Characterization of *Campylobacter* isolates from a South African population

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DECLARATION

The experimental work described in this dissertation was conducted under the supervision of Dr Anthony M. Smith and Dr Karen H. Keddy at the Centre for Enteric Diseases, National Institute for Communicable Diseases (NICD) and University of the Witwatersrand, Johannesburg, South Africa.

I, Mandile Samantha Thobela, student number 374653 declare that this dissertation is my own independent work. It is being submitted for the degree of Master of Science in Medicine at the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination purpose at this or any other university.

Mandile Samantha Thobela

Day --------31st---------- of -------May---------- 2017

DEDICATION

To my grandmother,

Linah Mhloyisi Khoza

To my father,

Tommy Nhlanhla Thobela

To my mother,

Thandiwe Qochiwe Khoza

PRESENTATIONS

Thobela, M. S. Molecular surveillance for *Campylobacter* in RSA. Presented at the NICD Scientific Research Forum, Johannesburg, South Africa, 25 March 2015.
(Oral presentation)

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(Poster presentation)

Smith, A. M., **Thobela, M. S.,** Ismail, A., Keddy, K. H. Molecular epidemiology of *Campylobacter* isolates from South Africa investigated using web-based bioinformatics pipelines developed at the Centre for Genomic Epidemiology of the Technical University of Denmark. Presented at Washington DC, United States of America, a conference hosted by American Society for Microbiology (ASM), 24th -27th September 2015.
(Poster presentation)

ABSTRACT

In South Africa, there is a lack of national surveillance for *Campylobacter*. *Campylobacter* infections particularly those caused by *Campylobacter jejuni* and *Campylobacter coli,* cause a significant proportion of all diarrhoeal cases. Although diarrhoea caused by *Campylobacter* is short-lived, belligerent clinical manifestations include *C. jejuni*-induced Guillain-Barré Syndrome (GBS) which can cause considerable morbidity. Improvements in molecular subtyping methodologies, such as multi-locus sequence typing (MLST) and whole genome sequencing (WGS) based applications have been successfully applied in the field of molecular epidemiology of *Campylobacter*. Our study proposed to improve the molecular epidemiological understanding of *Campylobacter* in South Africa using polymerase chain reaction (PCR), WGS, and MLST. Antimicrobial susceptibility profiles of *Campylobacter* were also investigated. Furthermore, the prevalence of *Campylobacter* in stool specimens of children (<5 years) with acute diarrhoea and from patients with symptoms of acute flaccid paralysis (AFP) was investigated. A multiplex real-time PCR targeting *C. jejuni* and *C. coli* was used to screen a total of 2,341 samples from clinical isolates and clinical specimens associated with various existing surveillance programs. Antimicrobial susceptibility was performed using the Etest method. *C. jejuni* isolates were further subtyped using MLST based on the analysis of WGS data. In all surveillance programs, *C. jejuni* was the most predominant species of *Campylobacter* detected (>77%). Isolates had low prevalence of antimicrobial resistance to erythromycin (10%) and azithromycin (14%), however, ciprofloxacin (53%) and tetracycline (53%) resistance was notable. It was male (54%) children of less than five years of age (35%) who were more frequently associated with campylobacteriosis. A total of 33 different sequence types (STs) were identified through MLST; ST-227 and ST-572 were the most common STs. A total of five different clonal complexes grouped all typable isolates, with ST-206 complex and ST-21 complex being the most common clonal complexes. Strains with new unassigned STs of novel combinations of known alleles (*n*=8) and novel alleles were identified (*n*=1). Multiplex real-time PCR proved effective to detect and discriminate *Campylobacter* species. *C. jejuni* was the most isolated species during the course of the study. The application of

MLST reflected the genetic diversity that exists in *C. jejuni* strains. The current underrepresentation of *Campylobacter* burden of disease in South Africa may improve if better diagnosis and surveillance are introduced in South Africa.

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CONTENTS

DECLARATION ... iii

DEDICATION ... iv

PRESENTATIONS ... v

ABSTRACT ... vi

ACKNOWLEDGEMENTS ... viii

CONTENTS ... x

NOMENCLATURE ... xiv

LIST OF FIGURES ... xix

LIST OF TABLES ... xxii

Chapter 1: Introduction and Literature review ... 1

1.1. Introduction ... 1

1.2. The historical emergence of the genus *Campylobacter* ... 2

1.3. Taxonomy ... 3

1.4. Microbiology ... 3

1.5. Species of *Campylobacter* ... 4

1.6. Epidemiology ... 5

1.6.1. Burden of diarrhoeal diseases ... 5

1.6.2. Prevalence of *Campylobacter* diarrhoeal diseases ... 6

1.6.3. *Campylobacter* and coinfections ... 10

1.6.4. Risk factors and risk groups ... 10

1.6.5. Pathogenesis ... 13

1.6.6. Clinical characteristics of campylobacteriosis ... 16

1.7. Diagnosis, isolation, and identification of *Campylobacter* species ... 16

1.7.1. Phenotypic characterization of *Campylobacter* ... 16

1.7.2. Molecular characterization of *Campylobacter* ... 21

1.7.3. Whole genome sequencing (WGS) ... 26

1.8. Treatment ... 29

1.8.1. Antimicrobial mode of action ... 29

1.9. Antimicrobial Resistance ... 31

1.9.1. Epidemiology of antimicrobial-resistant *Campylobacter* ... 32

1.9.2. Prevalence of antimicrobial resistance in *Campylobacter* 33
1.9.3. Mechanisms of antimicrobial resistance in *Campylobacter* 35
1.10. Postinfectious manifestations of *Campylobacter* infection 36
1.10.1. Guillain-Barrè Syndrome (GBS) 36
1.10.2. *Campylobacter*-induced GBS 37
1.10.3. Clinical and electrophysiological subtypes of GBS 39
1.10.4. Mechanism by which *C. jejuni* induces GBS 39
1.10.5. Prevalence of *Campylobacter*-induced GBS 39
1.10.6. General clinical presentation of GBS 41
1.10.7. Management and treatment of GBS 41
1.11. Prevention and control measures of *Campylobacter* infections 42
1.11.1. Vaccination 43
1.12. Study objectives 44
Chapter 2: Materials and Methods 45
2.1. Human bacterial isolates and stool specimens 45
2.1.1. Bacterial isolates for *Campylobacter* surveillance 45
2.1.2. Stool specimens from the Rotavirus Surveillance Program 45
2.1.3. Stool specimens from the AFP Surveillance Program 46
2.2. Bacterial isolates of animal origin 46
2.3. Detection of *Campylobacter* from bacterial isolates 47
2.3.1. Phenotypic identification of *Campylobacter* 47
2.3.2. Genomic DNA extraction for the detection of *Campylobacter* 47
2.3.3. Genotypic detection of *Campylobacter* using multiplex real-time PCR 49
2.4. Antimicrobial susceptibility testing of *Campylobacter* isolates 50
2.5. Molecular subtyping of *C. jejuni* isolates using MLST 51
2.5.1. Genomic DNA extraction 51
2.5.2. Quantification of extracted genomic DNA 52
2.5.3. WGS and analysis of WGS data 52
2.5.4. MLST from WGS data of *C. jejuni* isolates 53
2.6. Data analysis 53
Chapter 3: Results 55
3.1. *Campylobacter* Surveillance Program 55
3.1.1. Submission of bacterial isolates during 2014 and 2015 55

