation over the years led scientists to believe that this organism differed greatly from *Vibrio* related bacteria due to their microaerophilic growth requirements, the low base compositions of their DNA and also their metabolism which lacked fermentative abilities; this resulted in the establishment of the genus *Campylobacter* by Sebald and Vernon in 1963. Since then *Campylobacer* spp. have been identified as Gram-negative, non-spore forming, spiral and small bacteria which are between 0.2-0.9 μm wide and 0.5-5.0 μm long. This organism is known to be highly motile with the use of a single flagellum or two flagella, however some *Campylobacter* spp. may have no flagellum (Man, 2011). Currently a number of important animal and human pathogens have been classified, belonging to the genus *Campylobacter*, the family *Campylobacteraceae*, the order *Campylobacterales*, to the class *Epsilonproteobacteria* and to the phylum *Proteobacteria*. These classifications have primarily been achieved through phylogenetic means and as of December 2014 the genus *Campylobacter* contains 26 species, 2 provisional species as well as 9 subspecies (Kaakoush et al., 2015).

Hydrogen enriched atmospheric conditions and growth at 42°C as well as time for incubation can be used during isolation procedures to support the growth or revival of *Campylobacter* species (Kaakoush et al., 2015). Laboratories worldwide have developed successful selective and filtration culture methods in order to isolate and identify *Campylobacter* spp., these bacteria are microaerophilic and thermophilic thus they essentially exhibit best growth in a low oxygen atmosphere preferably 5% oxygen, 10% carbon dioxide and 85% nitrogen, with an optimum temperature of 42°C (Garénaux et al., 2008; Gharst et al., 2013). Campylobacteriosis is mainly confined to the very young, usually children under the age of five years or to elderly patients and patients suffering from HIV/AIDS. This presents as a major threat in South Africa due to the extremely high rate of infection with immunocompromising diseases such as HIV/AIDS and therefore infection by *Campylobacter jejuni* or *Campylobacter coli* could prove to be fatal (Kovács, 2014).

Antibiotics have been used for decades as in poultry production systems and other livestock for prevention and control of infections and to enhance growth rates (Cheng et al., 2014). This unregulated use of antimicrobial agents has resulted in increased resistance against multiple antibiotics and also in microbes with human importance. The unwarranted use of antibiotics as an enhancement in animal feed is estimated to represent more than a half of the total antimicrobial use around the world (Verraes et al., 2013). Research has demonstrated that the administration of fluoroquinolones to poultry in production systems has caused an increase in the resistance of *C. jejuni* against these agents, isolated from both animals and humans (Colles et al., 2016). Growing antibiotic resistance in campylobacter is an emerging public health concern around the world recognized by the World Health Organization. *Campylobacter* species was previously considered susceptible to various antimicrobial agents; however in recent years, both animal and human isolates of this bacterium have shown growing resistance to several antibiotics such as macrolides (erythromycin), tetracycline, and fluoroquinolones, which are the most frequently prescribed antibiotics against *Campylobacter* infection in humans (Iovine, 2013) Fluoroquinolone-resistant *Campylobacter* has also become prevalent in Africa and Asia (Kaakoush et al., 2015). The occurrence of *Campylobacter* is much higher in poultry than any other categories of food products of animal origin and therefore resistance data regarding *Campylobacter* spp. is primarily based on broiler meat and poultry products. Other sources of infection can also contribute to campylobacteriosis in humans namely pets, contaminated water and raw milk; these, however, do not play as much of a role compared to the incidence of this pathogen in poultry (Kovács, 2014). Due to *Campylobacter* being concentrated in the intestinal tract of food animals, fecal contact during processing regularly contaminates foods and aids in transmission of this pathogen via the food-chain. Antimicrobials are commonly used in animal production systems prophylactically or therapeutically; this causes selective pressure on the bacteria which infect food animals and in turn infect humans who consume contaminated meat. The chain of transmission of antimicrobial resistant bacteria especially from poultry to humans is very difficult to grasp because the transmission routes are often complex (Luangtongkum et al., 2010).

It is therefore important to understand the resistance mechanisms and the transmission of resistance determinants from animal to humans in order to treat *Campylobacter* infections (Verraes et al., 2013). The pathogenicity of *Campylobacter* spp. is challenging and poorly understood as compared to other enteric pathogens (O Cróinín & Backert, 2012) though advancements have been made in order to understand the importance and role of key factors associated with molecular mimicry processes in Guillain-Barré Syndrome (GBS) (Iovine, 2013) as well as bacterial virulence mechanisms such as cytolethal distending toxin (CDT), which is encoded by three linked genes namely *cdtA, cdtB* and *cdtC* (Ge et al., 2008). The 3 *cdt* genes (cytolethal distending toxin), which form a polycistronic *cdt* operon, are responsible for the

expression of cytotoxins which are lethal for host cells (Carvalho et al., 2013). Other important genes involved in pathogenicity, virulence and colonization such as *cadF* responsible for adhesion to Fibronectin F, which is also a genus-specific virulence gene (Konkel et al., 1999) conserved in all pathogenic *Campylobacter* species and is a well known marker for the detection of *Campylobacter* species. The hippuricase gene (*hip*O) is specific for *C. jejuni* because it is not detected in any other *Campylobacter* species (Al Amri et al., 2007). The *asp* gene encoding the enzyme aspartokinase only found in *C. coli*, *ciaB* (*Campylobacter* invasive antigen B) involved in host cell invasion, *dnaJ* which enables *Campylobacter* species to cope with diverse physiological stresses and plays a role as a chaperone protein. The *pldA* gene is involved in host cell invasion and is determined by an outer membrane phospholipase A. Each *ciaB, pldA* and *dnaJ* genes play a significant role in caecal colonization (Rizal et al., 2010). Entry of these isolates, harboring such virulence genes and antimicrobial resistance genes, into the food chain could represent a significant threat to public health. Furthermore, antibiotic resistance genes have been given a low priority in most developing and many developed countries. Most important, developing countries such as South Africa have received very limited attention regarding this problem.

**1.2. Justification**

Campylobacteriosis is an under-reported foodborne disease all over the world and this pathogen is difficult to culture successfully. As a result many laboratories steer away from such investigations and rather research the more common commensal pathogens such as *E. coli* and *Staphylococci*. This study was undertaken in order to broaden our understanding of antimicrobial and virulence genes associated with pathogenicity in *Campylobacter* spp. this is due to a huge gap which exists in our knowledge and because this pathogen is constantly evolving and acquiring new genes for survival. It is also important to differentiate the prevalence rates of *C.jejuni* and *C.coli* and which species is responsible as the main source as the cause of infection. There is a literature dearth on antimicrobial resistance and virulence of *Campylobacter* spp. in developing countries such as South Africa, therefore an investigation such as this study will contribute to the existing body of knowledge.

## 1.3. Hypothesis

It is hypothesized that *Campylobacter* spp. (*C. jejuni* and *C. coli*) are prevalent in the feces of broiler chickens and that most human infection is attributed mainly to them. It is further hypothesized that a variety of virulence genes such as *cadF, hipO, asp, ciaB, dnaJ, pldA, cdtA, cdtB* and *cdtC* which are associated with pathogenicity, adherence, cytolethal distending toxin production and colonization are present in *Campylobacter* spp. isolated from human and chicken. Finally it is hypothesized that the *Campylobacter* spp. isolated from human and chicken, harbor antibiotic resistance genes such as *gyrA*, $bla_{OXA-61}$ and *tetO* which confer resistance to antibiotics ciprofloxacin, ampicillin and tetracycline.

## 1.4. Aims and Objectives

**1.4.1.** To microbiologically isolate *Campylobacter* spp. from broiler chicken feces and human clinical samples using selective media techniques and specific growth conditions.

**1.4.2.** To isolate total DNA using the boiling method.

**1.4.3.** To differentiate between *C. jejuni* and *C. coli* by PCR detection of *hipO* and *asp* genes, specific to *C. jejuni* and *C.coli*, respectively.

**1.4.4.** To detect the presence of virulence genes: *cadF, ciaB, dnaJ, pldA, cdtA, cdtB* and *cdtC* in both chicken feces and human clinical isolates.

**1.4.5.** To confirm the presence of antimicrobial resistance genes such as *tetO*, *gyrA* and $bla_{OXA-61}$ in *C. jejuni* and *C.coli*.

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# CHAPTER 2
# LITERATURE REVIEW

The literature reviewed in this chapter will focus on the some of the main aspects surrounding *Campylobacter* spp., regarding the prevalence of this foodborne pathogen and where current gaps in our knowledge exist. Evaluation of the various sources and molecular typing methods which are available for isolation, identification and diagnosis of this pathogen will be reviewed. The main focus of this study will review virulence genes, antimicrobial resistance genes and also explore the multitude of virulence and antibiotic resistance mechanisms which drive *Campylobacter* fitness as a foodborne pathogen.

## 2.1. Incidence and prevalence of Campylobacteriosis

Campylobacteriosis is a significant public health concern worldwide with most infection cases in humans caused by *Campylobacter jejuni* subsp. *jejuni* (from here on referred to as *C. jejuni*) and *Campylobacter coli* (Zhao et al., 2010). *Campylobacter* spp. has been classified as being the leading cause of bacterial foodborne enteric disease in humans as well as one of the most important bacterial foodborne pathogens in countries such as Europe and the United States (EFSA, European Food Safety Authority, and ECDC et al., 2012, Wieczorek et al., 2012). Research over the years has demonstrated that there was a 14% increase in the occurrence of campylobacteriosis in 2012 compared to the period between 2006–2008, whereas the incidences of pathogens such as *Salmonella*, Shiga-toxigenic *Escherichia coli* (STEC) O:157, *Yersinia*, *Cryptosporidium*, *Shigella*, and *Listeria* infections notably decreased over the same time period (Kaakoush et al., 2015). Surveillance of *Campylobacter* spp. according to the U.S. Centers for Disease Control and Prevention (CDC) has demonstrated that in 2012 35% of laboratory-confirmed bacterial and parasitic infections were due to *Campylobacter* infections (CDC, 2013). Campylobacteriosis has increased in and around Europe; reports by the European Centre for Disease Prevention and Control (ECDC) and European Food Safety Authority (EFSA) have demonstrated that campylobacteriosis has turned out to be the most commonly reported zoonotic disease in Europe with a confirmed 45 per 100 000 cases closely followed by other zoonotic diseases such as salmonellosis and shigellosis (Efsa, 2011). *Campylobacter* was reported as the second leading cause of acute gastroenteritis in Australia in 2010, after the norovirus (Kaakoush et al., 2015). Reported cases of campylobacteriosis are observed to be around 200 000 per year; however, this does not reflect a true representation because campylobacteriosis is very much an underreported occurrence and the true estimation is assumed to be approximately 9 million cases per year (Havelaar et al., 2012).

Data on an epidemiological scale regarding campylobacteriosis in Asia and the Middle East are very restricted. In Beijing, China, 14.9% (142/950 cases) of patients in the period between 2005 and 2009, with gastroenteritis in a hospital were confirmed to be positive for *Campylobacter* species as the cause of infection, 127 were infected with *C. jejuni* and the remaining 15 with *C. coli* (Havelaar et al., 2012). Based on detection rates of *Campylobacter* in raw chicken and the consumption of chicken products in China from 2007 to 2010, Wang and colleagues calculated that 1.6% of the urban and 0.37% of the rural population are affected by campylobacteriosis every year (Wang et al., 2013). From January 2008 to December 2010 in India, an infectious disease hospital in Kolkata reported that 7.0% (222 of 3186 cases) of hospitalized patients who presented with gastroenteritis were positive for *Campylobacter* species as the source of infection and 70% of the isolates examined were identified as *C. jejuni*. A case-controlled study conducted between 1 December 2007 and 3 March 2011 was initiated to identify the basis of diarrhea in children aged between birth to 5 years (59 months) findings demonstrated that *C. jejuni* was significantly related to children presenting with moderate to severe diarrhea from Kolkata, India, Mirzapur, Bangladesh, and Karachi, Pakistan. Important data collected between March and April of 2011 from the Netherlands revealed that 71.4% of 493 gastroenteritis reported cases were PCR positive for *Campylobacter* DNA (Bouwknegt et al., 2013).

Ground-breaking work carried out by Lastovica and colleagues in South Africa, implemented culture methods which were optimal for the isolation of most *Campylobacter* species, this revealed a more complete and realistic epidemiological landscape regarding *C. jejuni* and emerging *Campylobacter* species. The research conducted isolated 5443 strains of *Campylobacter* species between the period of 2005 to 2009. This was carried out at the Red Cross Children's Hospital situated in Cape Town; these strains were isolated from stools of children suffering with diarrhea. Findings of the study demonstrated that 40% were determined positive for *C. jejuni* with 32.3% *C. jejuni* subsp. *jejuni* and 7.7% *C. jejuni* subsp. *doylei*, the second most prevalent organism responsible for infection was found to be *C. concisus* (24.6%) (Lastovica, 2006). A 10-year study from 1997 to 2007 from Blantyre, Malawi, reported that *C. jejuni* and *C. coli* were detected by real-time PCR in 21% (415 out of 1941 children) of hospitalized children with diarrhea, with *C. jejuni* responsible for the majority (85%) of all campylobacteriosis cases reported (Mason et al., 2013). Samples collected between May 2011 to May 2012 at a hospital in Kisiri situated in Kenya, were tested for the detection of a range of enteric pathogens the findings demonstrated that 5.8% (9 of the 156 samples) of the diarrheal samples examined from patients proved to be positive for *Campylobacter* species (Swierczewski et al., 2013).

With the use of diverse detection techniques, which included culture, enzyme immunoassay and PCR, for the detection of *C. jejuni* or *C. coli* another study sampled 138 children in Tanzania and in 34.8% of gastroenteritis cases were attributed to *Campylobacter* species which was positively detected in diarrheal samples (Komba et al., 2013). Therefore, it is not unreasonable to assume that *C. jejuni* and other *Campylobacter* spp. are endemic to children in most investigated regions of Africa. *Campylobacter* spp. are highly unpredictable organisms and can adapt to a wide range of ecological niches. The distribution of this pathogen in the environment can vary according to species and conflicting prevalence rates of *C. jejuni* and *C. coli* have been observed in various water, food and animal sources (Ragimbeau et al., 2014). Human bacterial enterocolitis or more specific *Campylobacter*-enteritis is of great concern globally; in developed countries, the most prevalent species which cause human infections are *Campylobacter jejuni* and *Campylobacter coli*, however *C. upsaliensis* is the most important species for the cause of human infections in developing regions. Species and subspecies which are recognized in the genus *Campylobacter*, their common hosts and the typical diseases they are associated with in humans and animals are demonstrated in Table 2.1 (On, 2013).

**Table 2.1:** List of valid species and subspecies in the genus *Campylobacter* and their common hosts and disease associations in animals and humans (On, 2013)

| Taxon | Host animal species | Human disease association | Animal disease association |
|---|---|---|---|
| *Campylobacter avium* | Poultry | None as yet | None as yet |
| *Campylobacter canadensis* | Whooping cranes | None as yet | None as yet |
| *Campylobacter coli* | Pigs, poultry, ostriches, cattle, sheep | Gastroenteritis | Gastroenteritis, infectious hepatitis |
| *Campylobacter concisus* | Humans, domestic pets | Gastroenteritis, periodontitis | None as yet |
| *Campylobacter cuniculorum* | Rabbits | None as yet | None as yet |
| *Campylobacter curvus* | Humans | Periodontitis, gastroenteritis | None as yet |
| *Campylobacter fetus subsp. fetus* | Cattle, sheep, reptiles | Gastroenteritis, septicaemia | Spontaneous abortion |
| *Campylobacter fetus subsp. venerealis* | Cattle, sheep | Septicaemia | Infectious infertility |
| *Campylobacter gracilis* | Humans | Periodontitis | None as yet |
| *Campylobacter helveticus* | Dogs, cats | Periodontitis | Gastroenteritis |
| *Campylobacter hominis* | Humans | None as yet | None as yet |
| *Campylobacter hyointestinalis subsp. hyointestinalis* | Cattle, deer, pigs, hamsters | Gastroenteritis | Gastroenteritis |
| *Campylobacter hyointestinalis subsp. lawsonii* | Pigs | None as yet | None as yet |
| *Campylobacter insulaenigrae* | Seals, porpoises | None as yet | None as yet |
| *Campylobacter jejuni subsp. doylei* | Humans | Septicaemia, gastroenteritis | None as yet |
| *Campylobacter jejuni subsp. jejuni* | Poultry, cattle, pigs, ostriches, wild birds | Gastroenteritis, Guillain-Barré syndrome | Spontaneous abortion, avian hepatitis |
| *Campylobacter lanienae* | Cattle | None as yet | None as yet |
| *Campylobacter lari subsp. concheus* | Shellfish | Gastroenteritis | None as yet |
| *Campylobacter lari subsp. lari* | Wild birds, dogs, poultry, shellfish, horses | Gastroenteritis, septicemia | Avian gastroenteritis |
| *Campylobacter mucosalis* | Pigs | None as yet | None as yet |
| *Campylobacter peloridis* | Shellfish | Gastroenteritis | None as yet |
| *Campylobacter rectus* | Humans | Periodontitis | None as yet |
| *Campylobacter showae* | Humans | Periodontitis | None as yet |
| *Campylobacter sputorum* | Humans, cattle, pigs, sheep | Gastroenteritis, abscesses | Spontaneous abortion |
| *Campylobacter subantarcticus* | Birds in the subantarctic | None as yet | None as yet |
| *Campylobacter upsaliensis* | Dogs, cats | Gastroenteritis | Gastroenteritis |
| *Campylobacter ureolyticus* | Humans | Gastroenteritis, Crohn's disease | None as yet |
| *Campylobacter volucris* | Black-headed gulls | None as yet | None as yet |

## 2.2. Isolation Methodologies and Laboratory Diagnosis of *Campylobacter* spp.

The isolation of all *Campylobacter* species from clinical samples has no solitary or common method that exists. *Campylobacter* species which are usually investigated tend to be thermotolerant and specific isolation techniques are required such as selective media containing blood or blood free agar containing specific growth supplements, these are however only effective for some species. In 2006 Lastovica formulated a robust protocol named the Cape Town protocol; this involves homogenization of clinical samples through membrane filters with pore sizes of either 0.45 or 0.65 µm directly onto blood agar media either containing vancomycin supplement or without supplement (Lastovica, 2006). Once samples have filtered through, the plates are then subjected to incubation at a temperature of 37°C under microaerobic conditions which have 5% $O_2$ enriched with $CO_2$ and $H_2$, the hydrogen is not a requirement, however, it does enhance the growth of some *Campylobacter* species. The Cape Town protocol has achieved successful isolation of a range of *Campylobacter* species a wide range of sample types such as saliva, fecal and intestinal biopsies. Enrichment is a crucial step especially when samples contain a low number of *Campylobacter* cells, these samples are enriched in a selective broth prior to execution of the Cape Town protocol and this procedure has been effective for isolating of a number of *Campylobacter* species from patients with cases of chronic gastroenteritis and IBD (Man, 2011). Some of the common enrichment broths include Preston broth, Brucella broth, Bolton broth and modified charcoal cefoperazone deoxycholate (mCCDA), these have been used successfully to isolate *Campylobacter* species (Gharst et al., 2013).

Diagnosis of *Campylobacter* infection in a laboratory necessitates either culture independent or culture dependant methods which will lead to a definitive diagnosis. With the use of culture dependant methods, this would involve isolated single colonies being subjected to biochemical testing which determine phenotypic traits that the pathogen possesses. Following this analysis the biochemical profile can be matched to characteristics that have previously been identified in the *Campylobacter* genus or to the species level (Ghorbanalizadgan et al., 2014). Other culture dependant methodologies should also be performed in order to favor growth during isolation, these include time of incubation, optimal temperature (usually 42°C for *Campylobacter*) and proper atmospheric conditions such as an $H_2$ enriched atmosphere which favours growth of this organism (Gharst et al., 2013). Culture-independent methods involve isolation of DNA or RNA from a pure culture or from clinical samples which is followed by identifying specific genetic signatures present in the genome of the organism. This involves techniques such as PCR amplification of the gene of interest (eg. 16S rRNA gene) with the use of species specific primers (Marinou et al., 2012) or a different approach could be used which involves sequencing techniques after

isolation to determine genetic markers present. At present culture independent methods are being used more frequently for the detection of *Campylobacter* species, in many cases these tests enhance detection sensitivity and therefore will impact public health surveillance at a molecular level seeing that detailed analysis of pathogens such as *Campylobacter* spp. is required for monitoring the circulation of different strains (CDC, 2013).

### 2.2.1. Biochemical identification of *Campylobacter* spp.

The culture dependant methodology of biochemical tests are still being used in laboratories all over the world to ensure definitive confirmation of *Campylobacter* species, these tests can also differentiate and identify organisms in a genus and to the species level. Lastovica (2006) reviewed the common biochemical tests for differentiation between members of the *Campylobacter* genus demonstrated in Figure 2.1 below; this article outlined a biochemical flowchart for the identification of *Campylobacter* spp. the initial tests were growth of *Campylobacter* spp. either supplemented with $H_2$ or not, then use of an indoxyl acetate test, a hippurate hydolysis test, growth on MacConkey agar followed by an aryl sulfatase test and finally tests determining $H_2S$ production. These biochemical tests that have been outlined are regarded as standard diagnostic tests for identifying *Campylobacter* species, other useful tests include the lead acetate test, nitrate reductase test, pyrazinamidase test and catalase test. However, the most relevant test in clinical use is the hippurate hydrolysis test which is the only biochemical test that distinguishes between *C. jejuni* and *C. coli*, the latter species *C. coli* is unable to hydrolyze hippurate and thus will yield a negative test whereas *C. jejuni* is capable of hydrolyzing hippurate (Lastovica, 2006).