3.1.2. Proportion of real-time PCR positive isolates for *Campylobacter* 57
3.1.3. Detected species of *Campylobacter* by real-time PCR 57
3.1.4. Sex distribution of characterized species of *Campylobacter* 57
3.1.5. Detection of *Campylobacter* by age groups 59
3.1.6. Detection of *Campylobacter* by both sex and age groups 60
3.1.7. Invasive cases of *Campylobacter* 60
3.1.8. Annual occurrences of *Campylobacter* infections 60
3.1.9. Antimicrobial susceptibility profiles of *Campylobacter* 61
3.2. Animal isolates 65
3.2.1. Detected *Campylobacter* species 65
3.2.2. Antimicrobial susceptibility profiles of *Campylobacter* 65
3.3. MLST characterization of *C. jejuni* isolates in South Africa 67
3.3.1. Distribution of STs and clonal complexes of *C. jejuni* isolates 70
3.3.2. Province distribution of STs 71
3.3.3. STs of animal isolates 73
3.3.4. Diversity of *C. jejuni* isolates 73
3.4. Rotavirus Surveillance Program 75
3.4.1. Demographic information 75
3.4.2. Detection of *Campylobacter* from children <5 years of age with diarrhoea 75
3.5. Acute Flaccid Paralysis Surveillance Program 77
3.5.1. Polio-negative stool specimens of AFP patients 77
3.5.2. *Campylobacter* detected from AFP patients 77
Chapter 4: Discussion 80
4.1. *Campylobacter* Surveillance Program 80
4.1.1. Submission of bacterial isolates 80
4.1.2. *Campylobacter* positive isolates and the detected species 81
4.1.3. Sex-specific occurrences of *Campylobacter* infections 83
4.1.4. Age-related occurrences of *Campylobacter* infections 84
4.1.5. Gender and age distribution of positive *Campylobacter* cases 87
4.1.6. Province distribution of detected *Campylobacter* 88
4.1.7. Detection of invasive *Campylobacter* species 89
4.1.8. Antimicrobial susceptibility profiles of detected *Campylobacter* species 89
4.2. Animal isolates 93
4.3. Population structure of *C. jejuni* isolates defined by MLST 94

4.4. Rotavirus Surveillance Program 98
4.5. Acute Flaccid Paralysis Surveillance Program 100
4.5.1. Detection of *Campylobacter* from AFP patients 100
4.5.2. Annual occurrences of detected *Campylobacter* 101
4.5.3. Distribution of detected *Campylobacter* by sex 101
4.5.4. Distribution of detected *Campylobacter* by age category 102
4.5.5. Province distribution of detected *Campylobacter* 103
4.6. Limitations 104
4.7. Future prospects 105
Chapter 5: Conclusions 107
References 112
Appendices 139
Appendix A: Housekeeping genes for *Campylobacter* MLST 139
Appendix B: TE buffer for crude genomic DNA extraction 140
Appendix C: Preparation and validation of QIAamp Fast DNA Stool Mini Kit 141
Appendix D: *Campylobacter* real-time PCR 142
Appendix E: Extraction and quantification of DNA for WGS 145
Appendix F: Rotavirus Surveillance Program study sites 146
Appendix G: Ethics clearance certificate 147
Appendix H: Plagiarism declaration with Turnitin report 148

NOMENCLATURE

( )	Open bracket close bracket / parenthesis
[ ]	Open square bracket close square bracket
+	Plus
<	Less than
=	Equal to
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
°	Degree
°C	Degree Celsius
µg/mL	Microgram per milliliter
µL	Microliter
AFP	Acute flaccid paralysis
AIDP	Acute inflammatory demyelinating polyneuropathy
AIDS	Acquired immune deficiency syndrome
AMAN	Acute motor axonal neuropathy
AMSAN	Acute motor and sensory axonal neuropathy
BIGSDB	Bacterial Isolate Genome Sequence Database
bp	Base pair
CCDA	Charchoal cefoperazone deoxycholate
CDC	Centers for Disease Control and Prevention

CDT	Cytolethal distending toxin
CED	Centre for Enteric Diseases
CLSI	Clinical and Laboratory Standards Institute
CTON	Cytotonic toxin
CTOX	Cytotoxins
DMP	Diagnostic Media Products
DNA	Deoxyribonucleic acid
EDTA	Disodium ethylenediaminetetra-acetic acid
et al.	And others
Etest	Episolimeter test
EU	European Union
g	Gram
G+C	Guanine and Cytosine
GBS	Guillain-Barrè syndrome
GERMS-SA	Group for Enteric Respiratory and Meningeal Surveillance-South Africa
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
HS	Heat stable
IFT	Institute of Food Technologists
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IL8	Interleukin 8
IV	Four
IVIg	Intravenous immunoglobulin
L	Liter
LOS	Lipooligosaccharide
M	Molar
MCGH	Microarray comparative genomic hybridization
MDR	Multi drug resistance
mg	Milligram
MIC	Minimum inhibitory concentration
Min	Minute
mL	Milliliter
MLST	Multi locus sequence typing
mM	Millimolar
MST	Minimum spanning tree
NCTC	National Collection of Type Cultures
ng	Nanogram
NGS	Next generation sequencing
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
NTC	Non template control
ORF	Open reading frame
PCR	Polymerase chain reaction

PE	Plasma exchange
PFGE	Pulsed-field gel electrophoresis
pH	Percentage Hydrogen
qPCR	Quantitative polymerase chain reaction
QRDR	Quinolone resistance determining region
RM	Restriction Modification
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSA	Republic of South Africa
RT	Room temperature
S-layer	Surface layer
SNPs	Single nucleotide polymorphisms
sp.	Species
ST	Sequence type
T4SS	Type IV secretion system
TE	Tris-EDTA
TSA	Trypticase Soy Agar
UK	United Kingdom
UPGMA	Unweighted pair group method with arithmetic averages
USA	United States of America
VNC	Viable non-culturable
wgMLST	Whole genome multi-locus sequence typing
WGS	Whole genome sequencing/sequence

WHO	World Health Organization
WHO-PD-VAC	World Health Organization-Product Development for Vaccines Advisory Committee
xg	Gravitational force

LIST OF FIGURES

Chapter 1

Figure 1.1: Scanning electron micrograph showing the curved/rod shape and bipolar flagella of *C. jejuni*. Image adopted from Javid and Ahmed, (2016). 2

Figure 1.2: Incidence and prevalence data of campylobacteriosis extracted from literature published from studies conducted in developed and developing countries. Image adopted from Kaakoush *et al*., (2015). 7

Figure 1.3: Trends of *Campylobacter* outbreak cases from 2007 to 2013. Image adopted from Kaakoush *et al*., (2015). 8

Figure 1.4: Pathogenesis of *Campylobacter* infection and the associated clinical manifestations. Abbreviations: inflammatory bowel diseases (IBD), and irritable bowel syndrome (IBS).Question marks represent conditions associated with campylobacteriosis, however with some degree of uncertainties. Image adopted from Kaakoush *et al*., (2015). 14

Figure 1.5: A model representation of the mechanisms of *Campylobacter* invasion of human intestinal epithelial cells and bloodstream translocation of the organism which subsequently causes haemolysis. Abbreviations: Interleukin 8 (IL8), Type IV secretion system (T4SS), and Surface layer (S-layer). Image adopted from Man, (2011). 15

Figure 1.6: Model representation of a sequenced human *C. jejuni* NCTC11168 genome (1 641 481 bp). The outer most circle from the origin of replication represents coding sequences in both the clockwise (dark green) and anticlockwise (light green) directions. The positions of hypervariable sequences in the genome are represented in black. The genes involved in the biosynthesis of surface structures are represented in dark red (clockwise) and pale red (anticlockwise). Annotated in the innermost sphere are clusters of genes responsible for the biosynthesis of extracellular polysaccharide (EP), LOS, and flagella modification. Image adopted from Parkhill *et al*., (2000). 28

Figure 1.7: Mechanisms through which antimicrobial resistance can be achieved for the different classes of antimicrobials. Image adopted from Grasso *et al*., (2016). .. 32

Figure 1.8: Sectors where antimicrobial use is applied and the impact it has in the ecosystem which drives the epidemiology of antimicrobial resistance and subsequently implicate human health through multiple pathways. Image adopted from IFT, (2006). 33

Figure 1.9: A model representation of the mechanisms facilitating the pathogenesis of *C. jejuni*-induced GBS. Abbreviations: Alpha C protein (APC); acute motor axonal neuropathy (AMAN); acute inflammatory demyelinating polyneuropathy (AIDP). Image adopted from van den Berg *et al*., (2014). 38

Chapter 3

Figure 3.1: Antibiogram of the total viable *Campylobacter* species (*n*=229) tested against various antimicrobial agents during the years 2014-2015. Depicted on the vertical axis are the antimicrobial agents (erythromycin, azithromycin, ciprofloxacin, and tetracycline) tested against the *Campylobacter* isolates. The horizontal axis shows the generated (susceptible/intermediate/resistance) antimicrobial susceptibility profiles in percentage (%). 64

Figure 3.2: Dendrogram demonstrating phylogenetic relationships of 111 *C. jejuni* isolates on the basis of MLST profiles which were grouped by UPGMA clustering. The *C. jejuni* isolates were from human hosts and animal hosts (chicken, sheep, and bovine). For each isolate an allele profile, Sequence type (ST), clonal complex, province, source of isolate, and year of isolation was presented. Non-typable (NT) isolates and isolates with new combinations of previously known alleles (NST) were presented. An allele digit of zero (0) represented an incomplete allele sequence. ϕProvinces: Gauteng (GP), Western Cape (WC), KwaZulu-Natal (KZN), North West (NW), Free-State (FS), and Mpumalanga (MP)..... 69

Figure 3.3: Phylogenetic analysis (minimal spanning tree) of South African *C. jejuni* isolates from humans and of veterinary origin (chicken, sheep, and bovine). Each coloured node (circle) represents one ST along with the ST number. STs representing one strain appear small in diameter, with or without the nodes being

shaded in colour. The diameter of the node represents the number of strains within one ST; the bigger the node, the higher the number of strains found within the ST. The connecting lines between the different STs depict the number of allelic differences between them; one allele difference (black bold lines); two allele difference (grey bold lines or thin lines]); three alleles difference (grey hatched lines); more than three alleles difference (grey dotted lines)..... 71

Figure 3.4: Minimal spanning tree of 111 *C. jejuni* isolates based on STs and the province distribution thereof. Each node represents a ST and the segments found within some of the nodes represent ≥2 *C. jejuni* strains belonging to the same ST, while un-segmented nodes represents a single *C. jejuni* strain. The shade of colour found in each node represents the province of the isolate(s) as stipulated in the keys. 72

Figure 3.5: Minimal spanning tree of 111 *C. jejuni* strains illustrating shared STs between human hosts and animal hosts. Each node represents a ST and the shade of colour within each node represent the sources, depicted in the keys, from which *C. jejuni* strains belonging to a specific ST were isolated. 74

LIST OF TABLES

Chapter 1

Table 1.1: Properties of *Campylobacter* exploited for phenotypic identification of species. 20

Chapter 3

Table 3.1: Demographic information of the study population collected during the years 2014 and 2015..... 56

Table 3.2: Distribution of real-time PCR confirmed *Campylobacter* isolates during the 2014-2015 study period..... 58

Table 3.3: The overall distribution of real-time PCR positive *Campylobacter* isolates submitted in the years 2014-2015 by age-group and sex..... 59

Table 3.4: Species-specific antimicrobial susceptibility profiles of detected *Campylobacter* in the years January 2014 to December 2015 (*n*=229). 63

Table 3.5: *Campylobacter* species of animal origin recovered in Western Cape and Gauteng Provinces during January 2014 to December 2015. 66

Table 3.6: Diversity indices of South African *C. jejuni* isolates. 74

Table 3.7: Stool specimens of children under five years of age with diarrhoea screened for the presence of *Campylobacter* in the years February 2014 to December 2015. 76

Table 3.8: Stool specimens from polio-negative AFP case patients screened for the presence of *Campylobacter* species during October 2014 to December 2015. 79

Chapter 1: Introduction and Literature review

1.1. Introduction

Campylobacteriosis is a disease caused by species of *Campylobacter,* particularly *Campylobacter jejuni and Campylobacter coli.* These infections are a major leading cause of gastrointestinal disease globally, with recent incidence rates that are higher than those caused by *Salmonella* in developed countries (Acheson and Allos, 2001; Samuel *et al*., 2004). Although gastrointestinal diseases associated with species of *Campylobacter* are rarely fatal, significant proportion of diarrhoeal cases are reported annually, which result in significant burden on health care systems and a major cause of economic loss globally (Sheppard *et al*., 2009). Common sources of *Campylobacter* infection have been defined, however, how these sources contribute to human infection remains incompletely addressed and reported cases of infection continue to increase (Tenkate and Stafford, 2008; Sheppard *et al*., 2009).

Belligerent clinical manifestation of *Campylobacter* includes post-infectious manifestation of *C. jejuni* infection resulting in Guillain-Barrè Syndrome (GBS) which can cause considerable morbidity (Acheson and Allos, 2001; Friedman *et al*., 2004). Another emerging issue is the global increase of *Campylobacter* species resistant to clinically recommended antimicrobials (Wimalaratha *et al*., 2013). National *Campylobacter* surveillance programs established in developed countries are not as they should be in many developing countries; a situation that compromises incidence values for a defined population (Coker *et al*., 2002). Irrespective of the lack of incidence data from national surveys, case-control community-based studies showed incidence estimates of 40, 000 to 60, 000 per 100, 000 for children of less than five years of age (van Vliet and Katley, 2001; Coker *et al*., 2002; Galate and Bangde, 2015). The report demonstrated that campylobacteriosis is often a paediatric disease in developing countries (van Vliet and Katley, 2001; Coker *et al*., 2002; Galate and Bangde, 2015).