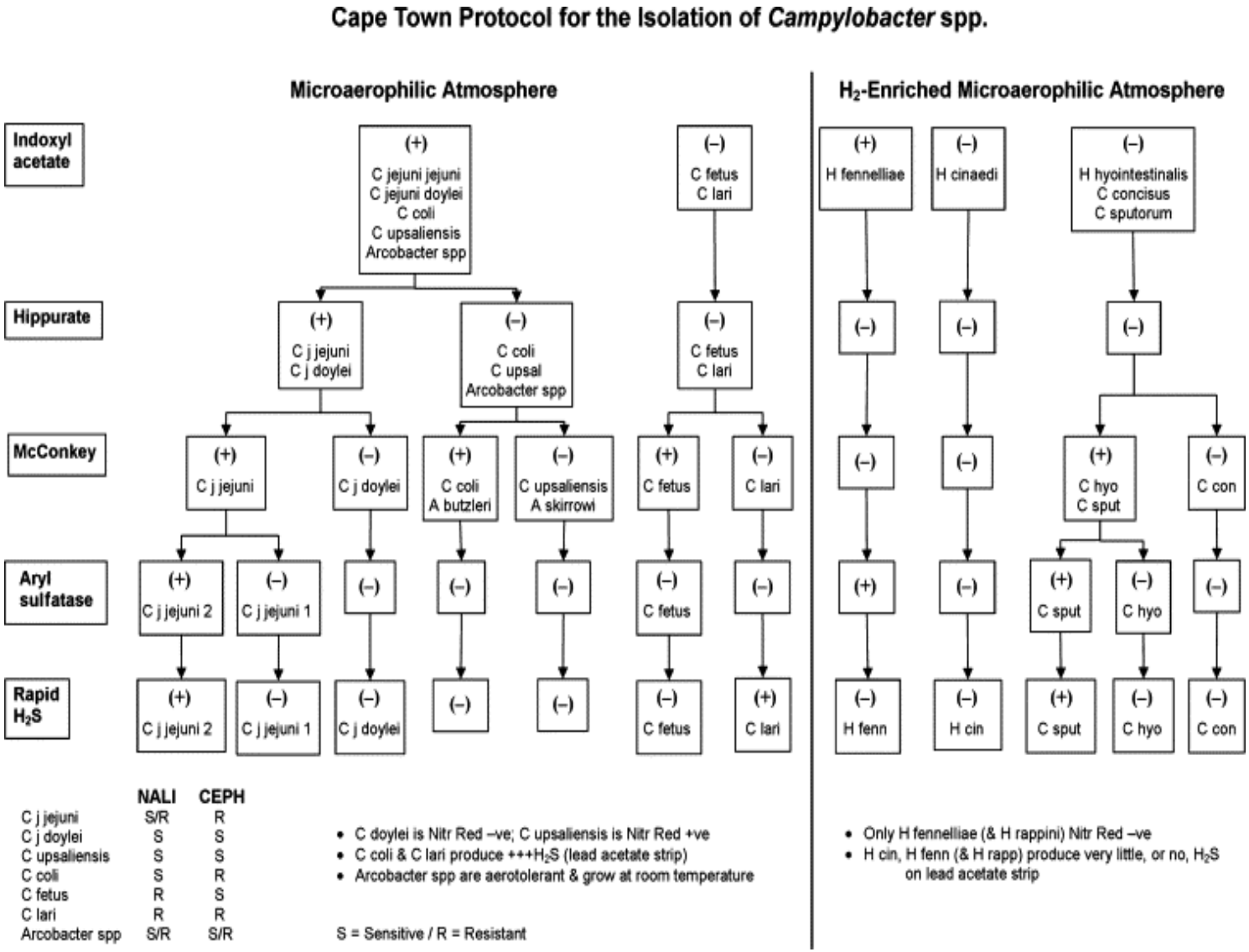


**Figure 2.1:** Biochemical flowchart using the Cape Town protocol for the isolation and identification of *Campylobacter*, *Helicobacter*, and *Arcobacter* species from clinical specimens (Lastovica, 2006).

### 2.2.2. Molecular identification

*Campylobacter* species and many other bacterial species have made use of the 16S rRNA gene for rapid detection and identification of organisms. This gene, however, fails to differentiate between closely related species such as *C. jejuni* and *C. coli*. The differentiation between *Campylobacter* species and strains relies on a larger 23S rRNA gene and a region between the 16S and 23S rRNA genes called the internal transcribed spacer (ITS) region which is the most discriminatory region and therefore this is used to differentiate between *Campylobacter* species (Lehtopolku et al., 2011). A phylogenetic tree was generated and resulted in the highest resolution for differentiating between members of the *Campylobacter* genus when all three of these regions were combined. Enzyme immunoassays and real-time assays are available to detect *Campylobacter* species and some are even capable of detecting more than one species at a time (Oporto et al., 2009). The cost of sequencing bacterial genomes have become more affordable recently and therefore many genomes of *Campylobacter* species and strains have been completed and sequenced, these exceed a 100 *Campylobacter* spp. as well as *C. jejuni* and *C. coli* strains sequences which are currently available in the National Center for Biotechnology Information (NCBI) database. The sequencing of genomes provides a deeper understanding at the molecular level for the characterization of isolates and therefore this will most probably become a routine application in diagnostic laboratories in the future (Man, 2011).

## 2.3. Molecular genotyping

Over the years phenotypic methods such as serotyping, antibiogram, biotyping and phage typing have been implemented for epidemiological studies of *C. jejuni*. These methods have, however, failed to provide the discriminatory power, reproducibility and typability considered necessary for optimal gain of information; they are also expensive and extremely labor intensive (Ahmed et al., 2012). This is where molecular genotyping assays such as genome based methods have the potential to overcome these difficulties. Molecular genotyping assays which include pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST), to name just a few, can be employed for relative evaluation of genomic polymorphism in *Campylobacter* strains (Abay et al., 2014). Currently, genotyping assays developed for *Campylobacter* species can be separated into three groups based on the assessment of genetic polymorphisms: (a) in whole genome; (b) in a single locus of the genome and (c) in multiple loci of the genome (Ahmed et al., 2012).

### 2.3.1. Pulsed-field gel electrophoresis (PFGE)

Microrestriction profiling or more commonly known as PFGE was developed in 1984, as well as one of the first DNA-based techniques developed for typing *Campylobacter* spp. The basis of this technique was for the separation, electrophoretically, of large segments of DNA in *Campylobacter* chromosomes in order to construct genomic maps and estimate the genome size of organisms. However, even in closely related strains the instability of a genome and integration of mobile genetic elements such as bacteriophages is known to negatively influence PFGE profiles (Abay et al., 2014). Over the years this technique has, however, evolved to become an excellent choice for use in studying the molecular epidemiology of infectious pathogens such as *Vibrio cholerae*, *Streptococcus* spp., *Escherichia coli*, *Neisseria meningitides*, *Staphylococcus* spp., *Campylobacter* spp. and *Bordetella pertussis*. The CDC employs the use of a surveillance program called the PulseNet system; this program uses pulsed-field gel electrophoresis (PFGE) in order to detect sporadic cases of campylobacteriosis but more importantly for the detection of case-clusters of food-borne disease outbreaks (CDC, 2013). PFGE is accepted as the most discriminatory and sensitive genotyping methods available for genotyping of *Campylobacter* spp., these features are however dependant on the subtyping of the organism as well as the choice of restriction enzymes such as *SmaI*, *KpnI*, *SacII*, *SalI* and *BamHI* used in the technique (Gharst et al., 2013) and it is for the most part valuable for differentiating closely linked strains (Ahmed et al., 2012).

Recently, PFGE was used in the exposure of a *C. jejuni* clone which was extremely virulent in the United States (Sahin et al., 2012). This method basically utilizes restriction enzymes for the digestion of genomic DNA; first bacterial cells embedded in agarose plugs are treated with enzymes in order to release the cells so genomic DNA can be accessed, and then restriction enzymes digest recognition sites in the DNA. This is followed by periodically changing the electric field for the intention of separation of large DNA molecules which produces a macrorestriction pattern. Detection of microevolution in isolates can be achieved using the PFGE technique, other methods such as multilocus enzyme electrophoresis (MLEE) or multilocus sequence typing (MLST) fail to detect such sensitive changes in the DNA. Even though the analysis takes about 4-5 days and the technique is expensive, PFGE still remains as an extensive discriminatory typing method used for *Campylobacter* spp. (Datta et al., 2003). This method has been broadly made use of in epidemiological studies of *C. jejuni* ranging from the comparison of genotypes within and between hosts (Hein et al., 2003), for outbreak investigations in humans and animals/ retail meats, also for diversity investigation of sporadic disease isolates, distribution of antibiotic resistant strains and perseverance of genotypes in a human populations (Xia et al., 2009; Sahin et al., 2012; Di Giannatale et al., 2014).

### 2.3.2. Flagellin typing: flaA typing

The major flagellin genes of *Campylobacter* spp. *flaA* and a minor gene, *flaB* are the genes which encode the *Campylobacter* flagella. PCR amplification of the *flaA* gene, which contains short regions known to be highly variable, is used to provide a fingerprint of the isolate of interest and this allows prediction of clonal groups (Djordjevic et al., 2013). Following amplification the PCR product is digested with restriction endonucleases and separated by means of agarose gel electrophoresis which results in a distinctive microrestriction pattern for the isolate under investigation (flaA-RFLP)(Merchant-patel et al., 2010). *Campylobacter* spp. are capable of high rates of DNA uptake and the flagellin genes have a recombination ability which results in intragenomic and intergenomic recombination this produces *flaA* alleles which lack stability and are not species-specific, some alleles are also found in both *C. jejuni* and *C. coli*. Even though it is low cost, easy and fast to use flaA-RFLP this technique is not suitable for epidemiological surveys. This technique (flaA-RFLP) in combination with PFGE is more appropriate for the differentiation among *Campylobacter* isolates of different origins. However, a modified flagellin-based method (flaA-SVR) using single locus genotyping has been developed for standardization and a higher level of discrimination, this technique involes sequencing analysis of a 321bp short variable region of the *flaA* gene, there is no need for a combination of techniques as this technique alone possesses high discriminatory power (Huang et al., 2009).

### 2.3.3. Multilocus sequence typing (MLST)

The molecular typing method, *Multilocus sequence typing* (MLST) has become the most commonly used method for *Campylobacter* spp. This technique is based on the principle of *multilocus enzyme electrophoresis* (MEE) (Kittl et al., 2013). The primary *Campylobacter* MLST design, for *C. jejuni* and *C. coli*, requires sequencing of short DNA fragments found in several of the housekeeping genes such as *uncA*, *gltA*, *asp*, *tkt*, *glnA*, *pgm* and *gltA*; however, recently upto eleven housekeeping genes can be employed within the technique. Basically bacterial isolates are digested with different enzymes and during electrophoresis the mobility of genes result in a variation of different electrophoretic types which is driven by the mutations present in their gene locus. PCR products are then sequenced and alleles are assigned a match in allelic profiles present in a global PubMLST (http://pubmlst.org/) database. Once this is achieved sequence types in accordance with the database are linked to allele numbers and selected sequence types which correspond to four or more alleles which fit into the equivalent clonal complex. Taboada et al. (2013) reported that the pubMLST database contained over 6500 sequence types and many of the sequence types represented in the database only appeared once indicating that extensive genetic

diversity exists in *C. jejuni* and *C. coli* (Taboada et al., 2013). Identification of major sources linked to human disease has been achieved with the use of the MLST technique, the procedure also provides valuable insights in epidemiological studies, population structure and evolutionary pathways of *Campylobacter* spp. through genetic transfer (Sheppard et al., 2009). Regardless of the high diversity in clonal complexes and sequence types from a single source, a small amount of these actually structure the majority of isolates which leads to a certain recognition of dominant strains between studies, this has enabled source attribution to different hosts and a better understanding of *Campylobacter* epidemiology, especially in clinical cases (Sheppard et al., 2009). The poultry industry implemented intervention strategies and MLST was applied to assess the effects of these strategies, a significant decline in human campylobacteriosis due to poultry being the main cause of infection was observed in New Zealand as well as an overall decline in the reported number of *Campylobacter* cases (Lake, 2006). MLST can be used for phylogenetic studies as it measures variations that occur without selective pressure as well as variations that occur slowly within *Campylobacter* isolates. This technique can also detect intra and inter species recombination which takes place in MLST loci, these recombination events allow *Campylobacter* to adapt to constant changes in environmental conditions/ stresses and also to new hosts once infection is achieved. There is always a need for further discriminatory power in any method; therefore PubMLST has extended its database to include an extended MLST (eMLST) which contains variable regions in *flaA*, *flaB* and *porA* genes resulting in a 10 loci MLST system which is highly discriminatory and appropriate for use in short term and long term investigations (Ragimbeau et al., 2014). Many challenges exist with implementation of MLST, the time consuming nature as well as the elevated cost reflects a need for more streamlined processes, with the decreasing cost of whole genome sequencing (WGS) it is becoming more practical and cost effective to rather perform insilico MLST based on analysis from whole genome sequencing instead of sequencing individual eMLST/ MLST loci (Chen et al., 2013).

### 2.3.4. Amplified fragment length polymorphism (AFLP)

This technique was originally developed for use in plant genome analysis, however, successful adaptation has made AFLP possible for DNA fingerprinting of bacterial genomes. Since then AFLP has been implemented for *C. jejuni* epidemiological analysis, for detection, classification and association of infection, in studying persistence of *C. jejuni* clones within the environment as well as in different types of hosts, strain diversity and differentiation among species, subspecies, and strain isolates (Hein et al., 2003; Garénaux et al., 2008; Habib et al., 2009; Melero et al., 2012; Epps et al., 2013; Zhou et al., 2013; Khoshbakht et al., 2014). AFLP is a useful method for the differentiation of genetically related strains, whole genomic DNA from strains are digested with two restriction enzymes (4 and 6bp recognition sites)

and ligated with oligonucleotide adapters resulting in selective amplification of restricted fragments, these amplified products are then subjected to gel electrophoresis, producing multiple bands from the entire genome. The discriminatory power of AFLP is dependant on the primers labeled with radioactive or fluorescent dyes used(*HindIII* +A and *HhaI* +A), as well as the set of restriction enzymes (Merchant-patel et al., 2010). High resolution AFLP for *C.jejuni* and *C.coli* is driven by a combination of whole genome analysis and automated data, with this method there is no need for prior knowledge of genome sequences such as PFGE and RAPD-PCR which require such knowledge for the technique. Absolute requirements for the AFLP are double stranded DNA which is intact and purified, specific equipment and software (Ahmed et al., 2012). AFLP has been successfully used for epidemiological studies of *Campylobacter* spp. from the food chain and for genetic diversity studies of Campylobacter spp. detected on broiler farms as well as commercial broiler flocks (Havelaar et al., 2012).

## 2.4. Sources of *Campylobacter* infection

In poultry the colonization by *Campylobacter* spp., of the intestine does not cause any symptoms, thus detection, tracking and control of this pathogen is very difficult. A European Union wide baseline survey on the prevalence of *Campylobacter* in broiler chicken and carcasses revealed that *Campylobacte*r was present in an alarming 71% of live chickens and 75% of carcasses. Nevertheless, the prevalence of *Campylobacter* colonized broilers varied widely between countries: it ranged from the minimum 2% (Estonia) to a maximum of 100% (Luxembourg), and in Hungary it was observed at 50% (EFSA and ECDC et al., 2012). Campylobacteriosis is a zoonosis, therefore, significant reservoirs of the organism include companion and farm animals and they may even be carried on the exoskeleton of insects, which have the potential for transmission to humans (Kittl et al., 2013). Different *Campylobacter* species have been linked with different sources of infection, *C. jejuni* is the most prevalent in poultry and cattle, *C. coli* in swine and *C.upsaliensis* isolated from dogs (Kittl et al. 2013). *C. fetus* is known as an opportunistic pathogen and has often been isolated in systemic infection in humans, also from swine, birds, reptiles and has been the cause of bovine reproductive disorders (Bessède et al., 2014).

Other *Campylobacter* spp. such as *C. hyointestinalis* is also known to cause gastroenteritis in humans and infect animals such as bovine and reindeer (Kaakoush et al., 2015). *C. jejuni* and *C. coli* are the two most common thermotolerant pathogens associated with campylobacteriosis in humans and *C. jejuni* is responsible for 80 to 90% of these infections (CDC, 2013). Raw or undercooked poultry which is contaminated with *Campylobacter* spp. has been recognized as a major vehicle for the transmission of campylobacteriosis this source is responsible for 50 to 80% of human infection ( Efsa, 2011; Mossong et

al., 2016). Poultry includes broilers, laying hens, turkeys, ducks, ostriches and to a lesser extent other sources of infection include undercooked beef or unpasteurized milk, raw vegetables, environmental water and dairy products also play a role in transmission of this pathogen (Sheppard et al., 2009). When *Campylobacter* spp. are detected in the environment this is usually an indication that contamination with avian and animal feces or sewage effluent as well as agricultural runoff has occurred (Djordjevic et al., 2013). From animal farms to the commercial production of food commodities there are several possibilities for transmission of *Campylobacter* infection through cross-contamination of meat. Broiler flocks are regularly contaminated with *C. jejuni* and *C. coli*. Carcass contamination occurs as a result of direct contact with infected intestinal tissue and fecal matter or as a result of contact with contaminated surfaces such as conveyer belts, rubber gloves, cutting equipment and cutting tables (Callicott et al., 2008). An entire flock of chickens can be infected within two to four weeks from initial infection and the flock will remain infected until slaughter as a result of the avian immune system being too weak to eliminate the infection naturally (Hermans et al., 2011). *Campylobacter* infection from consumption of poultry, beef, and pork products are considered leading causes of human food borne illness as demonstrated below in figure 2.2 (EFSA and ECDC et al., 2012).

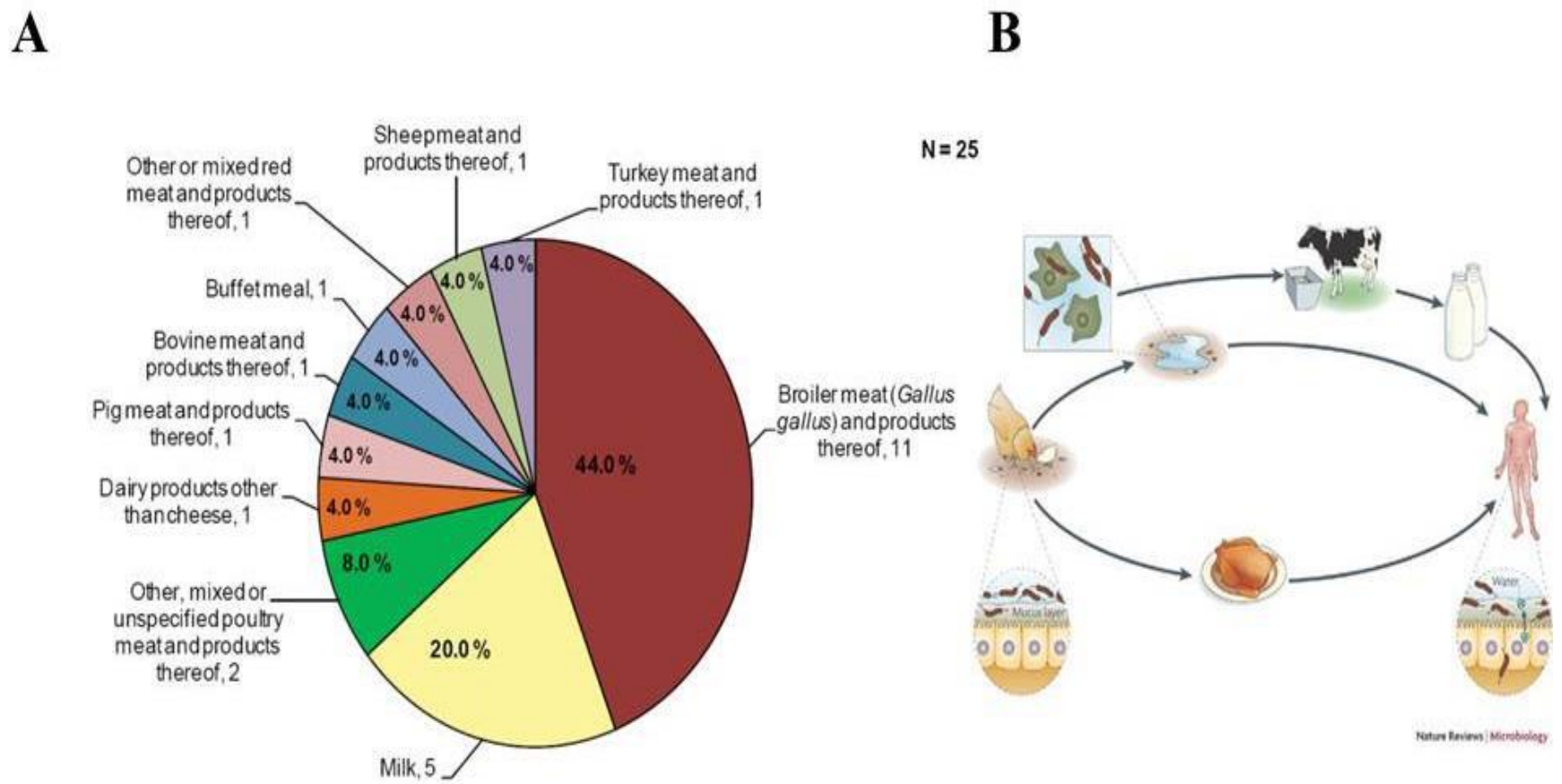


**Figure 2.2:** (A) Distribution of food vehicles in strong-evidence outbreaks caused by *Campylobacter* in the EU, 2012; (B) Environmental routes and reservoirs of *C. jejuni* infection (EFSA and ECDC et al., 2012).

### 2.4.1. Poultry

Poultry is recognized as a primary cause of food-associated transmission of *Campylobacter* species to humans. A major contributing factor of this pathogen is the high carriage rate of *Campylobacter* within the gastrointestinal tract of broiler chickens (Hermans et al., 2011). Therefore, *Campylobacter* species are found in abundance on poultry farms and their surrounding environment, including the soil, water sources, dust, building surfaces, and in the air. In addition to chickens, commercial turkeys and ducks can also serve as reservoirs of *C. jejuni* and *C. coli* (Noormohamed & Fakhr, 2014). Furthermore, poultry is also a significant reservoir for other *Campylobacter* species, such as *C. lari*, *C. upsaliensis*, and *C. concisus*. Domesticated broiler chickens and imported chickens both contribute to the overall burden of *Campylobacter* infections. It has been estimated that 71% of human campylobacteriosis cases in Switzerland between 2001 and 2012 were attributed to chickens (Di Giannatale et al., 2014). A study investigating the incidence of *Campylobacter* in crop which is the enlarged part of the digestive tract in avian species and serves as a short-term storage space for food as well as cecal samples from broiler chickens found 62% of crop samples to be positive, compared to only 4% of cecal samples, suggesting that the crop is an important niche and reservoir of *Campylobacter* spp. and may possibly contribute as a major source of contamination during processing at the abattoir. Given that *C. jejuni* strains are able to survive in chicken feces for up to 6 days subsequent to excretion, thus chicken feces are definitively a probable source of transmission to the environment and or humans when poultry manure is made use of as a fertilizer (Sheppard et al., 2009). The UK Food Standards Agency reported findings indicating that close to 73% of fresh whole retail chickens examined for the duration of 2014 to 2015 were infected with *Campylobacter*, with almost 19% of these whole chickens harboring a level of 10 000 CFU/g, which is regarded as highly contaminated (Colles et al., 2016). Information from Canada also maintain the finding that broiler chickens are the most important source of *Campylobacter* species (Biswas et al., 2011).