1.2. The historical emergence of the genus *Campylobacter*

In 1886 Theodor Escherich first described non-culturable spiral shaped bacteria which were observed in the colons of infants who died of a disease he named “cholera infantum” (Samie *et al*., 2007). Following Theodor Escherich’s observations, *Campylobacter* was first identified in the uterine mucus of a pregnant sheep in 1902 by two British veterinarians (Silva *et al*., 2011). Initially campylobacters were referred to as “Vibrio like” organisms until 1963 when Sebald and Vèron proposed ‘*Campylobacter*’ as the genus based on their curved-rod like shape [Figure 1.1], low DNA base composition, microaerophilic growth requirement, and their non-fermentive metabolism (Hèbert *et al*., 1983; Vandamme and Ley, 1991; Samie *et al*., 2007; Silva *et al*., 2011; Galate and Bangde, 2015). With on-going advances in diagnostic arsenals, selective media founded the initial routine detection of *Campylobacter* in clinical laboratories (Acheson and Allos, 2001; Moore *et al*., 2005).

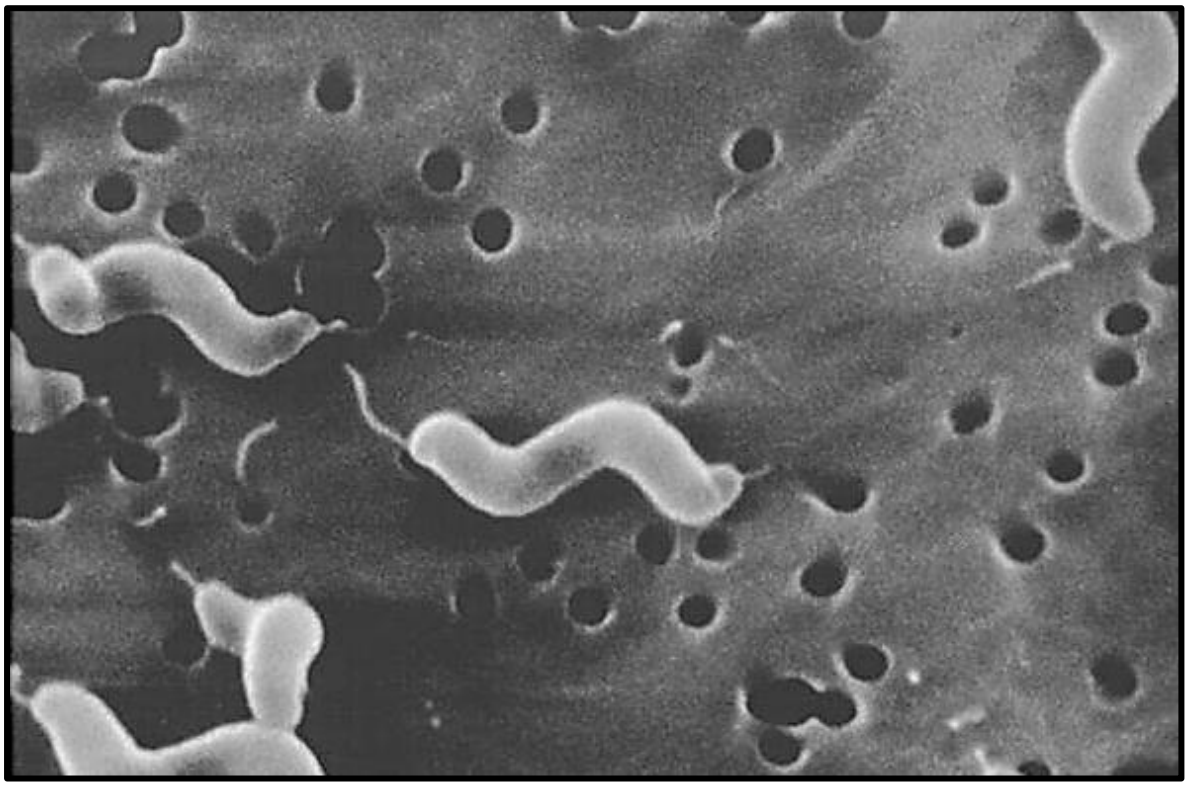


Figure 1.1: Scanning electron micrograph showing the curved/rod shape and bipolar flagella of *C. jejuni*. Image adopted from Javid and Ahmed, (2016).

1.3. Taxonomy

The family *Campylobacteraceae* which was first proposed in the early nineties consists of two genera; *Campylobacter* and *Acrobacter* (Vandamme *et al*., 1991; van Vliet and Katley, 2001; Silva *et al*., 2011; Galate and Bangde, 2015). The genus *Campylobacter*, first proposed in 1963 by Sebald and Vèron, was derived from a Greek word which described a curved rod (Moore *et al*., 2005; Samie *et al*., 2007). Currently, the genus *Campylobacter* consists of 26 species of which 19 have been isolated from humans and 10 subspecies of which nine are from humans, however, the taxonomic structure of the family *Campylobacteraceae* remains controversial with the on-going discovery of organisms that fall under the family (Lastovica, 2006; Debruyne *et al*., 2008; Galate and Bangde, 2015;). According to Debruyne *et al*., (2008), there are only 14 species of *Campylobacter* that are validly described (Silva *et al*., 2011; Galate and Bangde, 2015).

1.4. Microbiology

Campylobacter species are fastidious, gram-negative bacilli that are morphologically spiral or curved (Acheson and Allos, 2001; Mason *et al*., 2013). The morphology of *Campylobacter* is comparatively very small, about 0.2 to 0.5 µm wide and 0.5 to 8 µm long (Silva *et al*., 2011). *Campylobacter* species are motile by means of unipolar or bipolar flagellae [Figure 1.1] (Acheson and Allos, 2001). These thermophilic species of bacteria thrives well at higher body temperatures, ranging from 37 degrees Celsius ($^{\circ}$C) to 42°C (Pendleton *et al*., 2013). They require longer incubation periods for optimum growth, ranging from 48 hours to 96 hours microaerophilically, depending on the specific species (Perez-Perez and Blaser, 1996). *Campylobacter* species are non-fermenting organisms due to their incapability to ferment or oxidize common carbohydrate substrates (Galate and Bangde, 2015). However, they are oxidase positive and they reduce nitrates. Spirally shaped *Campylobacter* have been reported to transition into coccoid forms when exposed to atmospheric oxygen levels or other environmental stresses (Cappelier *et al*., 1997). Viable non-culturable (VNC) has been used to describe the coccoid transitioned state, and it is suggested to be a dormant state necessary for survival under un-optimal environmental conditions for

Campylobacter growth (Cappelier *et al*., 1997; van Vliet and Katley, 2001). Although *Campylobacter* requires special growth conditions, the bacterium may survive on food or in the environment for a couple of days (Schielke *et al*., 2014).