There are, however, indications that associated *Campylobacter* infections from ingestion of chicken may be more common in urban inhabitants than in rural populations. One study reported the levels of thermotolerant *Campylobacter* species to be three times higher in organic broilers than in conventional broilers (54% versus 20%), signifying that the probability of purchasing *Campylobacter* infected broiler meat is higher for organic sources than for conventional sources (Luangtongkum & Morishita, 2006).

### 2.4.2. Domestic Animals

Domesticated animals can also act as a source for many *Campylobacter* species. Commercially cooked foodstuffs tend to be less affected by *Campylobacter* compared to fresh and frozen meats which are frequently contaminated (Djordjevic et al., 2013). In addition to ingestion of infected meat from domestic animals, contact with domesticated and companion animals creates a considerable threat for the transmission of *Campylobacter* species. Recent studies indicated that 16 to 17% of the overall cases of reported campylobacteriosis from Denmark were due to cattle being the main source. Likewise, cattle in Switzerland, have been estimated to be responsible for 19% of *Campylobacter* infections, which is to a large extent higher than the contribution observed from pigs which was observed to be 1.2% (Kittl et al., 2013). Sheep and goats have also been investigated for their rates of carriage of *Campylobacter* species because these animals also serve as a source of food for human consumption. In Nigeria, almost 7% of intestinal samples isolated from sheep were found to be positive for *C. jejuni*, *C. coli*, or *C. lari* (Oporto et al., 2009). Only *C. jejuni* and *C. coli* were detected in cecal samples obtained from sheep in a Swiss abattoir and a higher incidence rate of 17.5% was documented in this investigation (Sáenz et al., 2000). In both studies, *C. jejuni* was determined as the main species of *Campylobacter* detected. Screening of fecal samples from 222 dairy goats on 12 farms in Spain failed to detect any *Campylobacter* species. Cats and dogs have also been implicated as carriers of *Campylobacter* species. An alarming 97% of dogs suffering with diarrhea and about 58% of healthy dogs have been determined to be positive for *Campylobacter* species. Interestingly, one of the major *Campylobacter* species colonizing dogs and cats is *C. upsaliensis*; however, *C. coli*, *C.concisus*, *C. fetus*, *C. gracilis*, *C. helveticus*, *C. jejuni*, *C. lari*, *C. mucosalis*, *C. showae*, and *C. sputorum* have all been identified in these pets. Other domestic pets that have been explored as prospective reservoirs of *Campylobacter* species consist of rabbits, hamsters, ferrets and pet reptiles. Due to the low rates of carriage of *Campylobacter* species in the domestic pets mentioned above, it is likely that they do not play a role as major sources of transmission of *Campylobacter* species to humans (Man, 2011).

### 2.4.3. Water is an effective vehicle of transmission of *Campylobacter* spp.

Intake of contaminated water or water that has not been sterilized properly is a leading risk factor for campylobacteriosis (Lake, 2006). This source acts as an efficient vehicle of transmission of *Campylobacter* species to humans and animals and is accountable for a number of outbreaks in different countries around the world. Individuals who make use of wells, rivers and lakes as drinking sources instead of using municipal surface water systems are at a much greater risk of becoming infected with

*Campylobacter* spp. rather than any other enteric pathogen (Swierczewski et al., 2013). In densely populated districts such as some rural areas in South Africa, groundwater is commonly used without disinfection. Some gastrointestinal infections are due to ingestion of this contaminated water which result in severe symptoms due to under-nutrition and lack of intervention strategies in most developing populations and nearly 90% account for infant deaths alone (On, 2013). Most farms utilize private water supplies for their cattle and domesticated animals, the animals which are exposed to this kind of water source are more likely to test positive for *Campylobacter* spp. (Huang et al., 2009). Some factors such as cleaning and emptying troughs on a regular basis have reduced the risk of *Campylobacter* colonization in cattle. During outdoor grazing period in spring cattle are more likely to test positive for *Campylobacter* spp. because the water supply comes from a lake and during winter when they are confined indoors and they are usually given municipal chlorinated tap water for drinking, therefore they are less likely to be exposed to *Campylobacter* (Huang et al., 2009).

Outdoor water can be contaminated by sources such as wild bird feces and waste overflow from domesticated animals which are contaminated with *Campylobacter*. The role of contaminated water in the transmission of *Campylobacter* spp. to different hosts could be a common factor as a source, however infection via waterborne outbreaks are extremely underestimated. A study conducted by Champion et al. (2005) indicated that *C. jejuni* isolated from different sources such as human, chicken, bovine, ovine, and environmental exhibit clustering into two clades identified as a “livestock clade” a “nonlivestock clade.”, demonstrated in Figure 2.3. Results grouped 55.7% of human and 11.4% of livestock *C. jejuni* isolates within the non-livestock clade proposing that the environment plays an important role as a reservoir but particularly contaminated water is underestimated as a source of transmission for *Campylobacter* species (Champion et al., 2005).

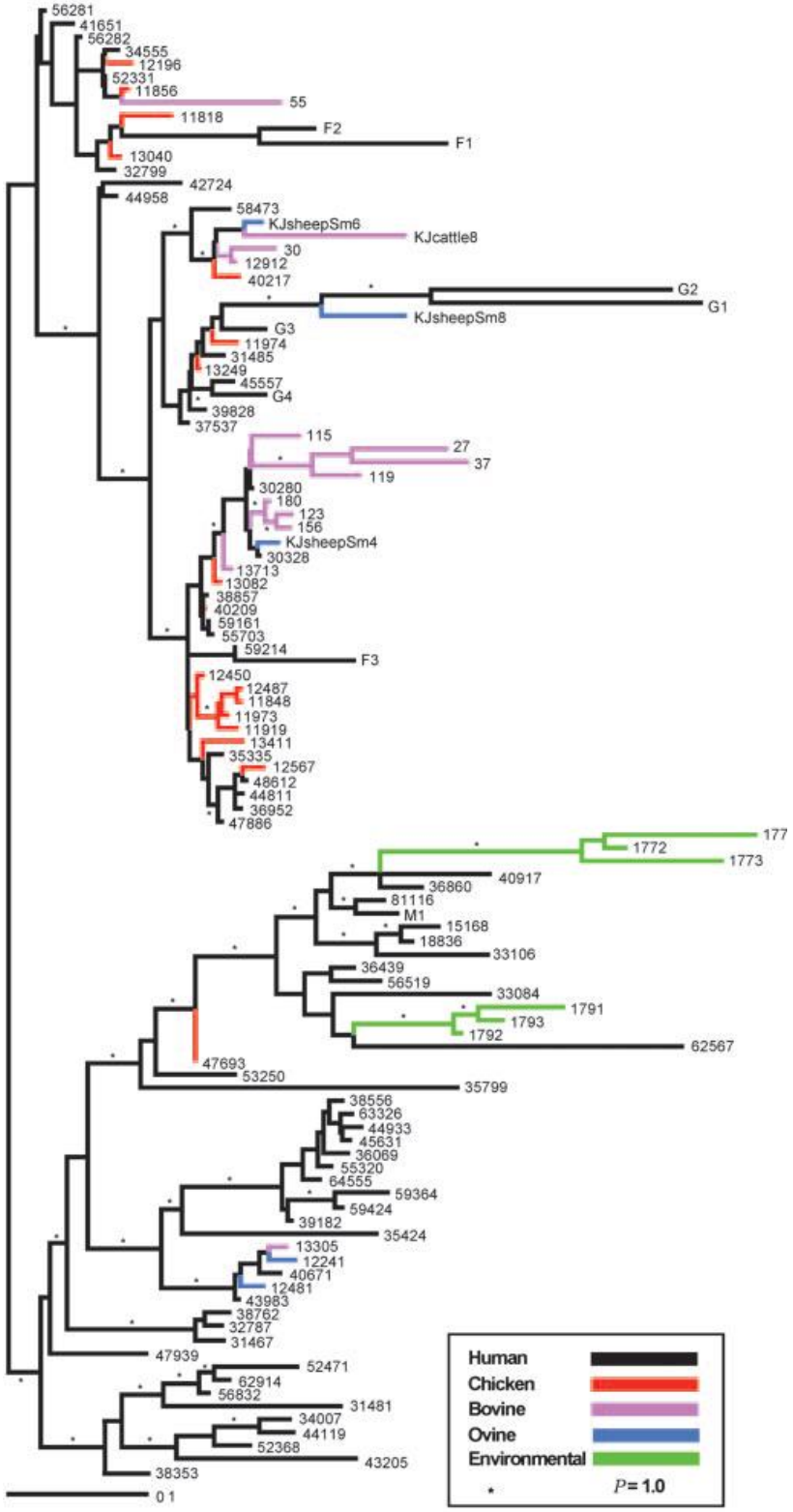


**Figure 2.3:** Phylogenomic relationship of strains associated with different ecological niches. Strains are designated at the end of branches and are colored according to the ecological niche from which the *C. jejuni* strain was isolated (Champion et al., 2005).

## 2.5. Reducing *Campylobacter* Transmission in Chickens

In the farm environment the usual route of transmission for *Campylobacter* is horizontal transmission. At the onset of *Campylobacter* being present in a chicken flock, it tends to spread rapidly and within 1 week most intestinal tracts of chickens are colonized with the pathogen (Thibodeau et al., 2015). The major locations of colonization for *Campylobacter* species are the small intestine, the crop and the ceca. Once colonization has occurred it persists until the chicken is slaughtered. Colonization by *Campylobacter* spp. can occur in large numbers of up to 109 bacteria per gram without affecting chickens in any way clinically or in any unfavorable way (Hermans et al., 2011). Poultry farms and their environment is recognized as an abundant source of *Campylobacter* species, therefore, strategies regarding strict hygiene have been introduced on the majority of farms in an effort to decrease *Campylobacter* colonization of chicks (Melero et al., 2012). Current literature has indicated that a few strategies can significantly reduce *Campylobacter* colonization of chicks; these include use of house-specific boots and overshoes, on entering poultry houses hygiene barriers are used, following routine boot dips and the requirement of hand-washing facilities (Workman et al., 2005; Callicott et al., 2008; ; Epps et al., 2013; Wang et al., 2013; Colles et al., 2016).

Factors such as exposure to fecal material in a flock from chickens already infected with *Campylobacter* and living on the same farm increases the risk of additional colonization in some of the healthy chickens, in some countries flies play a significant role in transmitting *Campylobacter* species from fecal sources to poultry. Another important factor is the number of chicken houses on a farm; this can increase the probability of chickens being colonized even though the correlation between the number of houses and the level of increased threat appears to fluctuate by geographic location (Collado et al., 2013). Birds from different hatcheries were found to have identical *Campylobacter flaA* types present in the isolates therefore proposing that when chicks are placed in broiler houses this is when colonization occurs and *C. jejuni* strains within the farm are passed from the previous flock to the new chicks (Merchant-Patel et al., 2010).When *Campylobacter* spp. are reduced in the gastrointestinal tract of broiler chickens it has been reported to considerably decrease the incidence of campylobacteriosis (O Cróinín & Backert, 2012). If chickens could be successfully vaccinated against *Campylobacter*, it would decrease *Campylobacter* levels in flocks as well as reduce transmission to humans and eliminate the need for some of the procedures needed after slaughtering. Investigations have attempted a range of vaccine candidates to prevent colonization in chickens; these include using formalin-inactivated *C. jejuni*, killed *C. jejuni* delivered intranasally, orally, with the use of a *Salmonella* carrier, and intraperitoneally, inner membrane antigens from *C. jejuni* and flagellar proteins, none of these however were successful in completely

preventing colonization (Tribble et al., 2009). With *Campylobacter* present in the intestinal tracts of chickens during slaughter, it can potentially contaminate the food processing environment, the food products purchased by consumers, and the slaughterhouse (Silva et al., 2011). For reduction of the number of *Campylobacter* bacteria at the processing level strategies have been implemented such as the use of chemical dip tanks for carcasses, treatment of meat products with trisodium phosphate, organic acids and acidified sodium chlorite, during primary processing UV light has also been used to decrease the microbial load (Bouwknegt et al., 2013). For direct eradication of the pathogen and reduction of bacterial numbers, irradiation or extremely low temperature for freezing the entire poultry supply would achieve decrease of the organism. While strategies mentioned above have been successful to an extent for reducing the transmission of *C. jejuni* to humans from poultry, they have not entirely eliminated the transmission of *Campylobacter* related gastroenteritis cases and in addition to this they are expensive and some investigations have shown that the quality of meat tends to degrade after such measures during processing (Mead, 2004; Humphrey et al., 2007; Sheppard et al., 2009; Hermans et al., 2011; Thibodeau et al., 2015). The use of multiple strategies at the primary level of processing seems to be the most cost-effective compared to use of a single method such as irradiation which is the least cost-effective approach for reducing *Campylobacter* organisms and poultry-related transmission (Lake, 2006).

## 2.6. Invasion by *Campylobacter* spp.

In order to maintain successful infectivity, various strategies need to be developed by microbial gut pathogens; firstly they need to attack tissues, find a way around or withstand the mechanism of the immune system, agitate the typical gut flora, cause injury to cells and maintain high numbers within the host. For successful infection to occur the most important step in campylobacteriosis is invasion when this has been initiated it results in disease outcome. Similarly to other intestinal pathogens, *C. jejuni* is also able to adhere and invade polarized cells such as T84, MKN-28 or Caco-2 (Melo et al., 2013) and non-polarized such as INT407, Hep-2 or HeLa cells. Enteropathogens use numerous mechanisms to invade and move across the intestinal cells of its host; however, there is still not much known about the *C. jejuni* invasion process. *Campylobacter* spp. have a favorable temperature range for growth; thermophilic *Campylobacter* spp. such as human pathogens *C. jejuni* and *C. coli*, which we will be focusing on in this research, have an optimal temperature of 42°C which reflects an adaptation to the intestines of warm-blooded avian species (Hermans et al., 2011) they can, however, persist at temperatures ranging from 34°C to 44°C. On the other hand non-thermophilic *Campylobacter* such as *C. fetus* and *C. hyonintestinalis* grow best at 25°C. For the transmigration of *C. jejuni* through polarized intestinal cells two routes are proposed (Figure 2.4). The transcellular route exhibited by *Campylobacter* follows

entering of the pathogen at the apical side and exiting at the basal portion of cells. The paracellular path taken by *Campylobacter* spp. enters at the basal region of the intestinal cells by crossing through tight and adherence junction linking epithelial cells or the pathogen could chose to continue their way through the lamina propria, this route enables the pathogen to travel to different parts of the human body such as the liver, spleen, lymph nodes and vessels (Man, 2011). The invasion process of *C. jejuni* is dependent on a number of factors which permit motility, glycosylation, capsular synthesis and adherence (O Cróinín & Backert, 2012).

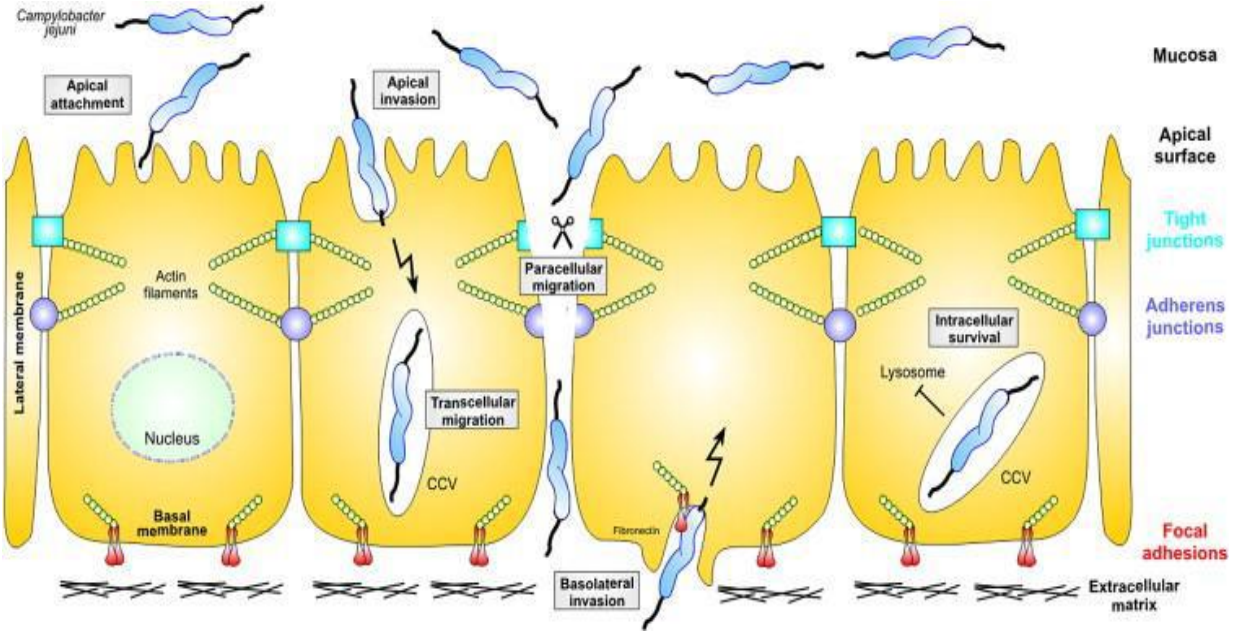


**Figure 2.4:** Hypothetical models for *C. jejuni* invasion mechanism (Backert & Hofreuter, 2013).

Figure 2.5 demonstrates two diverse mechanisms of access utilized by intracellular bacterial pathogens into target cells. The "zipper" and "trigger" invasion mechanisms allow pathogens to stimulate their own uptake into targeted cells; this is achieved by disturbance of the hosts' cell signaling pathways (Backert & Hofreuter, 2013). With reference to A (the zipper mechanism), the first step (infection) is colonization of the gastric epithelium by gastrointestinal pathogens, the second step (receptor binding) involves surface proteins such as adhesions of the bacteria which allow binding to their equivalent receptors found on host cells, the third step (membrane engulfment) initiates the zippering of the host cell plasma membrane around the pathogen; this is mediated by the cytoskeleton, the fourth stage (endosomal trafficking) involves internalization of the bacterium within a vacuole, the bacteria have adapted to either survive or escape from the vacuole (Backert & Hofreuter, 2013).

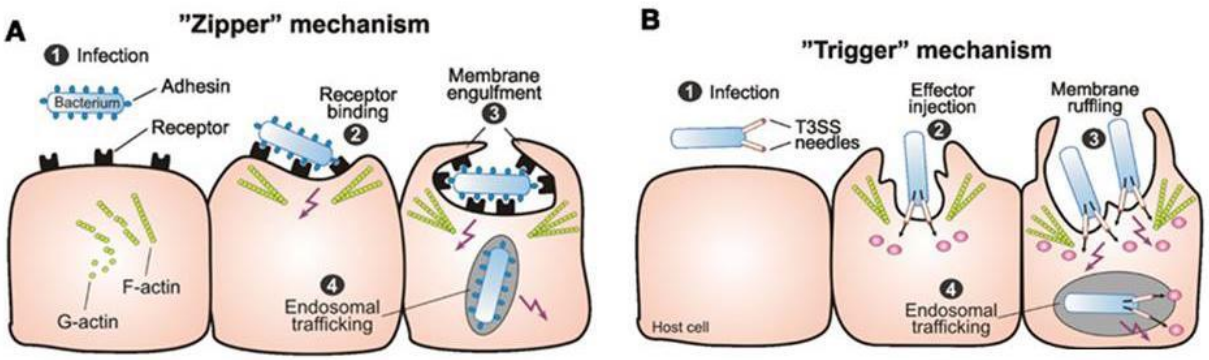


**Figure 2.5:** Mechanism of bacterial invasion into non-phagocytic host-cells (O Cróinín & Backert, 2012).

With reference to B in Figure 2.5, this is the "trigger" mechanism used by pathogens *Shigella* or *Salmonella* spp., intestinal epithelium is colonized in step 1(infection), followed by step 2 (Effector injection) where the pathogens inject a variety of effector proteins into the hosts cytoplasm via type-III or type-IV secretion system, once effector proteins are inside the cytoplasm the third step (Membrane ruffling) begins which involves triggering bacterial uptake as well as initiating a number of signaling events such as cytoskeletal rearrangements which triggers membrane ruffling and finally stage 4 (endosomal trafficking) where bacteria are internalized within a vacuole where they can survive or escape (O Cróinín & Backert, 2012).

## 2.7. Host colonization and Clinical manifestations

Campylobacteriosis is impossible to differentiate from other bacterial gut infection clinical expressions include fever, abdominal cramping and diarrhea, with or without the presence of blood in stools (Epps et al., 2013). In the developed world, most human *Campylobacter* infections are caused by *Campylobacter jejuni*; however, *Campylobacter coli* is fast becoming more prevalent in clinical cases (CDC, 2013). *Campylobacter* infection for humans has a very low infectious dose; it takes as few as 500 to 800 organisms to initiate infection. *Campylobacter* spp. are able to colonize host cells through three different stages of infection. The first stage entails colonization of the host's intestinal epithelial cells following ingestion of the pathogen and transmission through the intestinal gut leading to the production of cytotoxins by *cdt* genes which then result in diarrhea. The second stage involves invasion and colonization of the hosts intestinal cells and damage to the mucosal surface cells in the jejunum, ileum and colon. The last stage involves *Campylobacter* crossing the intestinal epithelium through translocation and migration to diverse extra-intestinal sites through the lymphatic system (Epps et al., 2013). The incubation time for this organism after ingestion usually ranges between 24 – 72 hours however if the

inoculum number for infection is low it may take a week or more for symptoms to begin such as fever, headaches and myalgia which happens in the initial 24hrs (Kovács 2014). Following infection during the next few days heavy cramping which causes severe abdominal pain results, this can often be mistaken for appendicitis. Self-limiting diarrheal disease is the leading cause of most campylobacter infections, this lasts for two days and generally resolves over a week, without treatment however a relapse can occur which can result in death usually in patients under the age of 5 or elderly patients, people with underlying diseases and also including those who suffer from immunocompromising diseases like Acquired Immune Deficiency Syndrome (AIDS) (Silva et al. 2011).

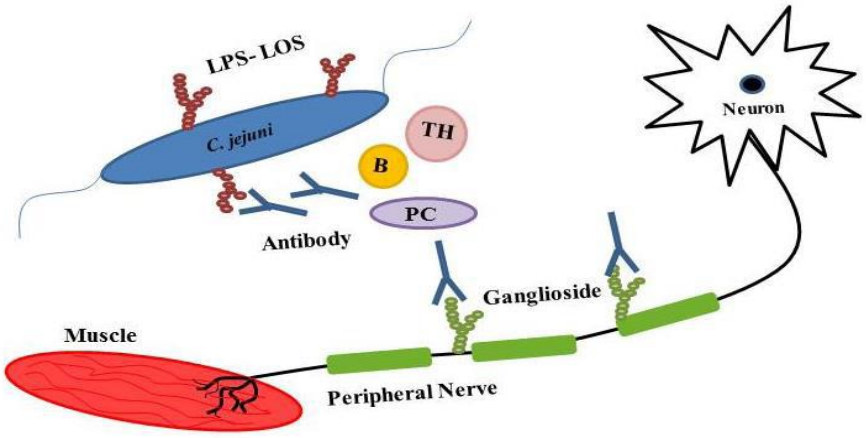


**Figure 2.6:** The molecular background of the *C. jejuni* induced autoimmune GBS infection process. LPS-LOS: Lipopolysaccahrides-Lipooligosaccharides; TH: T helper lymphocyte, B: B lymphocyte; PC: Plasma cell (Kovács, 2014).

Extraintestinal infections are most likely to appear in immunocompromised hosts, following infection, these include meningitis, reactive arthritis, pancreatitis, bacteraemia, infections of nearly any organ and hemolytic uremic syndrome to name a few (Hauser et al., 2013). An acute inflammatory polyneuropathy known as Guillain-Barré syndrome (GBS) has been frequently associated with *Campylobacter* infections, 20–50% are attributed to a *C. jejuni* infection (Humphrey et al., 2007). GBS is serious but also rare and occurs on 1 out of a 1000 cases, it is an immune-mediated polyneuropathy of the peripheral nervous system (PNS) which results in neuromuscular paralysis. GBS develops 1 to 3 weeks subsequent to infection with *Campylobacter* and results on account of molecular mimicry, as demonstrated in Figure 2.6 above, the host produces *Campylobacter* antibodies during infection development and these identify the surface polysaccharides of the bacterium. The lipopolysaccharide structures LOS resemble neural glycolipids (gangliosides) of the pathogen and this cross reacts with the antibodies inducing an attack on

the peripheral nervous system of the host (O Cróinín & Backert, 2012). It is also interesting to note that symptoms of patients depend on which part of the world they reside in, this highlights the significance of the role host immune status plays on the degree of infection; disease outcome has also been suggested to be reliant on the virulence of *Campylobacter* species (Rodrigues et al., 2015).

## 2.8. Virulence and Pathogenisis of *Campylobacter* spp.

The pathogenic mechanism by which *Campylobacter* species causes disease is mysterious, in addition to pathogenic factors, the role of host dependent factors should also be considered (Bouwknegt et al., 2013). The virulence factor which lead to infection is thought to be unique when compared to other enteric pathogens, however, the molecular background of virulence mechanisms which contribute to campylobacteriosis is not well understood (Rodrigues et al., 2015). Chemotaxis, production of toxin, motility, invasion, adhesion and intracellular survival are some of the potential virulence properties which have been reported (Kovács, 2014). An crucial step for establishing early infection of *Campylobacter* species is colonization of mucus; serine and threonine repeats are harboured by mucin glycoproteins; these are associated with variable numbers of tandem repeats (VNTR) which are essential components toward which *Campylobacter* displays chemotaxis. This results in widespread contamination rates in poultries even though the pathogen invades and adheres to their intestinal epithelial cells, it does not cause any disease in them. Studies have demonstrated that mucus colonization modulates the pathogenicity of *Campylobacter* in a species-species manner, which could explain why some of the same *Campylobacter* strains are pathogenic in humans yet commensal in chickens (Iraola et al., 2014).

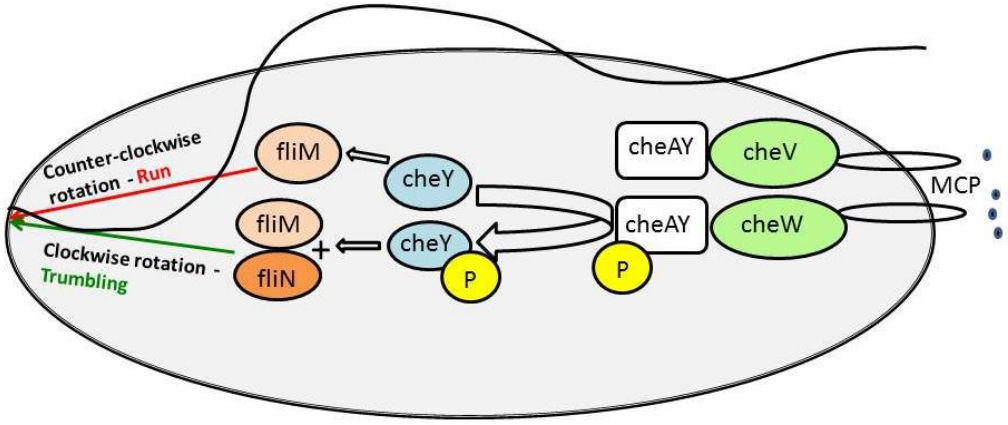


**Figure 2.7**: A model of chemotaxis signal transduction pathways in *C. jejuni* (Reuter et al., 2015).

There are ten methyl-accepting chemotaxis proteins (MACPs/MCPs) present in *C. jejuni*, these behave as chemoreceptors which sense sugars and amino acids that give off extracellular signals conferring *C. jejuni* with the ability to rotate their flagella and maneuver the pathogen to change direction (Reuter et al., 2015). The model of chemotaxis is driven by MACP which transmits a signal to autophosphorylate CheA and to phosphorylate CheY which results in the flagella rotating in a clockwise direction. When chemo attractant ligands occupy the majority of receptors this leaves more of the CheY non-phosphorylated inducing flagella in a counterclockwise direction, this pushes the cell forward in a fast movement resulting in a run by the bacteria. Tumbling of bacteria results when the concentration of the ligands decrease and phosphorylated CheY increase and bind to FliM causing a clockwise flagellar rotation (Figure 2.7); (Reuter et al., 2015). When mutation arise in MACPs/MCPs this results in reduction of colonization of chicken's gut, mutations such as *docB* and *docC* provide suitable chemotaxis depending on the environmental component, this is achieved by methylation of *docB* and *docC* (Thibodeau et al., 2015).

## 2.9. Virulence genes responsible for host infection

In campylobacteriosis, invasion is the key step of the infection in order to develop disease; this is dependent on the ability of *Campylobacter* species to bind to the *Cad*F protein which is encoded by the *cadF* gene responsible for the binding to the extracellular matrix of human intestinal cells (Nayak *et al*., 2005). *C. jejuni* which lack *Cad*F has revealed reduced ability to adhere to chicken intestine; experiments have demonstrated the same result with human and chicken cell lines (Monteville et al., 2003; Flanagan et al., 2009). Another gene essential for establishing human infection is the *fla*A gene encoded by a flagella protein this protein mediates motility, colonization, and invasion of the gastrointestinal tract. Three linked genes *cdt*A, *cdt*B, and *cdt*C are responsible for the cytolethal distending toxin (CDT) released by *Campylobacter* species (Kovács, 2014).

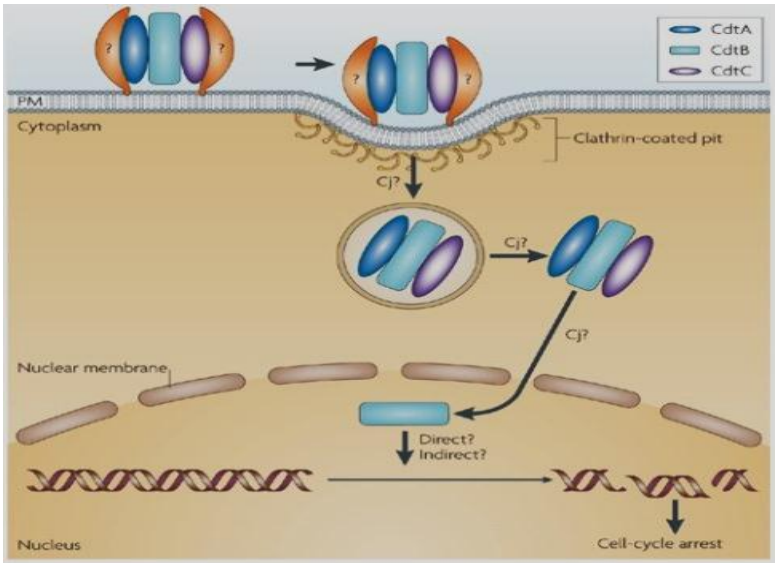


**Figure 2.8:** Uptake and activity of cytolethal distending toxin (Young et al., 2007).

The cytolethal distending toxin is the only verified toxin known to be released and expressed during colonization and results in distension of the epithelial cells, which manifests in bloody diarrhea from an infected patient (Dasti et al., 2010). The cytopathic effect of the cytotoxin activity is connected with damage to nuclear DNA which results in the inhibition of the cell cycle in G1/S or G2/M phase depending on the type of cell which induces cellular distension and cell death. The genes *cdt*A and *cdt*C are thought to play a role in binding the bacteria to host cells (Whitehouse et al., 1998) and the enzymatically active subunit encoded by *cdtB*, shares homology with *DNase I* of *E. coli* (Lara-Tejero & Galán, 2000). All three of these subunits are required for the cytotoxin activity known to be lethal for host enterocytes (Purdy et al., 2000; Lara- Tejero & Galan, 2001). Cytolethal distending toxin (CDT) is also involved in inducing the release of proinflammatory cytokine IL-8 from intestinal epithelial cells in humans (Hickey et al., 2000). Microarray analysis of the CDT cluster has demonstrated that these genes were present in all *C. jejuni* isolates from human samples (Dorrell et al., 2001; Volokhov et al., 2003). Studies have also analyzed the distribution of the CDT cluster as well as the separate *cdt*A, *cdt*B and *cdt*C genes in *C. jejuni* and *C. coli* and indicated that their prevalence in isolates from poultry and other sources exceeds 90% (Bang et al., 2001; Datta et al., 2003).

## 2.10. Antibiotic resistance and associated mechanisms in *Campylobacter* species

Antibiotics have been used for decades as in poultry production systems and other livestock for prevention and control of infections and to enhance growth rates (Igimi et al., 2008; Rozynek et al., 2009). This unregulated use of antimicrobial agents has resulted in an increased resistance against multiple antibiotics and also in microbes with human importance. The unwarranted use of antibiotics as an enhancement in animal feed is estimated to represent more than a half of the total antimicrobial use around the world (Moore et al., 2005). Research has demonstrated that the administration of fluoroquinolones to poultry in production systems has caused an increase in the resistance of *C. jejuni* against these agents, isolated from both animals and humans (Smith & Fratamico, 2010). Growing antibiotic resistance in campylobacters is an emerging public health concern around the world recognized by the World Heath Organization (McDermott et al., 2006). *Campylobacter* species was previously considered susceptible to various antimicrobial agents; however in the recent years, both animal and human isolates of this bacterium have shown growing resistance to several antibiotics such as macrolides (erythromycin), tetracycline, and fluoroquinolones, which are the most frequently prescribed antibiotics against *Campylobacter* infection (Iovine et al., 2013) Fluoroquinolone-resistant *Campylobacter* has also become prevalent in Africa and Asia. The only alternative treatment considered is gentamicin (Aarestrup & Engberg, 2001).

The overuse of antibiotics in animal husbandry for prevention of secondary infection, off-label uses and as a growth promoter, as opposed to therapeutic purpose, in order to sustain the increasing need for human consumption of poultry has gotten out of control. The other aspect which is of great concern is the antibiotic usage in the human population which has led to an increase in antibiotic-resistant *Campylobacter* infections (Maćkiw et al., 2012). Luangtongkum et al. (2010) suggested that resistance patterns in poultry may possibly predict human resistance patterns, in their study this was most clearly shown with fluoroquinolones (FQ) (Luangtongkum et al., 2010). When low levels of antibiotics are used on poultry farms over long periods of time this initiates the emergence of resistant bacteria. In some regions veterinary antibiotics can be obtained with no prescription or additional controls such areas include Indonesia, Thailand, India (Sinha et al., 2013) and some parts of Africa, which is alarming due to the persistent emergence of resistant bacteria (Moyane et al., 2013). The European Union and Japan have banned the general use of antibiotics for growth promotion and Australia have implemented that FQ should not be used in any food producing animals (Colles et al., 2016). Australia primarily authorizes the use of ionophores and flavophospholipol as nonprescription antimicrobials in poultry, whilst chlortetracycline is registered for the treatment of birds that produce eggs, and a wider range of antibiotics

(including amoxycillin, neomycin, lincomycin, spectinomycin and oxytetracycline) are approved for use in chickens. Many acute diarrhea cases in humans are treated with fluoroquinolones, serious systemic infection can be treated with an aminoglycoside such as gentamicin, although not all cases of *Campylobacter* infection require antibiotic treatment, in severe or recurring cases susceptibility testing is important to ensure appropriate and timely treatment (Swierczewski et al., 2013).

Macrolides still remain the means of choice and resistance rates to erythromycin continue to be comparatively low for the moment (Wieczorek & Osek, 2013). Tetracyclines are scarcely used in the treatment of clinical *Campylobacter* enteritis due to high rates of tetracycline resistance (Abdi-Hachesoo et al., 2014). Effective therapy against most enteric pathogens is achieved by use of fluoroquinolones and ciprofloxacin which are used broadly as prophylaxis for travelers in the treatment of acute bacterial diarrhea (Simaluiza et al., 2015). An increasing number of *Campylobacter* isolates have developed resistance to this class of antibiotics and made their efficacy less certain; they are also now being cultured from both clinical and food samples in a number of European countries, Canada and the United States (EFSA & ECDC et al., 2012). These resistance rates were reported to develop among patients after treatment with fluoroquinolones and were also found to overlap with the introduction of these agents in veterinary medicine (Nelson et al., 2007).

*Campylobacter* is naturally transformable even though it possesses restriction modification systems that usually decrease the uptake of foreign genetic material such as mobile genetic elements. This has not however, restricted the pathogen from acquiring resistance genes from other organisms (Iovine, 2013). Therefore investigating the resistance mechanisms present in *Campylobacter* is imperative to both human and animal health. Mobile genetic elements represent a combination of endogenous and acquired genes which are chromosomal or plasmid-borne and these induce antibiotic resistance mechanisms in *Campylobacter* (Hein et al., 2013). General mechanisms of antibiotic resistance are demonstrated in Table 2.2 and include: (1) antibiotic's target modification and/or its expression (such as DNA gyrase mutations), (2) lack of ability of the antibiotic to arrive at its target (such as expression of the major outer membrane protein or MOMP) (3) antibiotic efflux by means of the multidrug efflux pumps such as CmeABC and (4) alteration or inactivation of the antibiotic such as β-lactamase production (Luangtongkum et al., 2010).

**Table 2.2:** Mechanisms of Antibiotic resistance (Iovine 2013)

| Antibiotic class | Resistance mechanisms |
|---|---|
| Aminoglycoside | Modification of the antibiotic by aminoglycoside-modifying enzymes (AphA, AadE, Sat) Contribution of efflux is not clear |
| Beta-Lactam | Enzymatic inactivation of the antibiotic by β-lactamase (penicillinase, OXA-61) Decreased membrane permeability of most anionic and MW > 360 kDa antibiotics due to MOMP, Efflux through CmeABC and possibly others |
| Fluoroquinolone | Modification of the DNA gyrase target (Thr-86-Ile; also Asp-90-Asn, Ala-70-Thr) Efflux through CmeABC |
| Macrolide | Mutations in 23S rRNA, Contribution of mutations in ribosomal proteins L4/L22 is likely minor, Efflux through CmeABC and possibly others, Decreased membrane permeability due to MOMP |
| Tetracycline | *tetO* gene, plasmid-borne in the majority of cases,<br>resistance mediated through ribosomal protection<br>Modification of the target ribosomal A site by *TetO* binding<br>Efflux through CmeABC and possibly others<br>Contribution of decreased membrane permeability due to MOMP is not clear |
| Multidrug-resistance (MDR) | Efflux pump with a broad specificity; preventing accumulation of antibiotics |

On ihe ribosome, shown in Figure 2.9 below, two major resistance mechanisms are indicated in blue at the left of the diagram, the antibiotic tetracycline is prevented from occupying that site where the protein *TetO* is binding to the A site (demonstrated in brown and dark purple), however access of the aminoacyl tRNA is still allowed in order for protein synthesis to continue (Iovine, 2013). The binding affinity for macrolides is decreased by point mutations occurring in the 23S rRNA in the domain V region (indicated in black) at position 2075 principally and less often at position 2074 (indicated by the two red stars), mutations in this region lead to macrolide resistance (Iovine, 2013). In the figure an example is shown where the structure of the 552 kDa, dianionic antibiotic ceftriaxone is limited because it exceeds a molecular weight of 360 kDa, the major outer membrane protein (MOMP, in green) restricts the entry of most antibiotics that are negatively charged or with a molecular weight larger than 360 kDa. In the figure, in light purple, the Thr-86-Ile substitution in DNA gyrase is shown and this indicates the main means of fluoroquinolone resistance, and this single mutation also confers high level resistance to this class of antibiotics (Iovine, 2013). The multi-drug efflux pump CmeABC (blue squares in the figure) contribute to resistance against fluoroquinolones, macrolides, β-lactams and tetracyclines, and works with other resistance mechanisms to confer high-levels of resistance (Pumbwe et al., 2004). From the aminoglycoside phosphotransferase family, aminoglycoside-modifying enzymes (AME) are the main

factors that mediate aminoglycoside resistance. The Ambler class D OXA-61 contributes to β-lactam resistance, mediated by β-lactamases of the penicillinase type (Iovine, 2013).

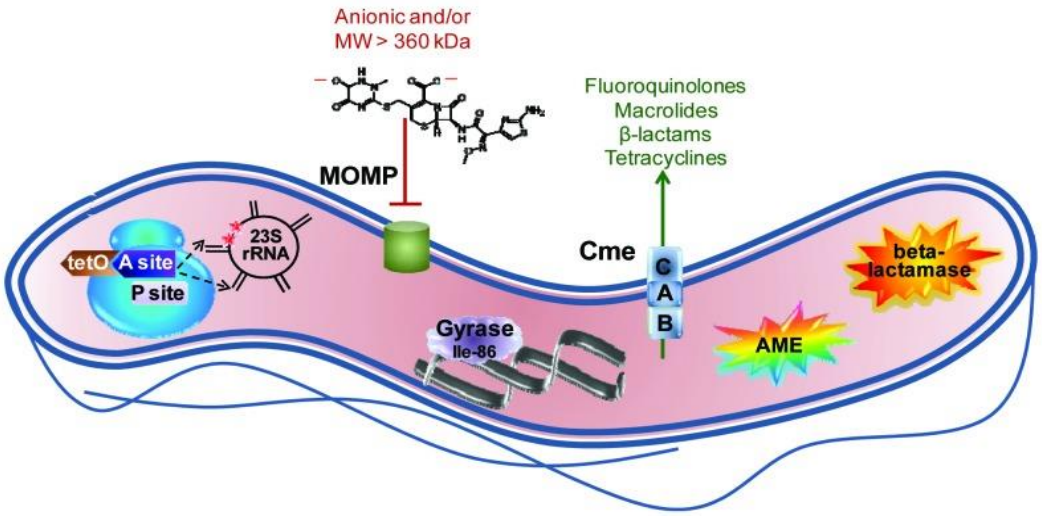


**Figure 2.9:** Summary of major antibiotic resistance mechanisms in *Campylobacter* (Iovine, 2013).

### 2.10.1. β-Lactam Resistance Mechanism

Currently, ampicillin is not considered useful in the treatment of campylobacteriosis; however, because its resistance is frequently related to β-lactamases, which can be can be transferred between different bacteria and is chromosomally mediated there is a need for its resistance to be monitored (Obeng et al., 2012). The occurrence of β-lactamase varies extensively in both poultry and human populations, but is generally found at greater than 20% in *Campylobacter*. There are three main mechanisms which mediate β-lactam resistance in *Campylobacter*, these are: (1) chromosomally encoded β-lactamases cause enzymatic inactivation, (2) alterations in outer membrane porins cause reduced uptake and (3) efflux helps remove any β-lactam antibiotics which enter the cell. In the presence of a penicillinase-type of β-lactamase in *Campylobacter* resistance to amoxicillin, ampicillin and ticarcillin is expressed this can be overcome with β-lactamase inhibitors tazobactam, clavulanic acid and sulbactam (Obeng et al., 2012). More recently, a class D β-lactamase OXA-61 was identified in *Campylobacter*; this is one of the resistance genes we will focus on in this study ($bla_{OXA\text{-}61}$) which mediates resistance to antibiotics such as penicillin, oxacillin, ampicillin, amoxicillin-clavulanate, piperacillin and carbenicillin. This enzyme also demonstrates associations to other OXA-type genes in other bacteria like *Fusobacterium*, *Acinetobacter* and

*Pseudomonas* (Djordjevic et al., 2013). The veterinary and human populations investigated have a high prevalence of OXA-61, however, the β-lactam class for *Campylobacter* is not included in the national data, on the prevalence of β-lactam resistance on the national antimicrobial resistance monitoring system (NARMS).