1.5. Species of *Campylobacter*

The emergence of species within the genus *Campylobacter* over the last three decades has indicated their pathogenic clinical importance (Acheson and Allos, 2001; Moore *et al*., 2005). *Campylobacter* species have been implicated as aetiological agents for veterinary diseases, such as diarrhoea in cattle and septic abortion in cattle and sheep, and more recently as significant clinical pathogens, particularly of human public health concern (Moore *et al*., 2005; Silva *et al*., 2011). *Campylobacter* is a major food and waterborne pathogen where foods of animal origin, particularly poultry, have been identified as significant sources of *Campylobacter* infection (Acheson and Allos, 2001). *C. jejuni* and *C. coli* are species of major public health importance of the genus *Campylobacter* (Perez-Perez and Blaser, 1996; Persson and Olsen, 2005; Chokboonmongko *et al*., 2013; Ioannidou *et al*., 2013; Mason *et al*., 2013; Moore *et al*., 2005; Wagenaar *et al*., 2013 Schielke *et al*., 2014).

Campylobacteriosis, the disease caused by a *Campylobacter* infection, which results in enteritis, is mainly caused by *C. jejuni* and to a lesser extent by *C. coli* (Wagenaar *et al*., 2013). *C. jejuni* has been found to account for over 80% of human cases, followed by *C. coli*, which accounts for most of the remainder (Gillespie *et al*., 2002; Vidal *et al*., 2016). *Campylobacter* species are invasive bacteria, capable of translocating and reaching the blood stream. In the 1950's, association of human *Campylobacter* infections with blood cultures was rare, rendering campylobacters to be deemed opportunistic human pathogens, most likely to occur in patients who are immunocompromised, among the very young or the elderly (Acheson and Allos, 2001; Moore *et al*., 2005). The rate of bacteraemia caused by *Campylobacter* remain as low as 1%, especially when contrasted with invasive infections caused by *Salmonella* (Acheson and Allos, 2001; Moore *et al*., 2005; Wagenaar *et al*., 2013). Reports on other *Campylobacter* species such as *C. lari*, *C. upsaliensis*, and *C. fetus*

have been documented as causative agents for human diseases. However, these reports of non-*C. jejuni*/*C. coli* infections globally accounts for a small fraction of all *Campylobacter* infections (Wagenaar *et al*., 2013).

This has resulted in studies conducted to only focus on epidemiological studies involving *C. jejuni* and *C. coli* (Acheson and Allos, 2001; Moore *et al*., 2005; Wagenaar *et al*., 2013). Biotypes and serotypes of *C. jejuni* and *C. coli* have been identified and the congruency between biotypes and serotypes extracted from humans and animals show that campylobacteriosis is zoonotic (Perez-Perez and Blaser, 1996; Colles and Maiden, 2012). Studies aimed at understanding the epidemiology of *Campylobacter* species have identified *C. jejuni* and *C. coli* as the major causative agents of sporadic gastroenteritis in humans (Lee and Newell, 2006). However, knowledge founding the pathophysiology of the organisms is poorly understood (Parkhill *et al*., 2000).

1.6. Epidemiology

1.6.1. Burden of diarrhoeal diseases

Diarrhoeal diseases pose a major public health problem in Republic of South Africa (RSA) making it the third leading cause of death in the country (Van Derslice and Briscoe, 1993; Nannan *et al*., 2012). In Africa, it is estimated that 3.552 million children of less than five years of age die each year, with diarrhoea accounting for 11 % of these deaths (Mason *et al*., 2013). The disease is associated with high morbidity, mortality, and economic loss for families and communities. The World Health Organization (WHO) report has estimated that around one billion people lack access to improved water and 2.5 billion have no access to basic sanitation globally (WHO, 2013).

1.6.2. Prevalence of *Campylobacter* diarrhoeal diseases

The increase in incidence of human campylobacteriosis globally has attracted the attention of the WHO (Coker *et al*., 2002). However, the true estimates of incidence of diarrhoeal diseases caused by *Campylobacter* are poorly known. Various approaches are being investigated and implemented which may potentially reflect the true incidence of human campylobacteriosis (WHO, 2013). The rate at which human campylobacteriosis cases are escalating have been reported to be numerically greater than those of salmonellosis and shigellosis (Acheson and Allos, 2001). In developing countries, *Campylobacter* is one of the commonly isolated bacteria from faeces of infants due to contaminated food and/or water (Mason *et al*., 2013). These findings suggest the need for more studies aimed at understanding the epidemiology and control of campylobacteriosis which is still far from clear (Coker *et al*., 2002; Nylen *et al*., 2002; Klena *et al*., 2004). The limiting factor in developing countries is the lack of established national surveillance programs for campylobacteriosis, regardless of the pervasiveness of the disease. National surveillance programs for *Campylobacter* have been established in developed countries (Tauxe *et al*., 1988; WHO, 2013).

I. Incidence of *Campylobacter* in developed countries

Campylobacter species fall among the most prevalent bacterial enteric pathogens isolated in developed countries (Tauxe *et al*., 1988). Majority of the published literature reports incidence and prevalence estimates of campylobacteriosis from developed countries, with a few reports from developing countries [Figure 1.2]. While the incidence of campylobacteriosis is high, most illnesses occur sporadically, and cases are rarely (typically <1%) associated with outbreaks (Taboada *et al*., 2013). It has been reported that outbreaks or smaller case clusters do occur far more frequently than previously conceived to occur [Figure 1.3] (Taboada *et al*., 2013; Kaakoush *et al*., 2015). Literature collected from the year 2007 to 2013 has shown majority of *Campylobacter* outbreak cases to be associated with poultry products and contaminated water [Figure 1.3] (Kaakoush *et al*., 2015). Other sources of *Campylobacter* outbreaks discovered during those years included ingestion of

mud/food, unpasteurized milk, animal contact, and sexual interactions (Kaakoush *et al*., 2015). Campylobacteriosis is a commonly reported zoonosis in the European Union (EU) and a commonly notified bacterial gastrointestinal disease in Germany (Schielke *et al*., 2014). The annual number of notified campylobacteriosis cases has increased in many European countries in recent years relative to other enteric pathogens such as *Salmonella* (Nylen *et al*., 2002; CDC, 2010; Schielke *et al*., 2014). In the United States of America (USA), *Campylobacter* infection is the second commonest cause of bacterial enteritis after salmonellosis, with 2.4 million cases estimated to occur yearly (CDC, 2010).