The β-lactam ring required for antimicrobial activity are found in a diverse class of compounds including penicillins, cephalosporins, carbapenems and monobactams belonging to the β-lactam antibiotics (Obeng et al., 2012).. Various side chains located on the β-lactam ring confer particular properties to individual members of this family such as pharmacokinetics, resistance to stomach acid and hydrolysis by β lactamases, basically the method entails binding to and thereby inactivating the bacterial penicillin binding proteins which are essential catalyzing the final cross linking step (Obeng et al., 2012). This mechanism results in a lack of structural integrity in the cell walls, osmotic swelling and lysis. As with macrolides, the MOMP (Figure 2.9) in *C. jejuni* and *C. coli* tend to reject most β-lactams with a molecular weight exceeding 360 or which are anionic however, some antibiotics such as imipenem (Molecular Weight (MW)  299), ampicillin (MW 333) and cefpirome (MW 347) are able to bypass MOMP. The CmeABC efflux pump may also contribute to β-lactam resistance and is the most effective efflux pump with regard to β-lactams (Obeng et al., 2012)

### 2.10.2. Tetracycline Resistance Mechanism

In *Campylobacter* tetracycline resistance is primarily mediated by a ribosomal protection protein (*tet*O), which is transferred as plasmid-encoded gene or in the chromosome where it is not self-mobile (Abdi-Hachesoo et al., 2014). The two most important mechanisms of tetracycline resistance in various genera of bacteria are the efflux pump and ribosomal protection genes and acquirement of new tetracycline resistance genes is mostly associated with mobile genetic elements such as plasmids or transposons, which are often conjugative elements in *Campylobacter* species and other bacteria (Ferro et al., 2015). The *tetO* gene is the most commonly reported determinant conferring resistance to tetracycline in the *Campylobacter* genus, and in many previous studies, this gene was detected in all tetracycline-resistant *Campylobacter* spp. isolates (Abdi-Hachesoo et al., 2014; Mazi et al., 2008; Obeng et al., 2012; Sahin et al., 2012; Wieczorek & Osek, 2013). The continuous use of tetracyclines, since being discovered in the 1940s, in human and veterinary populations has created widespread resistance and thus limited their use these days (Abdi-Hachesoo et al., 2014). Commonly used members of this class are tetracycline, doxycycline and minocycline. The tetracyclines are lipophilic protein synthesis inhibitors that likely use a combination of the hydrophobic pathway described for macrolides as well as outer membrane porins to

gain access to the bacterial ribosome; exactly how each pathway contributes to tetracycline entry in *Campylobacter* is not completely clear. The first mechanism of tetracycline resistance in *Campylobacter* is the alteration of tetracycline's ribosomal target by tetracycline reversibly binding to the 30S subunit which prevents the attachment of charged aminoacyl-tRNA to the ribosomal A site (Figure 2.9) by inhibiting the protein synthesis which is protection of an unoccupied A site by the binding of bacterial protein *tetO* to that site. This is a major mechanism in *Campylobacter* as well as in other Gram-negative bacteria for resistance to tetracyclines. The other mechanism is the contribution of efflux to tetracycline resistance; this is demonstrated by the increase in tetracycline MIC when efflux pumps are genetically inactivated. An example is the disruption of the *cmeG* efflux pump which makes the mutant strain 4 times more vulnerable to tetracycline compared with the wild-type strain. High-level resistance to tetracyclines can be mediated by *tetO* alone which can be found encoded on the chromosome or more commonly, on the plasmids *pTet* in *C. jejuni* and *pCC31* in *C. coli*. According to data collected during 2010 by NARMS, 43% of *C. jejuni* and 49% of *C. coli* isolates are tetracycline-resistant, making this class of antibiotic of little use in veterinary or human *Campylobacter* mediated disease (Simaluiza et al., 2015).

### 2.10.3. Fluoroquinolone Resistance Mechanism

Globally, fluoroquinolones (FQ) resistance was not popular in the late 1980s to early 1990s (Datta et al., 2003). However, the combination of indiscriminate use of FQ in humans and amplified FQ use in the poultry industry contributed to a rise in the prevalence of FQ-resistance in both animals and humans (Datta et al., 2003). Fluoroquinolones (FQ) are probably the most heavily-used class of antibiotic in humans, due to a number of qualities such as being well-tolerated, their evident concentration-dependent, bactericidal activity for both Gram-negative and Gram-positive organisms, they are also available in both oral and intravenous forms and can be taken once or twice daily (Man, 2011). The most commonly used antibiotic of the fluoroquinolone class is ciprofloxacin for treatment of acute bacterial diarrhea, however, if campylobacteriosis is very strongly suspected, which is difficult because campylobacteriosisis cannot easily be distinguished clinically from other causes of bacterial diarrheal infection, then the drug of choice for treatment would be macrolides. Therefore, FQ are used most of the time as treatment for most diarrheal cases, thus creating great clinical concern. Surveillance regarding FQ susceptibility in *Campylobacter* spp. in animals is imperative especially for food production purposes and because the emergence of resistant strains in animals threatens an increase in resistant human infections. Many countries including the United States, Denmark, and Australia have acknowledgment of this correlation and have therefore banned FQ use for veterinary purposes (CDC, 2013). As in many countries, antibiotics

are used both prophylactically and therapeutically in industrialized and commercial free-range poultry farms in South Africa, but not in small-scale family farming. The rates of FQ resistance were highest in commercial free-range broilers, at greater than 95%, but were lower in industrial broiler (16%) and lowest in poultry from family farms (8%) (Samie et al., 2007). In Europe variable resistant rates have been noticed from very low FQ-resistance (1.2%) in broilers in Norway, intermediate rates detected in Belgium and Poland, of 44% and 56%, respectively, to disturbingly high rate in Spain, where it was reported that in 2000, 99% of *Campylobacter* isolates from broilers were resistant to FQ (EFSA & ECDC et al., 2012). The increasing level of FQ-resistant *Campylobacter* in poultry was a sign of increasing FQ resistance in human isolates in the US, therefore in 2004 the Federal Drug Administration (FDA) reversed its prior authorization for the therapeutic use of the veterinary FQ enrofloxacin due to this (Simaluiza et al., 2015). Studies in a number of countries have revealed that FQ-resistant *Campylobacter* persists in poultry populations even after the removal of FQ, signifying that there is little or no fitness cost to FQ-resistance in *Campylobacter* species (Luo et al., 2004; Pollett et al., 2012; Iovine & Blaser, 2004; Nelson et al., 2007; Colles et al., 2016). Resistance to FQ in *Campylobacter* are mediated by two well-described mechanisms which work together synergistically: (1) the target of FQ is inactivated and (2) the efflux of FQ, basically the mechanism is mediated by two intracellular enzymatic targets of FQ which make up DNA gyrase, gyrase subunit A and gyrase subunit B (*gyrA* and *gyrB* genes) and the other enzymatic target is the structurally related topoisomerase IV (encoded by *parC* and *parE*) (Ragimbeau et al. 2014). Decreased DNA replication, transcription, and ultimately cell death is resultant of a stable complex formed by fluoroquinolones with the enzymes, these become trapped on the DNA and affect all these processes. However, some studies have confirmed that *C. jejuni* and *C. coli* lack the *parC* and *parE* genes; therefore, they cannot be a source of FQ resistance (Oporto et al., 2009; Iovine, 2013; Verraes et al., 2013; Chatur et al., 2014).

FQ-resistance in *C. jejuni* and *C. coli* arise via specific point mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene, with the Thr-86-Ile mutation being the most widespread. FQ resistance in *E. coli* or *Salmonella* requires the accumulation of quite a few point mutations in the QRDR before high-level resistance is accomplished, compared to the single mutation in *gyrA* of *Campylobacter* which leads to high-levels of resistance to ciprofloxacin (minimum inhibitory concentration (MIC) >16 g/ml) and therefore only a single point mutation is required for FQ-resistant mutants to emerge rapidly in animals and humans the less common mutation of Thr-86-Ala only confers resistance to nalidixic acid (Feodoroff et al., 2011). In a chicken model it is somewhat astonishing to observe that the Thr-86-Ile mutation in *gyrA* seems to enhance the fitness of *Campylobacter* and these resistant strains persist even after FQ are withdrawn from poultry flocks for more than a few years

(Iovine, 2013; Marinou et al., 2012; Melero et al., 2012; Nobile et al., 2013). The chromosomally encoded CmeABC multidrug efflux pump works in conjunction with *gyrA* mutations, this is resultant of a reduction of the intracellular concentration of a number of antibiotics including FQ (Pumbwe et al., 2004). Strains with DNA gyrase mutations alone lead to intermediate levels of FQ resistance however when CmeABC is also expressed they manifest high-levels of resistance, the CmeABC also assists in the emergence of *gyrA* mutants that otherwise could not endure selection by even low doses of FQ antibiotics. Interestingly, FQ-resistance has appeared on poultry farms even in the nonexistence of FQ administration (Marinou et al., 2012)

## 2.11. Impact of antibiotic usage in animals and fitness of antibiotic resistant *Campylobacter*

The discovery in 1950 that the addition of antibiotics to animal feed at subtherapeutic levels could lead to increased growth rates in husbandry animals resulted in research into methods to improve or stabilize meat supplies to the consumer. Indeed, by the turn of the 20th century, the majority of antibiotic usage in the United States was for agricultural purposes. This approach has led to a dramatic increase in antibiotic resistance in several human pathogens that originate from domesticated animals, including *Campylobacter* species. An increase in antibiotic resistance in *Campylobacter* isolates from poultry is not restricted to *C. jejuni*. Wieczorek & Osek (2013) found that *C. coli* had higher levels of resistance than *C. jejuni*, irrespective of the antimicrobial agents tested, including ciprofloxacin, nalidixic acid, tetracycline, and streptomycin. This increase in antibiotic resistance has also been observed in *Campylobacter* isolates from cattle (Wieczorek & Osek, 2013). While antibiotic use in agriculture is still ordinary in many countries, a number of countries have implemented bans on the nonmedicinal utilization of antimicrobials in livestock. It remains to be seen if such bans will result in a decline in resistance rates in these countries (Chen et al., 2013).

There have been numerous studies which have shown that fluoroquinolone resistant *Campylobacter* mutants which carry mutations in the *gyrA* gene can continue to persist on poultry farms after the withdrawal of fluoroquinolone antibiotics this may be due to the enhanced fitness of the fluoroquinolone resistant *Campylobacter* and these resistant mutants are stably maintained in the absence of antibiotic selection pressure (Nelson et al., 2007; Luangtongkum et al., 2010; Pollett et al., 2012; Chatur et al., 2014; Mäesaar et al., 2015; Colles et al., 2016). A study conducted by Luo et al. (2004) investigated the biological fitness and persistence of fluoroquinolone resistant *Campylobacter* spp. which harbored the Thr-86-Ile mutation in gyrase subunit A in chickens in the absence of antibiotic selection pressure, this was done using *in vivo*-derived clonally-related isolates and genetically defined mutants. The findings of

the study revealed that when fluoroquinolone resistant and fluoroquinolone susceptible strains of *Campylobacter* spp. were individually inoculated into chickens and both strains were able to, with equal efficiency, persist and colonize chickens in the absence of antibiotic usage (Luo et al., 2004). However, when the strains were co-inoculated into chickens, to evaluate competition and fitness, the fluoroquinolone resistant *Campylobacter* strains outcompeted the fluoroquinolone susceptible strains of *Campylobacter* and quickly became the dominant population in the chicken host which indicated that acquisition of FQ resistance mutants carrying the Thr-86-Ile mutation in GyrA enhances the *in vivo* fitness of FQ resistant *Campylobacter* instead of causing a fitness burden. And the prolonged colonization (upto 3 months) *in vivo* did not result in the loss of the resistance phenotype and the reversion/loss of the specific resistance associated mutation in GyrA (Luo et al., 2004). Since there are no compensatory mutations in GyrA and GyrB, which are the targets of FQ antimicrobials in *Campylobacter*, the enhanced fitness likely is likely due to the effect of the C257T mutation in *gyrA* (Luo et al., 2004). Antibiotic resistance in *Campylobacter* spp. is an increasing problem, as it is in many other microorganisms.

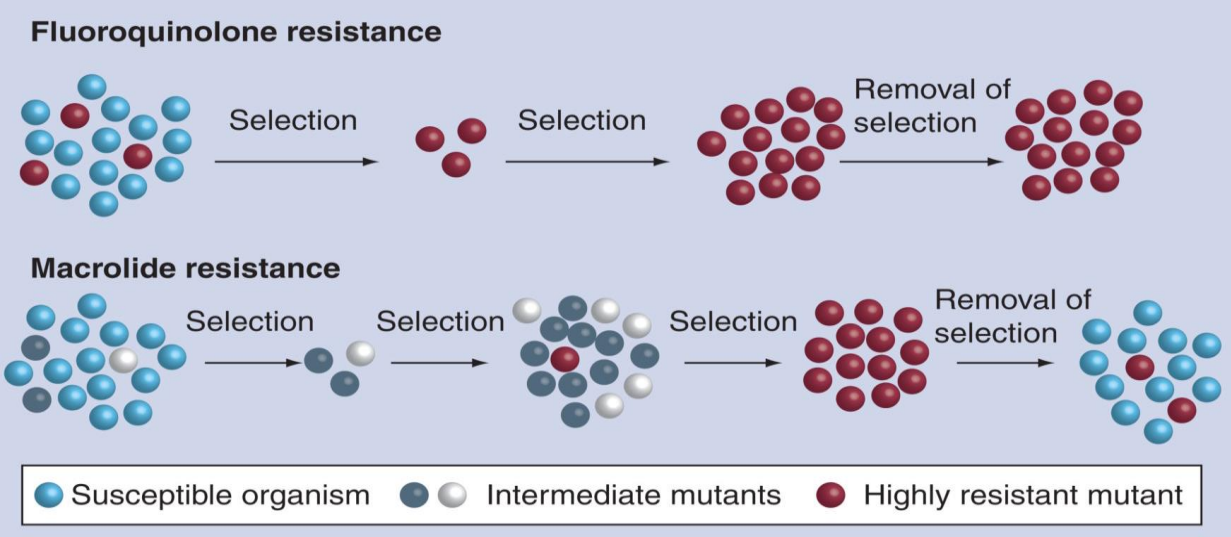


**Figure 2.10:** Model for the development and fitness of fluoroquinolone and macrolide resistance in *Campylobacter* (Luangtongkum et al., 2010).

Due to *Campylobacter*'s natural fitness and hypervariable genomic sequences, there is substantial genomic flexibility that supports the emergence of resistant mutants. *Campylobacter* is a commensal of many animal species which are exposed to veterinary antimicrobials; sufficient opportunities exist for *Campylobacter* to continue to evolve additional resistance mechanisms (Zhou et al., 2013). Furthermore, the over-use of antibiotics in the human population is an additional significant source of selective pressure. In this regard, the lack of a fitness cost of FQ-resistant *C. jejuni* is an issue that must be remembered when future resistance mutations arise in *C. jejuni* against other antimicrobials. Of greatest

clinical concern would be the emergence of widespread macrolide resistance, since this class is the current treatment of choice for campylobacteriosis. A better understanding of the mechanism of macrolide entry (possibly via the hydrophobic pathway) may be useful in eventually mitigating the impact of acquired macrolide resistance (Zhou et al., 2013). Also, the contribution of efflux pumps to antibiotic resistance necessitates further study, since this mechanism acts synergistically with other mechanisms of antibiotic resistance to confer high-levels of resistance in many instances. Furthermore, genome sequencing predicts 14 potential efflux pumps but only CmeABC and CmeG have been studied functionally, making this an abundant area for future research (Zhou et al., 2013).

## 2.12. Conclusion

Against this background this study was aimed to dectect the prevalence of several genes associated with virulence, cytotoxin production, adherence and invasion as well as detection of resistance genes which play significant roles in *C.jejuni* and *C.coli* resistance to antimicrobials such as ciprofloxacin, ampicillin and tetracycline. Furthermore, it was also aimed to evaluate if there is a statistically significant correlation amongst antimicrobial resistance and virulence genes isolated from both chicken and human clinical samples.

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# CHAPTER 3

## Prevalence of virulence genes related to adherence, invasion, and cytotoxicity in *Campylobacter* spp. isolated from commercial chickens and human clinical cases from South Africa


### 3.1. ABSTRACT

The pathogenicity of *Campylobacter* spp. depends on virulence factors which differ amongst strains of different origins. Several virulence-associated genes have been recognized and detected in *Campylobacter* spp., and the majority of them are associated with pathogenicity. The information regarding virulence genes and the prevalent genotypes of *Campylobacter* spp. is unclear in developing countries such as South Africa. This study aimed to detect the presence of genes associated with pathogenicity and responsible for invasion, expression of adherence, colonization and production of the cytolethal distending toxin (*cdt*) in *C. jejuni* and *C. coli*. Following ethical approval, 100 commercial chicken fecal samples were randomly collected from chicken farms within the Durban Metropolitan area in South Africa. Furthermore, 100 human clinical *Campylobacter* spp. isolates were randomly sampled from a collection which originated from a private pathology laboratory in South Africa. These samples were screened for the presence of the following virulence genes: *cadF, hipO, asp, ciaB, dnaJ, pldA, cdtA, cdtB* and *cdtC*. As expected the *cadF* gene was present in 100% of poultry and human clinical isolates. *C. jejuni* was the main species detected in both poultry and human clinical isolates whilst *C. coli* were detected at a significantly lower percentage ($p<0.05$). Eight percent of the *C. jejuni* from human clinical isolates had all virulence genes that were screened. Only one *C. coli* isolate demonstrated presence of all the virulence genes investigated, however, the *pldA* virulence gene was detected in 100% of the *C. coli* isolates in poultry and a high percentage (71%) in human clinical *C. coli* isolates as well. The incidence of *cdt* genes were detected at higher frequency in poultry than human clinical isolates. This could indicate that the presence of these genes are needed for successful infection in a host. The high prevalence rates of virulence genes detected in poultry and human clinical isolates, demonstrate the significance in the pathogenicity of *Campylobacter* spp. The high frequencies of the virulence genes can also explain the high prevalence of *Campylobacter* spp. in chickens and humans.


**Key words:** Adherence; expression; food borne pathogen; gastroenteritis; invasion; pathogenicity; toxin; virulence

## 3.2. Introduction

Campylobacteriosis is a significant public health concern worldwide with most infection cases in humans caused by *Campylobacter jejuni* subsp. *jejuni* (from here on referred to as *C. jejuni*) and *Campylobacter coli* (Zhao et al., 2010). Sources of infection of *Campylobacter* spp. in humans include house flies, migratory birds, wildlife, companion animals, livestock, milk, water and other environmental sources (Epps et al., 2013). However, poultry and retail meat products have been extensively reported and implicated as the major sources of infection in human cases of campylobacteriosis (Komba et al., 2013). *C. jejuni* and *C. coli* have continuously been the focus of study within the genus compared to other *Campylobacter* species (Iraola et al., 2014). Research over the years has demonstrated that *C. jejuni* is the primary cause of approximately 80-95% of *Campylobacter* infection in human cases and the remaining cause is usually due to *C. coli* (Ragimbeau et al., 2014). A range of gastrointestinal conditions such as colorectal cancer, inflammatory bowel disease (IBD) and Barrett's esophagus in humans have been associated with infection by *Campylobacter* species. Studies have also reported *Campylobacter* spp. in cases of extra-gastrointestinal manifestations such as reactive arthritis, bacteremia, brain abscesses and lung infections ( Swartz, 2002; Nielsen et al., 2010; Broaders et al., 2013; Iraola et al., 2014). However, the specific role of *Campylobacter* species in the development of all these clinical manifestations remains unclear and further investigation is required (Kaakoush et al., 2015). The epidemiology of *Campylobacter* is complex due to the wide distribution of the bacterium, its genetic variability and its interaction with the host (Khoshbakht et al., 2014). These factors regarding *Campylobacter* spp. has made studying the pathogenicity of this organism particularly challenging (Caro-Quintero et al., 2009).

Campylobacteriosis is mainly confined to the very young, usually children under the age of five years or to elderly patients and patients suffering with immunocompromising diseases (Silva et al., 2011). This presents as a major threat especially in South Africa due to the extremely high number of individuals that are infected with immune-compromising diseases such as HIV/AIDS (Kaakoush et al., 2015). Patients infected with *C. jejuni* or *C. coli* or both, experience severe watery or bloody diarrhea, extremely high fevers, major weight loss, and severe abdominal cramps which lasts on average for about 6 days. Symptoms usually begin in the period between 24 to 72 hours after ingestion of the bacterium depending on the dosage of the organism present in the contaminated food or liquid ingested (Zaidi et al., 2012). Avian species are important reservoirs for the transmission of *Campylobacter* species and their high body temperature provides an optimal growth for the organism (Noormohamed & Fakhr, 2014). *Campylobacter* are able to colonize the caecum of chicken in extremely high numbers of up to $10^9$ CFU/g of fecal matter, even though this pathogen is present in such high quantities the chicken rarely show symptoms of disease (Thibodeau et al., 2015). The illness in humans can last for a few days, depending

on the individual and the abdominal pain is usually mistaken for appendicitis. Understanding the molecular basis of the virulence associated with disease and knowledge of the genetic diversity amongst the different strains of *Campylobacter* is of great importance in the control of diseases syndromes associated with this organism (Fonseca et al., 2014). Studies have also revealed that infections due to *C. jejuni* and *C. coli* are more common during the summer months of the year (Garénaux et al., 2008; Chansiripornchai & Sasipreeyajan, 2009; Hermans et al., 2011; Fonseca et al., 2014; Garvis et al.,2016a). Although *C. coli* is less prevalent than *C. jejuni* in many geographic regions, *C. coli* infections is responsible for as many as 25% of all gastroenteritis clinical cases caused by *Campylobacter* species (Kaakoush et al., 2015). Gastroenteritis induced by *C. coli* is clinically impossible to differentiate from that by *C. jejuni* due to these organisms being fastidious in nature and their need for nutrient rich-based medium as well as anaerobic conditions for growth. This is the main reason *Campylobacter* spp. are not applied in routine diagnostic programs of clinical laboratories in most developing countries (Ghorbanalizadgan et al., 2014). The majority of *Campylobacter* infections are sporadic, and unlike for other food-borne pathogens, huge outbreaks are not very typical. Nevertheless, it is likely, that outbreaks or small case clusters occur far more frequently than previously suggested, due to cases of *Campylobacter* infection going unreported the true statistics fail to become recognized (Taboada et al., 2013).

Successful invasion and organization in host cells depends on the adhesin- and fibronectin-binding protein which is the product of the *cadF* gene, this gene plays a significant role during the infection process of *Campylobacter* binding to extracellular matrix of human intestinal cells (Khoshbakht et al., 2014). The presence of the *ciaB* gene plays a significant role due to the secretion of a *CiaB*, 73kDa protein which is important for the invasion of epithelial cells as well as colonization of intestines of avian species (Garvis et al., 2016b), *CiaB* proteins are also secreted in the presence of poultry serum and mucus (Biswas et al., 2011). The *pldA* gene is also related to cell invasion and is responsible for the synthesis of an outer membrane phospholipase which is important for caecal colonization (O Cróinín & Backert, 2012). The *dnaJ* virulence gene enables *Campylobacter* species to cope with diverse physiological stresses and is also considered to be a chaperone protein(Chansiripornchai & Sasipreeyajan, 2009), the *ciaB, pldA* and *dnaJ* which are recognized as heat shock protein genes are important for caecal colonization but mutations in these genes limit ability to achieve this (Jakee et al., 2015). The cytolethal distending toxin genes, *cdtA, cdtB*, and *cdtC* form a polycistronic *cdt* operon which are responsible for the expression of cytotoxins and lethal for host enterocytes (Carvalho et al., 2013). Against this background, this study aimed to isolate and identify *C. jejuni* and *C. coli* from chicken feces as well and human clinical isolates as well as to determine the prevalence of virulence genes related to adherence, invasion, and cytotoxicity.

## 3.3. Materials and Methods

### 3.3.1 Origin of samples and processing procedures

Human and animal studies have been approved by the appropriate ethics committee of the University of KwaZulu-Natal therefore; they have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments (REF:BE084/14).

A total of 100 human *Campylobacter* isolates cryopreserved in Brucella broth (Oxoid) with 15% glycerol were randomly sampled from a collection that was received from a private laboratory in Durban, KwaZulu-Natal during the year 2014. Cultures were revived on modified charcoal cefoperazone deoxycholate (mCCDA) agar (Oxoid, England) containing *Campylobacter* selective supplement SR0155 (Oxoid, England) A sterile loop was then streaked across the area of inoculation several times to achieve isolated colonies, then plates were incubated at 37°C for 48 hours under microaerobic conditions created by CampyGen (Oxoid, UK) gas generating packs in an anaerobic jar. A total of 100 chicken fecal samples were randomly collected between March and September 2016 from commercial farms within the Durban metropolitan area. Fresh broiler feces were aseptically collected with sterile swabs then directly inoculated into charcoal broth (Sigma-Aldrich, USA) and then transported to the laboratory for incubation at 37°C for 48 hours, under microaerophilic conditions (5% $O_2$, 10% $CO_2$ and 85% $N_2$) created by CampyGen (Oxoid, UK) gas generating packs in an anaerobic jar.

Template DNA for PCR was extracted via the conventional boiling method. In summary, characteristic colonies of *Campylobacter* spp. were isolated from plates and suspended in 300 µl TE buffer then vortexed for homogenization of cells. Suspensions were boiled at 100°C for 10 minutes then immediately cooled on ice. After centrifugation at 14,000 x g for 5 min, supernatants were transferred to a new tube and stored at -20°C until use in PCR (Datta et al., 2003). A positive *Campylobacter* spp. control was also prepared by isolating DNA from a reference strain of *C. jejuni* ATCC 29428 which was incubated under the same conditions and subjected to the same DNA extraction methods. The Thermo Scientific Nanodrop 2000, UV-Vis Spectrophotometer (USA) was used to check the concentration and quality of the isolated DNA.

### 3.3.2 Detection of virulence genes using PCR

PCR was performed on the DNA extracted from all samples which demonstrated positive *Campylobacter* growth. In order to differentiate between the species responsible for infection in patient samples as well as in poultry two species specific genes were used. The *hipO* gene region which is the hippuricase gene specific for *C. jejuni* (Marinou et al., 2012) and the *asp* gene region, the aspartokinase gene specific for *C.*

*coli* (Al Amri et al., 2013). The PCR (polymerase chain reaction) was used to detect nine virulence genes in the total DNA of *Campylobacter* isolates: *cadF, hipO, asp, dnaJ, ciaB, pldA, cdtA, cdtB* and *cdtC*. PCR primers were sourced from Inqaba Biotechnologies, South Africa, forward and reverse primers specific for the virulence genes under investigation were designed based on the gene sequence information in the GenBank database and in previously published studies (Al Amri et al., 2007; Chansiripornchai & Sasipreeyajan, 2009; Rizal et al., 2010). Target genes, primer sequences, product sizes and annealing temperatures are demonstrated in Table 3.1.

PCRs were carried out in the BIO-RAD, T100™ Thermal Cycler (Singapore) for a 25µl reaction using ThermoScientific DreamTaq Green PCR Master Mix (2X), A total of 12.5µl DreamTaq Green PCR Master Mix was used, with 1.5µl of each primer, 5µl template DNA and 4.5µl nuclease-free water making a total volume of 25µl. The amplification conditions for *cadF, hipO, asp, dnaJ, ciaB, pldA* consisted of an initial denaturalization at 95°C for 3 minutes, 45 cycles at 94°C for 30 seconds, specific Tm for each primer for 30 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes. The *cdt* genes (*cdtA, cdtB* and *cdtC*) were run using different amplification conditions according to Rizal et al., (2010), the conditions consisted of an initial denaturalization at 94°C for 15 minutes, 45 cycles at 94°C for 1 minute, specific Tm for each *cdt* primer for 1 minute, and 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes. PCR products were then electrophoresed on a 1.5% agarose gel run at 60V for 60 minutes, stained with ethidium bromide and then visualized using the BIO-RAD, ChemiDoc™ MP Imaging System.

### 3.3.3 Statistical analyses

Virulence genes detected in *C. jejuni* and *C. coli* were analyzed using IBM SPSS Statistics (version 23). Pearson's Correlation analysis, Fisher's Exact tests, Chi Square tests and Logistic Regression analysis were implemented to evaluate the relationship between the different PCR results obtained and the significance of the presence of virulence genes detected in both human and poultry samples. Every model included the presence/ absence of each virulence gene (0 = absent; 1 = present) and associations were considered significant when the P value was less than 0.05.

**Table 3.1:** Target virulence genes, primer sequences, amplicon sizes and annealing temperatures

| **Target gene** | **Primer Sequence (5' – 3')** | **Product Size (bp)** | **Annealing Temperature (°C)** | **References** |
|---|---|---|---|---|
| *cadF* | F-TTGAAGGTAATTTAGATATG | 400 | 43 | (Chansiripornchai& |
| | R-CTAATACCTAAAGTTGAAAC | | | Sasipreeyajan, 2009) |
| *asp* | F-GGTATGATTTCTACAAAGCGAGA | 500 | 53 | (Al Amri et al., 2007) |
| | R-ATAAAAGACTATCGTCGCGTG | | | |
| *hipO* | F-GAAGAGGGTTTGGGTGGT | 735 | 53 | (Al Amri et al., 2007) |
| | R-AGCTAGCTTCGCATAATAACTTG | | | |
| *ciaB* | F-TGCGAGATTTTTCGAGAATG | 527 | 54 | (Chansiripornchai& |
| | R-TGCCCGCCTTAGAACTTACA | | | Sasipreeyajan, 2009) |
| *dnaJ* | F-ATTGATTTTGCTGCGGGTAG | 177 | 50 | (Chansiripornchai& |
| | R-ATCCGCAAAAGCTTCAAAAA | | | Sasipreeyajan, 2009) |
| *pldA* | F-AAGAGTGAGGCGAAATTCCA | 385 | 46 | (Chansiripornchai& |
| | R-GCAAGATGGCAGGATTATCA | | | Sasipreeyajan, 2009) |
| *cdtA* | F-CCTTGTGATGCAAGCAATC | 370 | 49 | (Rizal et al., 2010) |
| | R-ACACTCCATTTGCTTTCTG | | | |
| *cdtB* | F-GTTAAAATCCCCTGCTATCAACCA | 495 | 51 | (Rizal et al., 2010) |
| | R-GTTGGCACTTGGAATTTGCAAGGC | | | |
| *cdtC* | F-CGATGAGTTAAAACAAAAAGATA | 182 | 48 | (Rizal et al., 2010) |
| | R-TTGGCATTATAGAAAATACAGTT | | | |

### 3.4. Results

#### 3.4.1 Prevalence of virulence genes

Virulence genes investigated in this study are shown in Figure 3.1 and species differentiation was confirmed by detection of *hip*O (Lane 1:735bp) and *asp* gene (Lane 2: 500bp). *C. jejuni* is the only species known to have the hippuricase gene (*hipO*) and it has not been detected in any other *Campylobacter* species and the aspartokinase gene (*asp*) for *C. coli* is specific for this species of *Campylobacter*. All isolates (100%), irrespective of the species were positive for the *cad*F gene based on PCR detection of a 400bp amplicon (Lane 3). This gene encodes *Campylobacter* species adhesion to fibronectin which is an important virulence factor for colonization of epithelial cells. The *dnaJ* gene is depicted in Lane 4 at 177bp and Lane 5 is the virulence gene *pldA* with an amplicon of 385bp, Lane 6 is *ciaB* at 527bp, Lane 7 is *cdtA* (370bp), Lane 8 is *cdtB*(495bp) which is a catalytic subunit in the CDT cluster and lastly Lane 9 is *cdtC* (182bp), *cdtA*, and *cdtC* are binding proteins responsible for delivering *cdtB* into target cells.

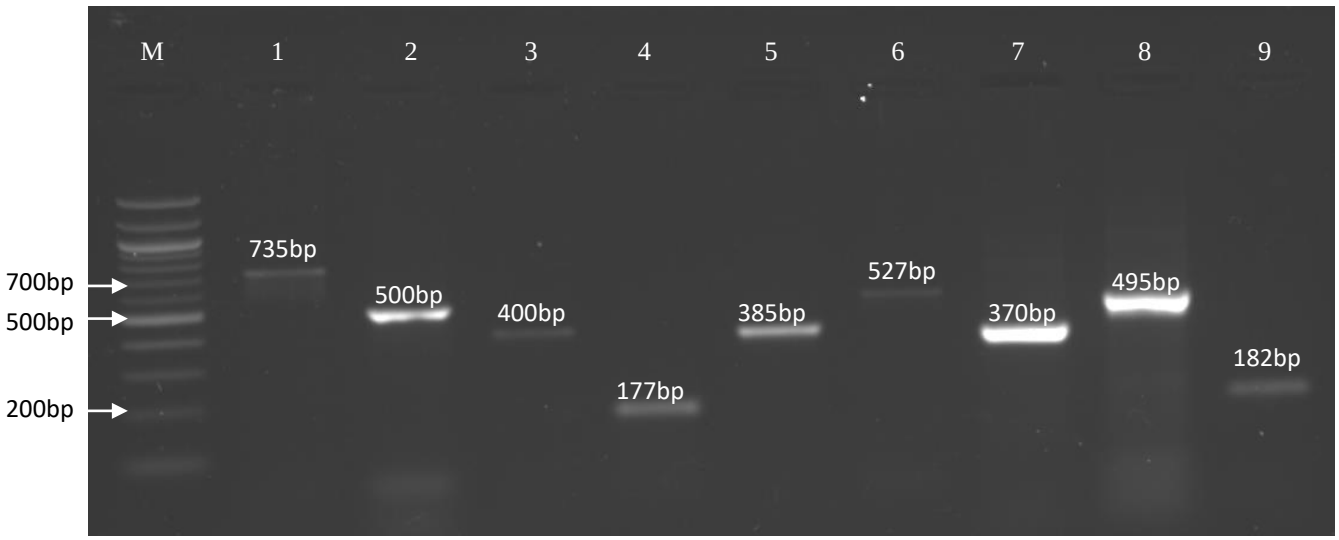


**Figure 3.1:** Representative gel of species identification and virulence genes investigated from *Campylobacter* spp. Lanes: M, 100-bp marker; 1, *hipO*; 2, *asp*; 3, *cadF*; 4, *dnaJ*; 5, *pldA*; 6, *ciaB*; 7, *cdtA*; 8, *cdtB*; and 9, *cdtC*.

Figure 3.2 demonstrates the prevalence of virulence genes found in human and chicken from *Campylobacter* species isolated (*C. jejuni* and *C. coli*). There were statistically significant differences ($p<0.05$) observed between the percentage of virulence genes found in chicken compared to the percentage observed in human clinical isolates.

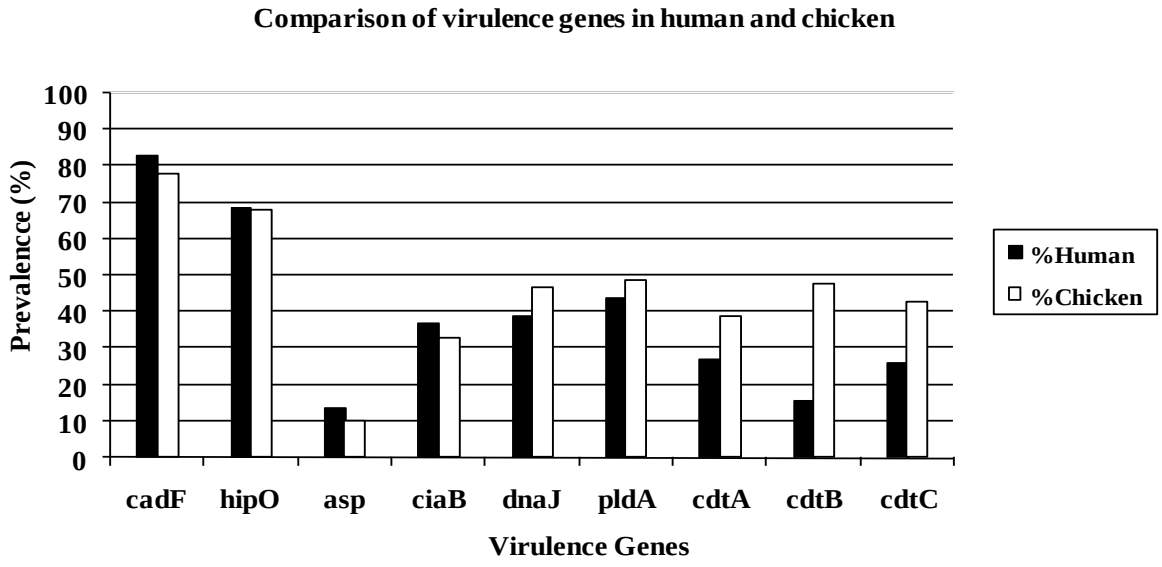


**Figure 3.2:** Prevalence of virulence genes in *Campylobacter* spp. inhuman clinical isolates and chicken feces.

Results in Figure 3.3 indicate that *C. jejuni* is responsible for the majority of infection (83%) in human gastroenteritis cases compared to the low incidence of *C. coli* found only in 17% of cases. Of the *C. jejuni* isolated from human, 45%, 46%, 49%, 33%, 20% and 30% were positive for *ciaB, dnaJ, pldA, cdtA, cdtB* and *cdtC*, respectively, and *C. coli* revealed 43%, 50%, 71%, 29%, 14% and 36%, respectively.

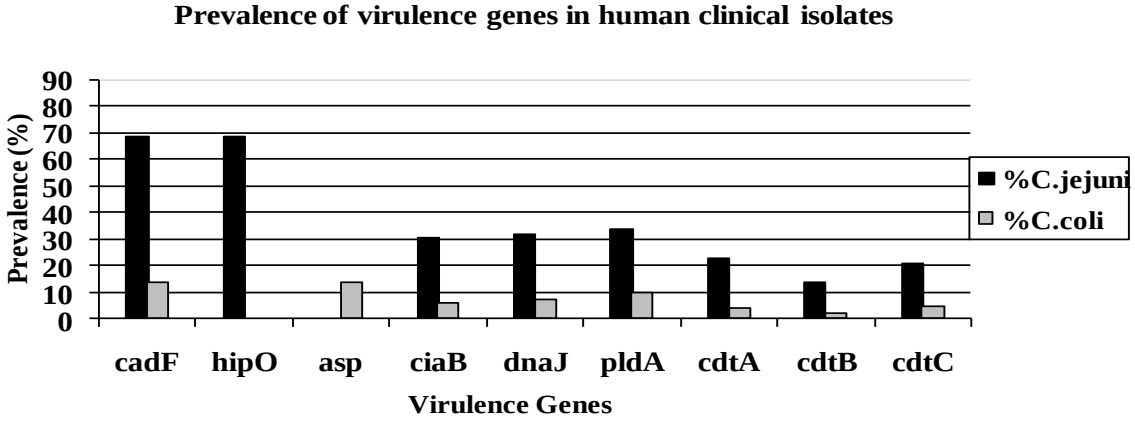


**Figure 3.3:** Prevalence of *C. jejuni* and *C. coli*, isolated fromhuman clinical isolates.

Isolates from chicken feces (Figure 3.4) indicate a high percentage of *C. jejuni* (87%) present as compared to only 13% of *C. coli*. The poultry isolates for *C. jejuni* demonstrated 47%, 59%, 57%, 56%, 63% and 56% presence of *ciaB, dnaJ, pldA, cdtA, cdtB* and *cdtC*, respectively, *C. coli* isolates revealed 10%, 70%, 100%, 10%, 50% and 50% for the presence of *ciaB, dnaJ, pldA, cdtA, cdtB* and *cdtC*, respectively.

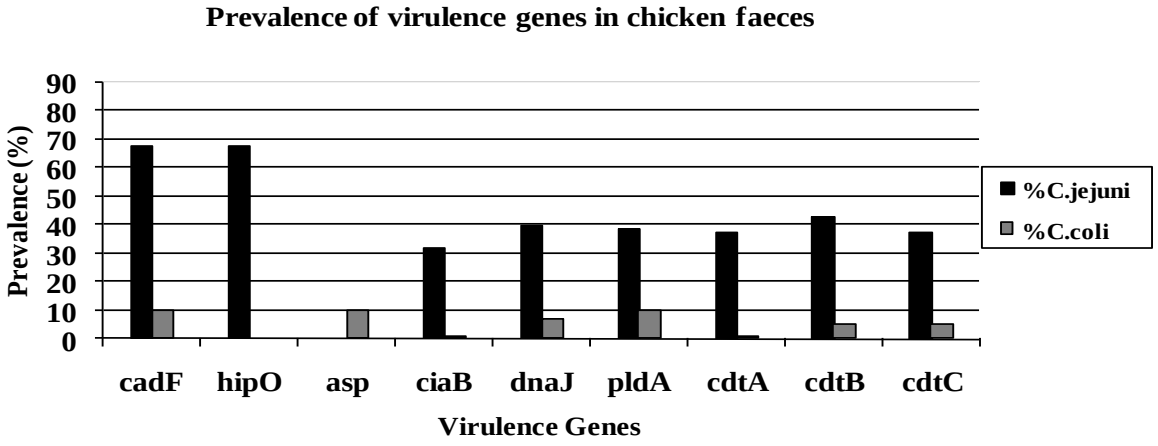


**Figure 3.4:** Prevalence of virulence genes in *C. jejuni* and *C. coli*, isolated from chicken faeces.

### 3.4.2 Statistical Outcome

Pearson correlations (Table 3.2) demonstrated significant ($P<0.05$) positive correlations between some virulence. The *asp* gene which identifies *C. coli* was the only gene which was not statistically significantly ($P>0.05$) correlated with some of the virulence genes. Results demonstrated strong correlation between the virulence genes. The presence of the *dnaJ* and *cdtC* gene was strongly correlated ($p<0.05$) with the presence of the *hipO* gene indicating that if the species is confirmed as *C. jejuni* by the *hipO* gene there is a high probability the *dnaJ* gene and *cdtC* genes will be present. Evidence of a positive correlation also exists between the *ciaB* gene and the *dnaJ* gene ($p<0.05$).

**Table 3.2:** Comparison of Pearson Correlations for virulence genes detected in *Campylobacter* species from human clinical isolates and chicken feces

| Genes | | *cadF* | *hipO* | *asp* | *ciaB* | *dnaJ* | *pldA* | *cdtA* | *cdtB* | *cdtC* |
|---|---|---|---|---|---|---|---|---|---|---|
| *cadF* | Pearson Correlation | 1 | .630** | .141 | .286** | .342** | .369** | .272** | .266** | .282** |
| | Sig. (2-tailed) | | .000 | .058 | .000 | .000 | .000 | .000 | .000 | .000 |
| *hipO* | Pearson Correlation | .630** | 1 | -.680** | .270** | .185* | .076 | .300** | .235** | .185* |
| | Sig. (2-tailed) | .000 | | .000 | .000 | .012 | .306 | .000 | .001 | .012 |
| *asp* | Pearson Correlation | .141 | -.680** | 1 | -.074 | .087 | .251** | -.125 | -.049 | .030 |
| | Sig. (2-tailed) | .058 | .000 | | .318 | .246 | .001 | .092 | .512 | .686 |
| *ciaB* | Pearson Correlation | .286** | .270** | -.074 | 1 | .157* | .209** | .484** | .340** | .406** |
| | Sig. (2-tailed) | .000 | .000 | .318 | | .035 | .005 | .000 | .000 | .000 |
| *dnaJ* | Pearson Correlation | .342** | .185* | .087 | .157* | 1 | .442** | .339** | .340** | .327** |
| | Sig. (2-tailed) | .000 | .012 | .246 | .035 | | .000 | .000 | .000 | .000 |
| *pldA* | Pearson Correlation | .369** | .076 | .251** | .209** | .442** | 1 | .349** | .329** | .334** |
| | Sig. (2-tailed) | .000 | .306 | .001 | .005 | .000 | | .000 | .000 | .000 |
| *cdtA* | Pearson Correlation | .272** | .300** | -.125 | .484** | .339** | .349** | 1 | .354** | .400** |
| | Sig. (2-tailed) | .000 | .000 | .092 | .000 | .000 | .000 | | .000 | .000 |
| *cdtB* | Pearson Correlation | .266** | .235** | -.049 | .340** | .340** | .329** | .354** | 1 | .421** |
| | Sig. (2-tailed) | .000 | .001 | .512 | .000 | .000 | .000 | .000 | | .000 |
| *cdtC* | Pearson Correlation | .282** | .185* | .030 | .406** | .327** | .334** | .400** | .421** | 1 |
| | Sig. (2-tailed) | .000 | .012 | .686 | .000 | .000 | .000 | .000 | .000 | |

**Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

Results in Table 3.3 indicate that there is a significant relationship between *cadF, hipO, dnaJ, pldA, cdtA, cdtB* and *cdtC* due to the presence of these genes in human and poultry samples investigated ($p<0.05$). The virulence genes *asp* and *ciaB* were not statistically significant from the results observed because the results demonstrated a P value greater than 0.05, for Chi-square and Fisher's Exact statistical tests when compared to the other genes investigated.

**Table 3.3**: Chi-Square and Fisher's Exact for virulence genes investigated

| | **Asymptotic Significance (2-sided)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *cadF* | *hipO* | *asp* | *ciaB* | *dnaJ* | *pldA* | *cdtA* | *cdtB* | *cdtC* |
| Pearson Chi-Square | .011 | .030 | .720 | .654 | .014 | .034 | .004 | .000 | .000 |
| Fisher's Exact Test | .011 | .038 | .827 | .760 | .017 | .038 | .005 | .000 | .000 |

A logistic regression analysis (Table 3.4) was conducted to predict the presence of virulence genes in chicken and human clinical isolates using the source of the isolates as a predictor. A test of the full model against a constant only model indicated that genes *ciaB, dnaJ* and *pldA* were not statistically significant (p>0.05). The Wald criterion demonstrated that *cdtA*, *cdtB* and *cdtC* virulence genes made a significant contribution to prediction of the presence of these genes in human clinical isolates and poultry with p = 0.025, 0.000 and 0.003 respectively (p<0.05).