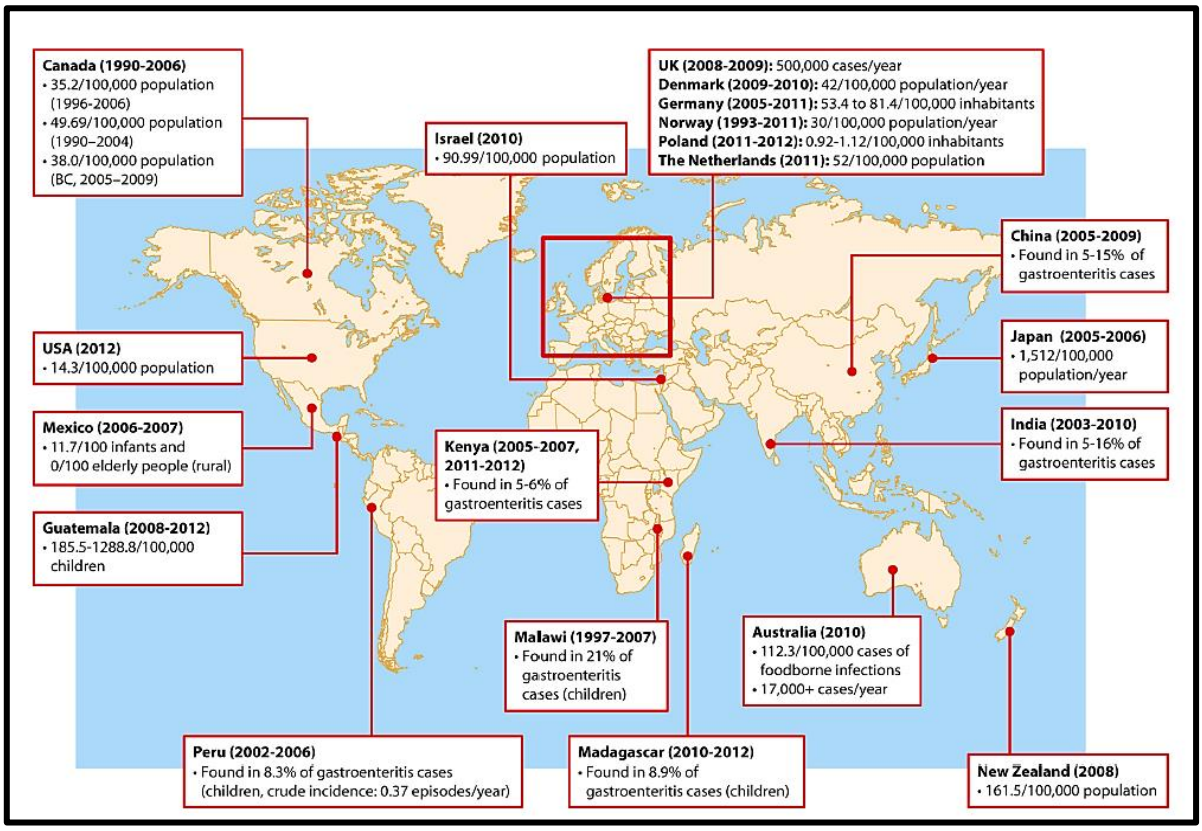


Figure 1.2: Incidence and prevalence data of campylobacteriosis extracted from literature published from studies conducted in developed and developing countries. Image adopted from Kaakoush *et al*., (2015).

II. Incidence of *Campylobacter* in developing countries

Campylobacter surveillance in developing countries is not what it should be as compared to developed countries; a situation that compromises incidence values for cases of campylobacteriosis for a population [Figure 1.2] (Coker *et al*., 2002;

Kaakoush *et al*., 2015). Compiled data from published literature has indicated research gaps existing in developing countries for *Campylobacter* (Kaakoush *et al*., 2015). For instance in Africa, there are few collected published data reports on cases of campylobacteriosis in Kenya and Malawi [Figure 1.2], as well as Tanzania, and South Africa (Kaakoush *et al*., 2015). The Global Enteric Multicenter Study (GEMS), which enrolled children with moderate-to-severe diarrhoea residing in Africa and in Asia, found the detection of *Campylobacter*, which was based on conventional culture methods, to be scarce and restricted to Asian countries (Kotloff *et al*., 2013). Incidence estimates in developing countries are based on laboratory confirmed cases of sporadic *Campylobacter* infections as well as outbreak cases, from which laboratory-based surveillance of *Campylobacter* and other enteric pathogens are founded (Coker *et al*., 2002). In 2000, Oberhelman and colleagues reported *Campylobacter* isolation to range from 5 to 20% in developing countries; although some countries have reported increase in isolation and incidence rates since their initial report, which has been linked to improved diagnostic methods (Coker *et al*., 2002; Galate and Bangde, 2015).

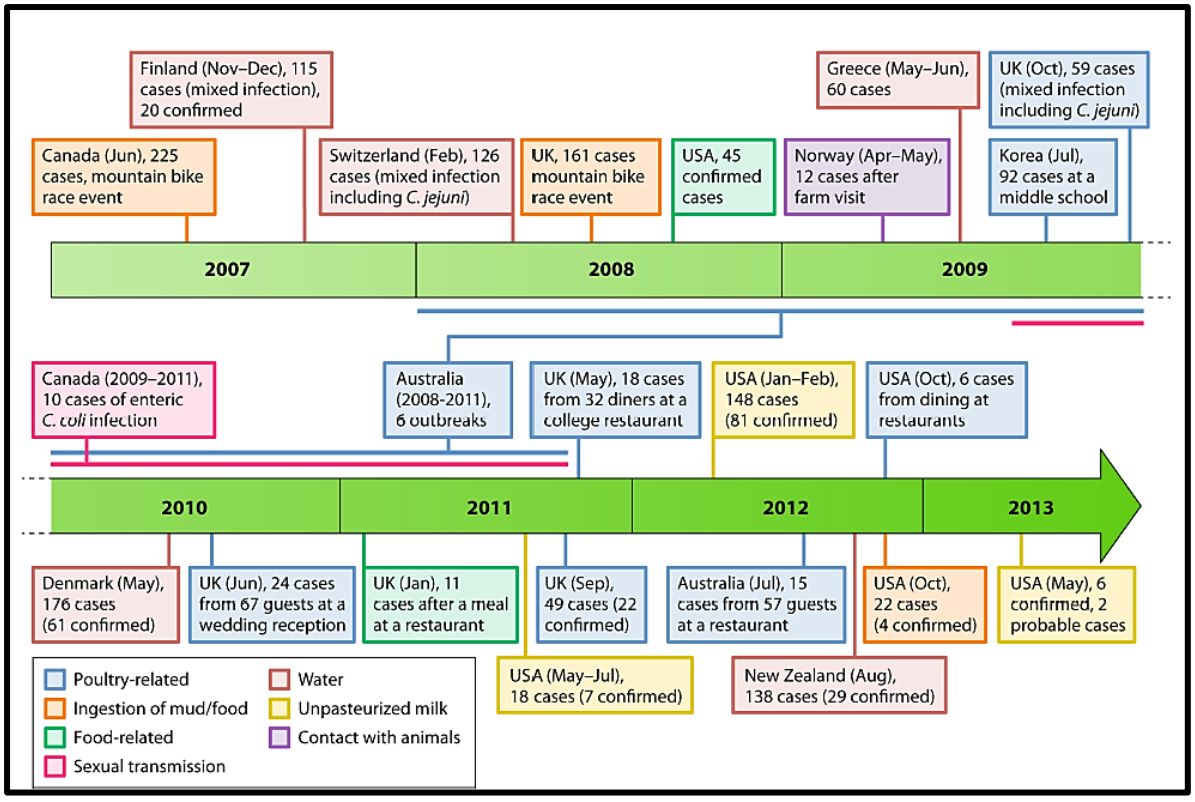


Figure 1.3: Trends of *Campylobacter* outbreak cases from 2007 to 2013. Image adopted from Kaakoush *et al*., (2015).

III. *Campylobacter* confirmed cases in RSA

There is insufficient literature describing the epidemiology of *Campylobacter* specifically in RSA, and as a result, values for burden estimates in the country do not exist. Studies that have been previously carried out are based on small sample size cases, carried out to give a snapshot of the prevalence of *Campylobacter* in RSA (Mason *et al*., 2013). In one of the studies, carried out by Mason *et al*., 2013, polymerase chain reaction (PCR) was used to examine *Campylobacter* species in adults and children with diarrhoea in RSA and the prevalence estimates were 12% and 7.5% respectively for *C. jejuni* & *C. coli* [*n*=225 samples; 34/225 children <5 years of age]. Another study was carried out in Venda, Limpopo province, RSA, where 322 stool samples (*n*=255 hospital patients; *n*=67 asymptomatic pupils) were analyzed to detect the prevalence of *Campylobacter* species in the Vembe district. The prevalence of *C. jejuni* and *C. coli* was 10.2% and 6.5% respectively and significantly associated with diarrhoea among all the organisms detected in the study (Samie *et al*., 2007). In 1983, Richardson found 30% (*n*=60 children <2 years of age with diarrhoea; 18/60) positivity of *C. jejuni* infected children of less than nine months in Soweto, RSA. Preliminary unpublished data from a Rotavirus surveillance project in RSA has found approximately 13% positivity for *Campylobacter* in stool specimens of children (<5 years of age) with acute diarrhoea (NICD annual review, 2014). These findings are much higher than previously thought, suggesting that the rates of *Campylobacter* infections have been previously underestimated in RSA.

A study in Cape Town, RSA, conducted by Lastovica (2009) gave a more comprehensive standing of the epidemiology of *Campylobacter*, where during a period of five years 5 443 strains of *Campylobacter* were isolated from diarrhoeagenic stools of children using developed culture methodologies for the isolation of *Campylobacter* species. Of the isolated species of *Campylobacter*, 40% were *C. jejuni* (32.3% *C. jejuni* subsp. *jejuni* and 7.7% were *C. jejuni* subsp. *doylei*), and 24.6% was accounted for by *C. concisus*. The developed culture method permitted the isolation of non-*C. jejuni*/*C. coli* such as *C. concisus* which was the second most detected species of *Campylobacter* (Lastovica, 2006) . The few studies conducted in African countries have shown campylobacteriosis to be prevalent in the paediatric population (Kaakoush *et al*., 2015). Lastovica and colleagues (2006)

extensively described some of the confounding factors affecting the successful isolation of *Campylobacter*, such as underestimating the relative abundance of *Campylobacter* species, fastidious nature of the organism that was not accounted for, and unoptimal growth conditions suppressing growth of *Campylobacter* (selective media, temperature, atmosphere, and incubation periods).

1.6.3. *Campylobacter* and coinfections

A study conducted by Mason *et al*., (2013) in Malawi demonstrated that in the non-diarrhoeagenic group, 16% of the children with *Campylobacter* in the specimen also had a viral coinfection. Poor hygiene and sanitation as well as close proximity to animals in developing countries intensifies frequent acquisition of any enteric pathogen, an observation which has been previously reported from studies of coinfection with *Campylobacter* (Coker *et al*., 2002; Lèvesque *et al*., 2013; Mason *et al*., 2013). Coinfection of flocks with more than one strain of *Campylobacter* has been reported, and as a result coinfection of humans with more than one *Campylobacter* strain is identified in 5 to 10% of sporadic cases (Forbes *et al*., 2009).

1.6.4. Risk factors and risk groups

I. Seasonality as a risk factor

Seasonality for campylobacteriosis in temperate and tropical countries differs (Acheson and Allos, 2001; van Vliet and Katley, 2001; Galate and Bangde, 2013). Summer and autumn *Campylobacter* peaks have been reported in temperate countries, although variation between and within countries have been reported (Nylen *et al*., 2002; Ekdahl and Andersson, 2004; Galate and Bangde, 2013). A study conducted in England and Wales showed *Campylobacter* peaks in spring, an observation which was consistent with results from previous studies (Louis *et al*., 2005). In temperate countries, campylobacteriosis is reported to increase with increasing temperature (Louis *et al*., 2005). This observation is contrary to tropical

countries, where *Campylobacter* infections do not have a marked seasonal pattern (Ekdahl and Andersson, 2004).

In the Southern part of Africa summer occurs from mid-October to mid-February, autumn from mid-February to April, winter from May to July, and spring from August to mid-October (Renssen, 2007). The weather in Africa can largely be classified as tropical; the lack of extreme temperature fluctuations, as well as the lack of surveillance for epidemics may be a contributing factor for the unrecognized *Campylobacter* seasonal peaks (Ekdahl and Andersson, 2004; Nylen *et al*., 2002; Galate and Bangde, 2013). In temperate regions, risk factors that are associated with campylobacteriosis in the summer months include animal contact, eating barbequed meals, drinking untreated water from streams and other natural sources, and most recently, flies have been postulated to be a risk factor, functioning as a mechanical vector (Nylen *et al*., 2002; Ekdahl *et al*., 2005). Studies have shown transfer of *Campylobacter* from the environment to flies with relative ease; in one such study, *Campylobacter* was isolated from 8% (4/49) of flies caught outside a broiler house in Denmark (Ekdahl *et al*., 2005).

II. Sex as a risk factor

Studies have reported that males and females differ in the way they respond to infections (Flanagan, 2014). The factors contributing to sexual dimorphism as far as infections are concerned include behavioural and physiological difference between males and females; however, there is scarcity of data supporting these viewpoints (Strachan *et al*., 2008). More recently, according to Olivier and William, (2010) the evolution of sex-specific immune defences play a role on how males differently perceive infections from females. As far as campylobacteriosis is concerned, tethered to paucity of data describing the epidemiology of the disease, explanations as to why males are more frequently implicated than females is less understood (Tauxe *et al*., 1988; Acheson and Allos, 2001). Although the significance to such findings has not yet an explanation, risk factors considered include differences in food handling practices and basic hygiene during food preparation, as well as the kind and amount of foods consumed (Samuel *et al*., 2004).

III. Age as a risk factor

Studies aimed at identifying risk groups for campylobacteriosis have reported variation in age distribution of infected individuals; being highest among those under five years of age, young adults, and the elderly (Sebbald and Sharp, 1985; Samuel *et al*., 2004; Ekdahl *et al*., 2005; Mshana *et al*., 2009; van Vliet and Katley, 2001; Cody *et al*., 2012; Weinberger *et al*., 2013). Age risk groups differ in industrialized to developing countries (Acheson and Allos, 2001; Coker *et al*., 2002). In industrialized countries, two age risk groups have been identified; those aged <1 in years and in young adults (11-44 years), with a prevalence estimate of 1-13% among children with acute diarrhoea and 1.5 % among non-diarrhoeaic children (Mshana *et al*., 2009).