**Table 3.4:** Logistic Regression analysis results demonstrating the significance of virulence genes found in human clinical isolates and chicken feces

| **Virulence genes** | **-2 Log Likelihood** | ***B*** | ***SE*** | **Wald** ***p Value*** | ***OR*** | ***95% C.I.*** |
|---|---|---|---|---|---|---|
| *ciaB* | 220.362 | 0.092 | 0.318 | .771 | 1.097 | 0.588 – 2.047 |
| *dnaJ* | 219.587 | - 0.537 | 0.319 | .093 | 0.585 | 0.313 – 1.093 |
| *pldA* | 217.706 | - 0.404 | 0.321 | .209 | 0.668 | 0.356 – 1.253 |
| *cdtA* | 212.844 | - 0.730 | 0.326 | .025 | 0.482 | 0.255 – 0.913 |
| *cdtB* | 185.315 | - 1.902 | 0.363 | .000 | 0.149 | 0.073 – 0.304 |
| *cdtC* | 210.508 | -0.991 | 0.328 | .003 | 0.371 | 0.195 – 0.707 |

### 3.5. Discussion

All isolates that were presumptively identified as *Campylobacter*, irrespective of the bacterial species, resulted positive for the *cadF* gene which facilitates adherence to fibronectin in contact regions (Colonization et al., 2009). These findings are in agreement with earlier studies with regard to the presence of the *cadF* gene in *Campylobacter* spp. isolated from human as well as chicken (Datta et al., 2003; Rozynek et al., 2005; Al Amri et al., 2008; Garvis et al., 2016). The presence of gene products from *cadF* (*Campylobacter* adhesin to fibronectin) has clearly demonstrated involvement in *Campylobacter* colonization and this has been shown by *in vivo* colonization using a chicken model (Thibodeau et al., 2015). This gene encodes a protein that interacts with a host extracellular matrix protein fibronectin, and is required for *Campylobacter* adherence to and colonization of the host cell surface (Ghorbanalizadgan et al., 2014). The high prevalence (100%) of the *cadF* gene in the present study demonstrates that many isolates originating from poultry faeces have pathogenic potential properties for humans. The ubiquitous existence of the highly conserved *cadF* gene in 100% of *Campylobacter* spp. was previously reported by Konkel et al. (1999) and was subsequently used by other investigators for successful detection of *Campylobacter* spp. (Rizal et al., 2010;Wieczorek & Osek, 2013).

The hippuricase gene (*hipO*) is specific for *C. jejuni* and was not detected in any other *Campylobacter* species. The correlation analysis demonstrated that there was a significant ($p<0.05$) strong positive correlation (63%) between the *cadF* and the *hipO* genes. Our study utilized the positive PCR amplification of *asp* gene in order to identify which samples belonged to the species *C. coli*. Furthermore, the correlation analysis indicated that the presence of the *asp* gene was negatively correlated (-0.63%) to the presence of the *hipO* gene ($p<0.05$). This is in agreement with other studies as *C. coli* is not as prevalent as *C. jejuni* as the cause of infection in humans (Samie et al., 2007; Feodoroff et al., 2011; Khoshbakht et al., 2013).

Many factors play significant roles in the varying isolation rates of *Campylobacter* species; these include whether samples are fresh or frozen, the type of sampling procedure used and the isolation protocol followed as well as the time of year for collection of samples. The *ciaB* (*Campylobacter* invasive antigen B) gene is known to be involved in the translocation of *Campylobacter* into host cells for the purpose of host cell invasion and also plays a significant role in caecal colonization in chicken (O Cróinín & Backert, 2012). The *ciaB* gene was detected in 45% and 42% in human and chicken samples respectively and had a significant low positive correlation (15.7%) with the *dnaJ* gene ($p<0.05$). With regard to the *dnaJ* gene which enables *Campylobacter* species to cope with diverse physiological stresses, there was a significant positive correlation when there was presence of the *hipO* and *ciaB* gene found in samples ($p<0.05$).

Another important factor for colonization of *Campylobacter* species in the intestine of chickens is the *pldA* gene (an outer membrane phospholipase A) which encodes proteins associated with increased bacterial invasion on cultured epithelial cells (Ghorbanalizadgan et al., 2014). The distribution of this gene was dissimilar among the two species investigated as *C. jejuni* showed a higher presence of this gene compared to *C. coli* in both human and chicken isolates. The percentage of *pld*A in chicken and human isolates was 53% and 63%, respectively. Research conducted by Datta et al. (2009) demonstrated enhancement in the presence of the *pldA* gene in chicken *C. jejuni* isolates ranging from 88% to 100% with age of broilers playing a major role. The results from this study indicated that *Campylobacter* spp. recovered from chicken faeces may have potential virulence consequences in humans. The occurrence *C. jejuni* and *C. coli* in chicken faecal samples from the slaughterhouse indicate a public health hazard due to this emerging food borne pathogen. The contamination of carcasses may occur from intestinal contents during slaughtering and or post slaughtering processes and if these products are not cleaned, stored or cooked properly, this could lead to outbreaks of infection in the public. Damage to nuclear DNA resulting in the inhibition of the cell cycle in G2 or M phase is the cytopathic effect of the cytotoxin released by *cdtA*, *cdtB* and *cdtC* (Carvalho et al., 2013).

The low percentage of the *cdtB* gene in humans found in this study (19%) is in agreement with a study conducted in India (Rizal et al., 2010) which reported a prevalence of 28%. However, chickens also demonstrated a low percentage of this gene (20%) in contrast to this study which found 62% which could be due to genetic factors or variation in isolates from different geographic areas. Translocation of *cdtB* to the nucleus of cells induces the genotoxic effects on host DNA; triggering DNA repair mechanisms that could lead to cell cycle arrest and eventually cause cell death (Ge et al., 2008). In addition it has also been suggested that CDT could also play a role in adhesion and invasion (Ge et al., 2008). Cell invasion could result in cellular injury, leading to reduced absorptive capacity of the intestine, whereas *cdt* production is important for interleukin-8 (IL-8) release by intestinal cells *in vitro* which plays an important role in the host mucosal inflammatory response caused by *Campylobacter* species (Hermans et al., 2011). Invasion of epithelial cells and *cdt* production are important bacterial virulence mechanisms that play a significant role for inducing enterocolitis. The presence of a single *cdt* gene does not have any effect on the bacterium virulence, however, if all three *cdt* genes are present together in a cluster then they are responsible for the release of a functional cytotoxin (Lapierre et al., 2016). Our study revealed that ten of the human clinical isolates had all three *cdt* genes compared to eighteen of the chicken isolates. The low percentage of *cdt* genes needs to be compared with cytolethal distending toxin production levels and tested on a variety of different cell lines in order to study the phenotypic characteristics of the isolates for a better understanding (Wieczorek et al., 2012).

### 3.6. Conclusion

The current study revealed that isolates of *C. jejuni* and *C. coli* from chicken and human clinical cases possess different virulence genes associated with invasion, expression of adherence, colonization and production of the cytolethal distending toxin. This demonstrates the pathogenic potential of isolates and this is a major public health concern. This necessitates the need for proper preventive measures to prevent contamination of food with *Campylobacter* spp. at the food production level in South Africa. This is the first study emanating from South Africa in which the virulence genes in Campylobacter spp. have been screened in both human and animal samples. Future studies should endeavor to ascertain the correlation between these virulence and antimicrobial resistance genes.

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# CHAPTER 4

## Detection and prevalence of antimicrobial resistance genes related to ciprofloxacin, ampicillin and tetracycline resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from chickens and human clinical cases in South Africa


### 4.1. Abstract

The indiscriminate use of antibiotics for treatment of human infection caused by a number of pathogens including *Campylobacter* spp. as well as in situations when they are used for growth-promotion purposes as opposed to therapeutic purposes in animal husbandry, has led to an increase in antibiotic-resistant *Campylobacter* infections. This study was conducted to investigate the prevalence of antibiotic resistance genes in *Campylobacter* spp. from chicken and human clinical isolates in South Africa; it is hypothesized that *gyrA*(235bp), *gyrA*(270bp), $bla_{OXA-61}$ and *tetO* resistance genes are present in both sources being tested, due to the misuse of antibiotics in human and chickens. A total of a 161 isolates of *Campylobacter jejuni* and *Campylobacter coli* were obtained from chicken fecal samples and human clinical samples following ethical approval and screened for the presence of antimicrobial resistance genes. We observed a wide distribution of the antimicrobial resistance gene *tetO* which confers resistance to the antibiotic tetracycline, *gyr*A genes in the quinolone resistance-determining region (QRDR) which is related to varying levels of ciprofloxacin resistance and in some cases naladixic acid resistance as well as $bla_{OXA-61}$ linked to ampicillin resistance. Our findings revealed that there was a higher ($P<0.05$) prevalence of antimicrobial resistance genes in chicken faeces compared to human clinical isolates. The *tetO* gene was the most prevalent resistance gene detected, this gene was isolated at an alarming percentage of 64% and 68% from human clinical isolates and chicken isolates, respectively. The *tetO* gene is most likely due to the transfer of plasmids carrying this gene between isolates for resistance to tetracycline. Chi- square statistical analysis indicated that *gyrA* made a significant contribution to the prediction of the presence of genes in human clinical isolates and poultry ($p<0.05$). In conclusion, it is difficult to distinguish an association between the presence of antimicrobial resistance genes in *Campylobacter* spp. and how they affect the fitness sof the pathogen as there are many factors and characteristics to consider at the genotypic and phenotypic level. The prevalence and combinations of various antimicrobial resistance genes isolated from poultry and human clinical samples in South Africa indicate that *Campylobacter* spp. possess the potential of resistance to a number of antibiotic classes.


**Key words:** Antimicrobial; quinolone resistance-determining region (QRDR); resistance; ampicillin; tetracycline; ciprofloxacin

**4.2. Introduction**

*Campylobacter* spp. (especially *Campylobacter jejuni* and *Campylobacter coli*) are currently recognized as being the most common causes of bacterial gastroenteritis in humans worldwide however, other *Campylobacter* spp. such as *Campylobacter lari* and *Campylobacter upsaliensis* have also been implicated in human gastrointestinal infections (Obeng et al., 2012; Centers for Disease Control and Prevention (CDC), 2013). *Campylobacter* cause diarrhea in an estimated 400 – 500 million people globally each year. In a number of developing countries the incidence of *Campylobacter* disease have been reported as higher than *Salmonella* and *Shigella* diseases (EFSA, European Food Safety Authority, and ECDC et al., 2012; Centers for Disease Control and Prevention (CDC) 2013). *Campylobacter* spp. are gram negative motile, spiral-shaped bacterium that exists as commensal organisms which commonly colonize the intestinal tract of food animals. Some of the main sources of infection have been associated with the ingestion of raw or uncooked meat (pig, sheep, cattle) but especially poultry meat and eggs, unpasteurized milk, contaminated drinking water as well as fresh produce (Topp & Villemur, 2016). Other sources of contamination also exist, such as direct contact with wild birds, animals, rodents or pets (Lapierre et al., 2016). Successful infiltration of *Campylobacter* spp. into the host depends on a number of important virulence factors involved in pathogenisis, such as the production of toxins, adherence to intestinal epithelial cells as well as the invasion and survival within host h,owever, continued infection depends on the survival of *Campylobacter* spp. in the presence of antibiotic treatment (Carvalho et al., 2013).

In addition to the virulence potential of *Campylobacter* spp., there is a significant concern about the increasing antibiotic resistance of this organism isolated from both humans and animals (Hein et al. 2013). Generally, when infections are caused by *Campylobacter* the symptoms are generally characterized as a mild enteritis and is usually self-limiting which rarely requires any antimicrobial treatment. Some severe cases do, however, result in prolonged enteritis and septicemia where antimicrobial treatment is often essential as well as in cases where patients are immunologically compromised (Chatur et al., 2014). Severe cases of *Campylobacter* infections are commonly treated with macrolides, such as erythromycin, and fluoroquinolones, such as ciprofloxacin are used to treat enteritis while aminoglycosides such as gentamicin are commonly prescribed to treat systemic infections (Noormohamed & Fakhr, 2014). An alternative treatment using tetracyclines have been suggested for clinical campylobacteriosis however they are rarely used (Wieczorek & Osek, 2013). Antibiotics in veterinary use vary greatly throughout the world. Situations in which antibiotics are of great concern are when they are used for growth-promotion purposes as opposed to therapeutic purposes. When low levels of antibiotics are used in this setting over long periods of time, this drives the emergence of resistant

bacteria. Areas such as India, Indonesia, Thailand as well as parts of Africa can obtain veterinary antibiotics without a prescription or any other controls in place which is of great concern (Iovine, 2013). Pathogens resistant to antimicrobials are a growing concern globally because they might compromise the effective treatment of infections in both animals and humans (Mäesaar et al., 2015). Molecular analysis of antibiotic resistance genes has shown that identical elements were found in bacteria that colonize both animals and humans, suggesting that bacteria originating from food of animal origin aid in the spread of resistant bacteria and resistance genes from animals to humans via the food chain (Moyane et al., 2013). Escalating numbers of *Campylobacter* isolates have developed resistance to fluoroquinolones and other antimicrobial classes such as tetracyclines, betalactams, aminoglycosides and macrolides. Resistance rates have also risen in *C. jejuni* and *C. coli* against penicillins and most of the cephalosporins as well as sulfamethoxazole, rifampicin, vancomycin, and trimethoprim (Maćkiw et al., 2012).

Although all these studies determined the resistance patterns exhibited by *Campylobacter* they have not investigated the resistance genes which are associated with resistant strains of *Campylobacter*. Ciprofloxacin and enrofloxacin are fluoroquinolones which have extensive application in both veterinary and human medicine; these antimicrobial agents have a wide spectrum of action over Gram-positive and Gram-negative bacteria (Colles et al., 2016). Ciprofloxacin is used for the treatment of human Salmonellosis and Campylobacteriosis, however, it is also used in the poultry production industry. Following exposure to fluoroquinolone residues these could remain in the animal body and produce resistant strains of bacteria. A number of studies have linked the therapeutic and prophylactic use to select for ciprofloxacin-resistant campylobacters in poultry that enters the food chain with the emergence and spread of antimicrobial resistance from this pathogen (Gallay et al., 2007; Habib et al., 2009; Marinou et al., 2012; Moyane et al., 2013; Zendehbad et al., 2015; Colles et al., 2016). Mutations play a major role in development of *Campylobacter* resistance, fluoroquinolone resistance is mediated by amino acid substitutions in the quinolone resistance-determining region (QRDR). The gyrase gene products are large enzymatic quaternary structures consisting of two pairs of subunits *GyrA* and *GyrB*. The *gyrA* gene, which encodes part of the *GyrA* subunit of DNA gyrase, confers a high-level of resistance to ciprofloxacin due to the point mutation Thr86Ile driven by the C257T change in the *gyrA* gene, other mutations of the *gyrA* gene region in *C. jejuni* include Thr86Ala which is responsible for high-levels of resistance to nalidixic acid and low-level resistance to ciprofloxacin (Colles et al., 2016). In *C. jejuni* and *C. coli*, when there is an absence of a secondary target for fluoroquinolones a unique modification in the *GyrA* subunit is adequate to confer the expression of a fluoroquinolone-resistant phenotype for the pathogen (Mäesaar et al., 2015).

Natural transformation may be a major mechanism for the transfer of chromosomally encoded resistance such as for fluoroquinolone and macrolide resistance while conjugation plays a major role for the transfer of plasmid mediated resistance such as for tetracyclines and aminoglycosides (Wieczorek & Osek, 2013). Tetracycline resistance in *Campylobacter* spp. is conferred by the *tet (O)* gene, which encodes ribosomal protection proteins (RPPs), this gene is extensively present in both *C. jejuni* and *C. coli* and has been shown to confer extremely high-levels of tetracycline resistance (512mg/L) by displacing tetracycline from its primary binding site on the ribosome and thus eliminating the action of the antibiotic (Abdi-Hachesoo et al., 2014). The high prevalence of conjugative *tet (O)* plasmids has made it possible to assume that conjugation has contributed significantly to the spread of tetracycline resistance in *Campylobacter*.

Against this background, this study was aimed to detect the presence of antimicrobial resistance genes in poultry and human clinical isolates, the 235bp and 270bp PCR products of the *gyr*A genes which confer resistance to fluoroquinolones, $bla_{OXA\text{-}61}$ gene which is linked to ampicillin resistance and *tetO* resistance gene which confers resistance to tetracycline. It is hypothesized that *gyrA*(235bp), *gyrA*(270bp), $bla_{OXA\text{-}61}$ and *tetO* resistance genes are present in both sources being tested, due to the misuse of antibiotics in human and chickens. This study was also undertaken to investigate the correlation and statistical significance of the antimicrobial resistance genes detected in isolates of *Campylobacter jejuni* and *Campylobacter coli* from chicken fecal samples and human clinical samples.

## 4.3. Material and Methods

### 4.3.1. Sampling procedure

Ethical approval was provided by the Biomedical Research Ethics Committee (BREC) for the use of human clinical isolates (REF:BE084/14). A total of 100 human *Campylobacter* isolates were analyzed, these cryopreserved samples in Brucella broth (Oxoid) with 15% glycerol were part of a collection that was received from a private laboratory in Durban, KwaZulu-Natal during 2014. Fecal samples from 100 broiler chickens were randomly collected in 2016 from a commercial broiler farm in the greater Durban area. Fresh broiler feces were sampled with sterile swabs then directly inoculated into charcoal broth (Sigma-Aldrich, USA) and transported back to the laboratory for incubation at 37°C for 48 hours, under microaerophilic conditions (5% $O_2$, 10% $CO_2$ and 85% $N_2$) created by CampyGen (Oxoid, UK) gas generating packs in an anaerobic jar.

### 4.3.2. Microbiological isolation and identification

*Human clinical samples*

From cryopreserved samples in Brucella broth (Oxoid) with 15% glycerol, cultures were revived on modified charcoal cefoperazone deoxycholate agar (mCCDA) (Blood-Free Agar) (Oxoid, England) containing *Campylobacter* selective supplement SR0155 (Oxoid, England). A sterile loop was then streaked across the area of inoculation several times to achieve isolated colonies and plates were incubated at 37°C for 48 hours under microaerobic conditions created by CampyGen (Oxoid, UK) gas generating packs in an anaerobic jar. Following incubation, species identification was confirmed by PCR targeting of the *hipO* gene region (Table 4.1) which is the hippuricase gene specific for *C. jejuni* (Marinou et al., 2012) and the *asp* gene region (Table 4.1), the aspartokinase gene specific for *C. coli* (Al Amri et al., 2013) following DNA isolation.

*Poultry Fecal Samples*

Following incubation, the fecal samples in charcoal broth (Sigma-Aldrich, USA) were filtered through a 0.65µm cellulose nitrate filter (Sartorius Stedim Biotech, Germany) onto mCCDA (Blood-Free Agar) (Oxoid, England). Approximately 500µl of the incubated charcoal broth was evenly distributed over the filter aseptically, once the liquid had been filtered through, forceps were used to aseptically remove the filter. The culture plates were then set in an inverted position in an anaerobic jar containing an atmosphere generation system (CampyGen sachet, Oxoid) and then incubated at 37°C for 48 hours. Following incubation, species identity was confirmed, after DNA isolation, by PCR targeting of the *hipO* gene specific for *C. jejuni* (Marinou et al., 2012) and the *asp* gene specific for *C. coli* (Table 4.1);(Al Amri et al., 2013).

### 4.3.3. DNA Isolation

Template DNA for PCR was extracted via the conventional boiling method which requires the following: characteristic colonies of *Campylobacater* species were isolated from plates and suspended in 300 µl TE buffer then vortexed for homogenization of cells. The suspensions were boiled at 100°C for 10 minutes then immediately cooled on ice. After centrifugation at 14,000 x g for 5 min, supernatants were transferred to new tubes and stored at -20°C until use in PCR for detection ofantibiotic resistance genes (Datta et al., 2003). A positive *Campylobacter* spp. control was also prepared by isolating DNA from a reference strain of *C. jejuni* ATCC 29428 which was incubated under the same conditions and subjected to the same DNA extraction methods. The Thermo Scientific Nanodrop 2000, UV-Vis Spectrophotometer (USA) was used to check the concentration and quality of the isolated DNA.

### 4.3.4. Detection of antibiotic resistance genes using PCR

PCR primers were synthesized and sourced from Inqaba Biotechnologies, South Africa, forward and reverse primers specific for the antibiotic resistance genes under investigation were designed based on the gene sequence information in the GenBank database and in previously published studies (Gibreel et al., 2004; Chatur et al., 2014). PCRs were carried out in the BIO-RAD, T100™ Thermal Cycler (Singapore) for a 25µl reaction. The amplification conditions for *gyrA*(235bp), *gyrA*(270bp) and $bla_{OXA-61}$ consisted of an initial denaturalization at 95°C for 5 minutes, 35 cycles at 95°C for 50 seconds, specific Tm for each primer (Table 4.1) for 30 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes. PCR conditions for the *tetO* gene, a 559bp product, were as follows: an initial denaturalization of 95°C for 1 min, and then 95°C for 1 min, 49°C for 1 min, and 72°C for 1 min, repeated for 35 cycles. PCR products were then electrophoresed on a 1.5% agarose gel run at 60V for 60 minutes, stained with ethidium bromide and then visualized using the BIO-RAD, ChemiDoc™ MP Imaging System.

### 4.3.5. Statistical Analysis of virulence genes and antibiotic resistance genes

The four antibiotic resistance genes and two virulence genes detected in *Campylobacter jejuni* and *Campylobacter coli* were analyzed using IBM SPSS Statistics (version 23). Pearson's Correlation analysis, Fisher's Exact tests, Chi Square tests and Logistic Regression analysis were implemented to evaluate the relationship between the different PCR results obtained and the significance of the presence of virulence genes and antibiotic resistance genes detected in human and poultry samples. Every model included the presence/ absence of each antibiotic resistance gene and each virulence gene investigated (0 = absent; 1 = present) and associations were considered significant when ($p<0.05$).

**Table 4.1**: Target virulence genes and antimicrobial resistance genes, Primer Sequences, Amplicon sizes and Annealing Temperatures

| **Target gene** | **Primer Sequence (5' – 3')** | **Product Size (bp)** | **Annealing Temperature (°C)** | **References** |
|---|---|---|---|---|
| *asp* | F-GGTATGATTTCTACAAAGCGAGA<br>R-ATAAAAGACTATCGTCGCGTG | 500 | 53 | (Al Amri et al., 2007) |
| *hipO* | F-GAAGAGGGTTTGGGTGGT<br>R-AGCTAGCTTCGCATAATAACTTG | 735 | 53 | (Al Amri et al., 2007) |
| *gyrA* | F-GAAGAATTTTATATGCTATG<br>R-TCAGTATAAC GCATCGCAGC | 235 | 53 | (Chatur et al., 2014) |
| *gyrA* | F-ACGCAAGAGAGATGGTT<br>R-TCAGTATAACGCATCGCAGC | 270 | 45 | (Chatur et al., 2014) |
| $bla_{OXA-61}$ | F- AGAGTATAATACAAGCG<br>R- TAGTGAGTTGTCAAGCC | 372 | 54 | (Obeng et al., 2012) |
| *tetO* | F-GGCGTTTTGTTTATGTGCG<br>R-ATGGACAACCCGACAGAAGC | 559 | 49 | (Gibreel et al., 2004) |

### 4.4. Results

PCR products of the antimicrobial resistance genes investigated in this study are demonstrated in Figure 4.1. The corresponding sizes of the *gyrA* genes depicted in lanes 1 and 2 have amplicons of 235bp and 270bp, respectively. Lane 3 is the $bla_{OXA-61}$ resistance gene which corresponds to a 372bp amplicon and Lane 4 is the plasmid-encoded *tetO* gene which has an amplicon of 559bp.

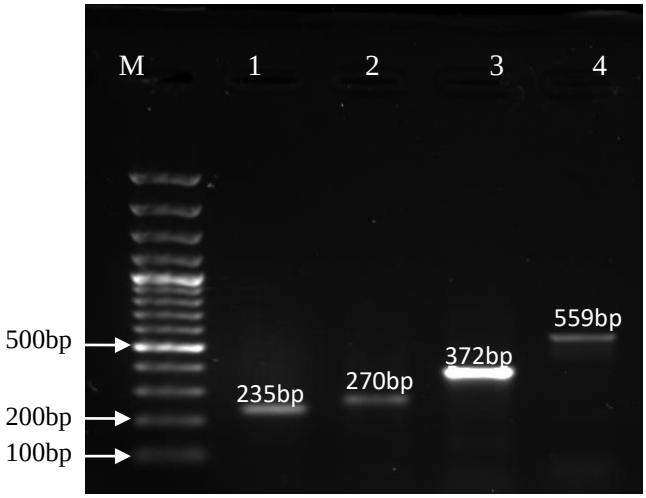


**Figure 4.1:** Representative gel of antibiotic resistance genes investigated from *Campylobacter* spp. Lanes: M, 100-bp marker; 1, *gyrA;* 2, *gyrA*; 3, $bla_{OXA-61}$; 4, *tetO*.

Results in Figure 4.2 indicate that *C. jejuni* isolated from human clinical samples as well as chicken fecal samples demonstrated similar presence of antimicrobial resistance genes. The gyrA (235bp) and gyrA (270bp) were detected at 49% and 36% in human clinical samples and 52% and 38% in chicken samples respectively for each gene. The resistance genes $bla_{OXA-61}$and *tetO* were detected at 58% and 56% in human clinical samples and 65% and 68% in chicken, respectively. Although *C. coli* was isolated at a low incidence the antimicrobial resistance genes in this species was detected at high percentages in chicken fecal samples. Detection rates for *gyrA*(235bp), *gyrA*(270bp), $bla_{OXA-61}$and *tetO* in chicken samples were 50%, 20%, 70% and 70%, respectively. A lower incidence was observed in human clinical samples which resulted in 50%, 36%, 29% and 57% for *gyrA*(235bp), *gyrA*(270bp), $bla_{OXA-61}$and *tetO*, respectively.

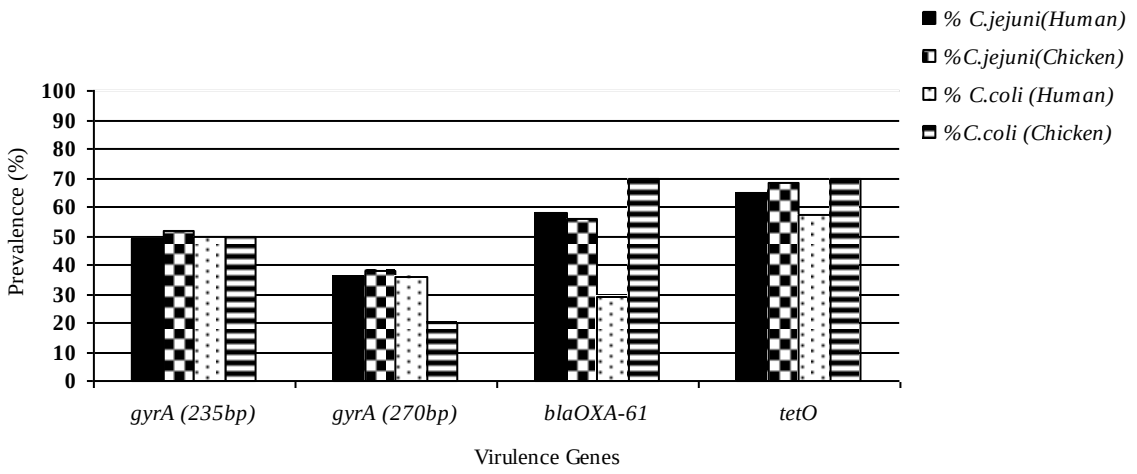


**Figure 4.2:** Percentage of *C. jejuni* and *C .coli*, from human clinical isolates (n = 83) and chicken feces (n = 78) which indicated presence of the antibiotic resistance genes under analysis.

Results in Table 4.2 indicate that there is a strong significant relationship (P<0.05) between all resistance genes (*gyrA*(235bp), *gyrA*(270bp), $bla_{OXA-61}$and *tetO*) in human clinical and poultry samples investigated in this study (p<0.05) for both Chi-square and Fisher's Exact statistical tests.

**Table 4.2**: Results of Chi-Square and Fisher's Exact tests indicating P values for virulence genes and antimicrobial resistance genes investigated

| | **Asymptotic Significance (2-sided)** | | | |
|---|---|---|---|---|
| | *gyrA*(235bp) | *gyrA*(270bp) | $bla_{OXA-61}$ | *tetO* |
| Pearson Chi-Square | 0.000 | 0.000 | 0.000 | 0.000 |
| Fisher's Exact Test | 0.000 | 0.000 | 0.000 | 0.000 |

A logistic regression analysis (Table 4.3) was conducted to predict the presence of antimicrobial resistance genes (*gyrA*, $bla_{OXA-61}$and *tetO*), in chicken and human clinical isolates using the source of the isolates as a predictor. A test of the full model against a constant only model indicated that genes *gyrA* (235bp), $bla_{OXA-61}$ and *tetO* were not statistically significant (p>0.05) from the data obtained. The p value of these genes in human clinical isolates and poultry exceed the 0.05 level of significance (p>0.05).

**Table 4.3:** Logistic Regression analysis showing the relationship between virulence genes and antimicrobial resistance genes detected in *Campylobacter* spp. from human clinical isolates and chicken feces

| | *B* | *SE* | *p Value* | *OR* | *95% C.I.* |
|---|---|---|---|---|---|
| *gyrA* (235bp) | -0.075 | 0.315 | 0.811 | 0.927 | 0.500 – 1.721 |
| *gyrA* (270bp) | 0.011 | 0.329 | 0.974 | 1.011 | 0.531 – 1.924 |
| $bla_{OXA-61}$ | -0.190 | 0.318 | 0.551 | 0.827 | 0.444 – 1.542 |
| *tetO* | -0.182 | 0.333 | 0.584 | 0.833 | 0.434 – 1.601 |

Pearson Correlation results (Table 4.4) demonstrated a strong positive correlation between the antibiotic resistance genes investigated in this study. All resistance genes investigated indicate that correlations between genes are significant at the 0.01 level ($p<0.01$). High correlations exist between the ampicillin resistance gene $bla_{OXA-61}$ and the tetracycline resistance gene *tetO* (64.3%), the second highest correlation exists between the *tetO* gene and the *gyrA* gene for fluoroquinolone resistance (56.8%).

**Table 4.4:** Comparison of Pearson Correlations for virulence genes and antimicrobial resistance genes

| | | *gryA*(235bp) | *gyrA*(270bp) | $bla_{OXA-61}$ | *tetO* |
|---|---|---|---|---|---|
| *gryA*(235bp) | Pearson Correlation | 1 | .461** | .430** | .568** |
| | Sig. (2-tailed) | | .000 | .000 | .000 |
| *gyrA*(270bp) | Pearson Correlation | .461** | 1 | .311** | .404** |
| | Sig. (2-tailed) | .000 | | .000 | .000 |
| $bla_{OXA-61}$ | Pearson Correlation | .430** | .311** | 1 | .643** |
| | Sig. (2-tailed) | .000 | .000 | | .000 |
| *tetO* | Pearson Correlation | .568** | .404** | .643** | 1 |
| | Sig. (2-tailed) | .000 | .000 | .000 | |

**. Correlation is significant at the 0.01 level (2-tailed).

**4.5. Discussion**

This study involved isolation of *Campylobacter* strains from human clinical samples and chicken fecal samples. *Campylobacter* species were revived from 83 of the 100 human clinical isolates that were chosen for this study and from the chicken feces 78 of the 100 samples that were collected indicated *Campylobacter* growth. All samples were subjected to PCR analysis of the *hipO* and *asp* gene for confirmation and differentiation of *Campylobacter* species. Following identification 83% of human clinical isolates and 78% of poultry isolates were subjected to detection of four antimicrobial resistance genes; PCR products of the antimicrobial resistance genes investigated in this study are demonstrated in Figure 4.1. The *gyrA* genes depicted in lanes 1 and 2 have amplicons of 235bp and 270bp conferring a moderate to high levels of resistance to fluoroquinolones due to mutations occurring in these genes (Chatur et al., 2014). Lane 3 is the $bla_{OXA-61}$ resistance gene which corresponds to a 372bp amplicon which confers ampicillin resistance (Obeng et al., 2012). Lane 4 is the plasmid-encoded *tetO* gene which primarily confers tetracycline resistance by displacing tetracycline from its primary binding site on the ribosome (Abdi-Hachesoo et al., 2014). Antimicrobial resistance genes isolated from human clinical isolates and chicken feces, in Figure 4.2, demonstrated similar presence in both these hosts. The 235bp and 270bp *gyrA* genes were present in 49% and 36% of human clinical isolates, respectively whereas the *Campylobacter* from chicken feces indicated a 51% and 36% presence of these genes respectively, regardless of differentiation of species. The 270bp *gyrA* gene was detected at the same incidence in both hosts. The $bla_{OXA-61}$ gene was detected at 53% and 58% in human clinical isolates, and chicken feces, respectively. The *tetO* gene which confers tetracycline resistance was the most prevalent resistance gene detected compared to the other resistance genes under investigation; this gene was isolated at 64% and 68% from human clinical isolates and chicken isolates, respectively.

Our results indicate that *C. jejuni* is responsible for the majority of infection (83%) in human clinical samples compared to the low incidence of *C. coli* found only in 17% of cases and in poultry samples *C. jejuni* was detected at 87% compared to *C. coli* found at 13%. Antibiotic resistance found in *Campylobacter* spp. is an emerging concern globally; many authors have described this as a problem of public health importance because many infections are becoming increasingly difficult to treat due to this issue. (Luangtongkum et al., 2010; Marshall & Levy, 2011; Djordjevic et al., 2013; Fonseca et al., 2014; Topp & Villemur, 2016). The increase of resistant bacteria has been linked to the overuse of antimicrobial agents in feed supplements used in the farming industry. The selective pressure produces microbial isolates that become resistant to these antibiotics and can easily become a health risk to humans if they reach the food chain (Luangtongkum et al., 2010). The use of antibiotics in the case of poultry, which is regarded as the main reservoir of *Campylobacter* spp., substantially reduces the number of safe antibiotics

available for treatment of human infections, and increases the risk of multidrug-resistant strains which may contaminate foods, this poses a serious health risk to those consuming these products (Ferro et al., 2015). Usually it is recommended that the drug for treatment of human *Campylobacter* infections is the macrolide erythromycin, followed by ciprofloxacin of the fluoroquinolone family and the third choice would be tetracycline (Ghunaim et al., 2015). Antibiotic resistance mechanisms for *Campylobacter* spp. have been suggested, they do, however, differ between the drugs involved, a mutation in the *gyrA* gene acts as one of the main mechanisms of resistance for fluoroquinolones, this gene encodes part of the DNA gyrase and in the presence of a single point mutation in quinolone resistance-determining region (QRDR), codon 86 is changed from Threonine to Isoleucine which results in a high level of resistance to the antibiotic ciprofloxacin (Wieczorek & Osek, 2013). *Campylobacter* carrying the Thr-86-Ile change in the GyrA subunit of DNA gyrase can persist in the absence of antibiotic selection pressure. Tetracycline resistance has been shown to be typically mediated by the presence of the *tetO* gene (Abdi-Hachesoo et al., 2014). Tetracycline has also been listed as an alternative treatment for *Campylobacter* gastroenteritis however it is used widely for therapeutic applications in livestock and poultry, which increases the risk of tetracycline resistant *Campylobacter* spp. (Zendehbad et al., 2015).

In this study we analyzed two *gyrA* genes involved in gyrase subunit A these genes play a role in DNA gyrase, however, mutations within the *gyrA* gene lead to phenotypic expression of resistance to fluoroquinolones. Unlike the fluoroquinolone resistance in other organisms such as *Echerichia coli* and *Salmonella*, accomplishment of high-level FQ resistance in *Campylobacter* does not necessitate stepwise accumulation of point mutations in *gyrA*. Rather, a single point mutation in the QRDR of *gyrA* gene is adequate to lead to clinically relevant levels of resistance to Fluoroquinolone antimicrobials (Wimalarathna et al., 2013). The *tetO* gene was analyzed as well, presence of this gene results in a resistance phenotype by the organism to the antibiotic tetracycline (Ferro et al., 2015). Lapierre et al. (2016) demonstrated that antimicrobial resistant strains of *Campylobacter* in their study were associated with the presence of the *cdtA* and *dnaJ* virulence genes; they also found high levels of ciprofloxacin and tetracycline resistance in many of the *Campylobacter* strains under investigation. This finding could be due to the *gyrA* and *tetO* resistance genes which confer resistance to the antibiotics ciprofloxacin and tetracycline. Oporto et al. (2009) conducted a study which reported moderate levels of resistance to ampicillin, ciprofloxacin and tetracycline in *Campylobacter* isolated from feces of free range birds in Spain (Oporto et al., 2009).

### 4.6. Conclusion

In summary, our work showed a disperse distribution of a number of resistance genes which play significant roles in resistance to fluoroquinolones, β-lactams and tetracycline antibiotics. Finally, we demonstrated statistically significant correlations between genes detected in human clinical and chicken samples ($p<0.05$). Extensive research needs to be implemented on this emerging pathogen to further establish proper control and preventative measures and to reduce contamination in order to alleviate emergence and transmission of resistant or multi-resistant strains of *Campylobacter* species. It is recommended that use of certain antibiotics be restricted in poultry production systems in South Africa in order to reduce the numbers of antibiotic resistant *Campylobacter* and limit transmission of multi-resistant strains to the food chain. This will ultimately improve use of antibiotics in the case of human infections when resistant strains are controlled at the critical level in food production industries.

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# CHAPTER 5
# GENERAL CONCLUSIONS

*Campylobacter* spp. are quickly escalating to becoming the number one most common food borne pathogen in the world. This is alarming considering that there is not much information available on them and even though we have uncovered some important information regarding virulence and antibiotic resistance in *Campylobacter* spp. there is still a great deal we do not know. Different regions around the world detect this pathogen at varying incidence, possibly due to the organisms' specific growth requirements and adaptation capabilities (Khoshbakht et al., 2013). How to reduce the occurrence and spread of *Campylobacter* has become more and more essential in the control of foodborne campylobacteriosis (Caro-Quintero et al., 2009). Data regarding *Campylobacter* spp. in South Africa is extremely scarce, therefore, any research with regard to this organism here could broaden our understanding and aid in proper control measures. *Campylobacter* isolated from food-producing animals, such as chicken, is a continuing concern for food safety and public health (Thibodeau et al., 2015). The presence and prevalence of virulence genes assist *Campylobacter* spp. in successful infection in a human host and when the pathogen harbors antimicrobial resistance genes this makes it difficult to treat and manage such infections in humans (Fonseca et al., 2014).

## 5.1. Significant Findings

In this project we conducted a series of analyses to isolate and detect *Campylobacter* spp. from chicken feces and human clinical samples; the aim was to isolate the two most prevalent species of *Campylobacter* which cause infection in humans, namely *Campylobacter jejuni* subsp. *jejuni* and *Campylobacter coli*. A higher incidence was noticed from the *Campylobacter* revived from human clinical isolates compared to the fresh chicken fecal samples examined this could possibly be due to a number of reasons: the isolation methodology used, the human *Campylobacter* specimens were isolated in a private laboratory probably with more sensitive equipment, also depending on the amount of fecal material collected this would affect the number of *Campylobacter* present and *Campylobacter* are extremely sensitive organisms, therefore, the time inbetween sampling and incubation could have affected some of the *Campylobacter* present in the sample thus hindering them from growth later on (Ghorbanalizadgan et al., 2014). The majority of *Campylobacter* spp. detected in human clinical samples and chicken fecal samples were identified as *C. jejuni* with hippuricase gene (*hipO*) (Marinou et al., 2012) and only a small percentage from the samples tested positive for *C. coli* with positive PCR amplification of *asp* gene(Al Amri et al., 2013). Furthermore, correlation analysis indicated that the presence the *asp*

gene was negatively correlated to the presence of the *hipO* gene ($p<0.05$). This finding is in agreement with other studies as *C. coli* is not as prevalent as *C. jejuni* as the cause of infection in humans (Kaakoush et al., 2015). Following identification and confirmation of *Campylobacter* spp. we employed monoplex PCR for the detection of seven virulence genes: *cadF, ciaB, pldA, dnaJ, cdtA, cdtB* and *cdtC.* All isolates resulted positive for the *cadF* gene which facilitates adherence to fibronectin and plays a significant role in colonization irrespective of the source or bacterial species (Khoshbakht et al., 2014). Presence of the *ciaB* gene indicated positive correlations with more than one of the other virulence genes, its correlation with the *dnaJ* gene was low, however, it was significant ($p<0.05$) according to our statistical analysis, there was a strong correlation with the *hipO* gene which identifies *C. jejuni,* this could possibly be the reason that drives *C. jejuni* to be the main organism as cause of infection in humans. The *ciaB* gene is known as the *Campylobacter* invasive antigen B gene which explains the invasiveness of *C. jejuni* as a pathogen compared to other *Campylobacter* spp. recognized, this gene is also involved in the translocation of *Campylobacter* into host cells for the purpose of host cell invasion and plays a significant role in caecal colonization in chicken (Garvis et al., 2016b). Another important factor for colonization of *Campylobacter* species in the intestine of chickens is the *pldA* gene (an outer membrane phospholipase A) which encodes proteins associated with increased bacterial invasion on cultured epithelial cells (O Cróinín & Backert, 2012), our study found a higher percentage of the *pldA* gene present in chicken fecal samples than in human clinical samples. Therefore, the results from this study indicate that *Campylobacter* species recovered from chicken feces have potential virulence consequences in humans because the occurrence of such genes such as *pldA* detected in *C. jejuni* and *C. coli* in chicken fecal samples from the slaughterhouse indicates a public health hazard due to this emerging food borne pathogen (Epps et al., 2013).

### 5.2. Importance of cytolethal distending toxin genes found in *Campylobacter* spp.

Cell invasion of epithelial cells and *cdt* production are important bacterial virulence mechanisms that play a significant role for inducing enterocolitis. The presence of a single *cdt* gene does not have any effect on the bacterium virulence, however, if all three *cdt* genes are present together in a cluster then they are responsible for the release of a functional cytotoxin (Jakee et al., 2015). Our study revealed that ten of the human clinical isolates had all three *cdt* genes compared to eighteen of the chicken isolates. This is very alarming as statistical analysis revealed the significance of these genes in both sources investigated in this study ($p<0.05$). The percentage of *cdt* genes needs to be compared with cytolethal distending toxin production levels and tested on a variety of different cell lines in order to study the phenotypic characteristics of the isolates for a better understanding. As this is an important virulence mechanism in

*Campylobacter* spp. for prolonged infection in a host further investigation of the *cdt* cluster is necessary (Carvalho et al., 2013). The initial phase of our study was to compare the detection of virulence genes amongst one another from the different sources, the second phase was to detect and evaluate antibiotic resistance genes present in these two sources of human and chicken samples and finally we investigated the statistical significance of the virulence genes found against the antibiotic resistance genes isolated from human clinical samples and chicken feces, using molecular analysis. Antimicrobial resistance genes examined in the poultry and human clinical isolates were: the 235bp and 270bp PCR product of the *gyr*A gene which confers fluoroquinolone resistance (Chatur et al., 2014), $bla_{OXA\text{-}61}$ linked to ampicillin resistance (Obeng et al., 2012) and *tetO* resistance gene which confers tetracycline resistance (Gibreel et al., 2004). Antimicrobial resistance genes isolated from human clinical isolates and chicken feces revealed a higher presence of genes in chicken isolates compared to human. The *tetO* gene was the most prevalent resistance gene detected. Statistical analysis indicated that the cytotoxin genes *cdtA*, *cdtB* and *cdtC* and the *gyrA* genes made a significant contribution to the prediction of the presence of genes in human clinical isolates and poultry ($p<0.05$).

Results demonstrated a strong correlation of a relationship between genes; the presence of virulence genes *cdtA* and *cdtB* were strongly correlated ($p<0.05$) with the presence of the antimicrobial resistance genes *gyrA* (235bp) and *gyrA* (270bp) indicating that if the toxin genes *cdtA* and *cdtB* are detected then there is a high probability the *gyrA* genes for antimicrobial resistance will also be detected in *Campylobacter* spp. under analysis. This was the only significant relationship observed between resistance genes and virulence genes from data we had obtained through this study; we possibly need to examine a larger sample set in order to make any definitive conclusions. The link between resistance genes and virulence genes relies on a number of factors and mechanisms, which mediate an overall expression by the organism. These include a diversity of the organism, the bacterial population present in a sample as well as the strain and origin of the species, all of these should be carefully considered to validate the observations within a study (Lapierre et al., 2016).

### 5.3. Conclusions and Recommendations

In summary, our study reveals a disperse distribution of virulence genes in *C. jejuni* and *C. coli* isolated from chicken feces and human clinical samples as well as the presence of a number of resistance genes which play a significant role in resistance to fluoroquinolones, β-lactams and tetracyclines. Finally, we demonstrated statistically significant associations between antimicrobial resistance genes and toxin genes in *Campylobacter* spp. isolated from chicken feces and human clinical samples. Based on the generally high level of PCR detected virulence genes isolated from poultry samples in this study, this raises the concern that this is potentially a major source of clinical *Campylobacter* infection in humans and transmission through the food chain is inevitable. Extensive research needs to be implemented on this emerging pathogen to further establish proper control and preventative measures and to reduce contamination in order to alleviate emergence and transmission of virulent and resistant or multiresistant strains of *Campylobacter* species. Together, the findings from this project have significantly improved our understanding of the molecular mechanisms underlying the development of virulence and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* from human as well as chicken and there should be more research conducted to increase our knowledge of this emerging pathogen.

## 5.4. References

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