In developing countries, the prevalence of *Campylobacter* infections in patients with diarrhoea range from 5-35%, and are reported to be hyperendemic among the young, particularly those aged less than two years of age (Ayyagari *et al*., 1984; Acheson and Allos, 2001; Mshana *et al*., 2009; Sheppard *et al*., 2009). According to Miller *et* al., (2005) and Weinberger *et al*., (2013) detection rates of campylobacteriosis in developing countries forms a U-shaped curve, a suggestive trend arising from repeated exposure to *Campylobacter* species in early childhood which results in acquisition of protected immunity at older age. Another aspect is that asymptomatic infections occur commonly in children and adults, an unusual trend in developed countries (Acheson and Allos, 2001; Coker *et al*., 2002). Plausible risk factors associated with *Campylobacter* infections for individuals of different age groups have been documented, which include the lack of a fully developed immune system (infants), poor hygiene, and close proximity to pets and farm animals, travel, consuming contaminated high risk foods such as poultry, stress, weakened immunity due to diseases, and age-related deteriorating of immunity (Tauxe *et al*., 1988; Acheson and Allos, 2001; Miller *et al*., 2005; Lund and O'Brien, 2011).

1.6.5. Pathogenesis

I. Source and mode of transmission

Meats, particularly of poultry origin, raw milk, and untreated water are well documented sources of human *Campylobacter* infections (Moore *et al*., 2005; Wagenaar *et al*., 2013; Fernandes *et al*., 2015). Approximately 50-70% of all *Campylobacter* infections are attributed to chicken consumption (Acheson and Allos, 2001); which is not surprising in light of the frequency with which poultry products are consumed (Acheson and Allos, 2001; Moore *et al*., 2005) as well as the nearly universal contamination of chicken carcasses with *Campylobacter* during slaughter processes (van Vliet and Ketley, 2001; Wagenaar *et al.,* 2013).

Cross-contamination in the kitchen from contaminated meat to items that will not be cooked is considered a major pathway for transmission (Acheson and Allos, 2001; Wagenaar *et al*., 2013). The infectious dose ranges from 500-800 organisms which can be carried in approximately one drop of chicken juice (Acheson and Allos, 2001; van Vliet and Ketley, 2001; Schielke *et al*., 2014). Person-to-person transmission, which occurs through the faecal-oral route is uncommon (Acheson and Allos, 2001; Schielke *et al*., 2014; Galate and Bandge, 2015; Kaakoush *et al*., 2015). Human exposure from reservoirs has been linked to multiple pathways which include: (1) food, particularly poultry; (2) the environment; and (3) direct animal contact [Figure 1.4] (Wagenaar *et al*., 2013; Kaakoush *et al*., 2015).

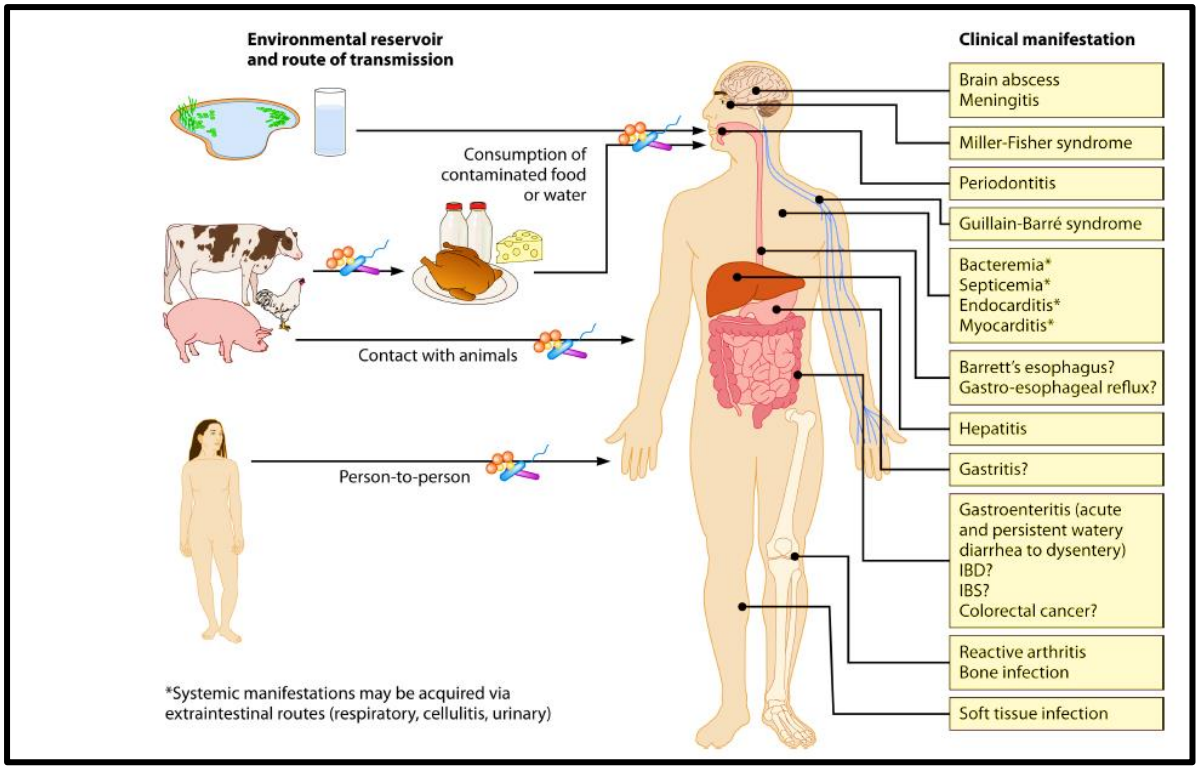


Figure 1.4: Pathogenesis of *Campylobacter* infection and the associated clinical manifestations. Abbreviations: inflammatory bowel diseases (IBD), and irritable bowel syndrome (IBS).Question marks represent conditions associated with campylobacteriosis, however with some degree of uncertainties. Image adopted from Kaakoush *et al*., (2015).

II. Virulence

Mechanisms by which *Campylobacter* species are virulent still remain largely undetermined (Perez-Perez and Blaser, 1996; Man, 2011; Galate and Bangde, 2015). A possible reason contributing to this stagnancy is the lack of pathogenesis similarity between *Campylobacter* species and other enteric pathogens (Galate and Bangde, 2015). Advances in medical model technologies aided in unraveling mechanisms by which *Campylobacter* causes infection [Figure 1.5]. According to Dastia *et al*., (2010) as well as Cróinín and Backert, (2012) flagella-mediated motility, bacterial adherence to intestinal mucosa (Man, 2001), invasive capability, and the ability to produce toxins (particularly Cytolethal distending toxin (CDT), an important virulence factor for *Campylobacter*) have been identified as virulence (Galate and Bangde, 2015). Flagella are required for the colonization of small intestine, followed by the migration to the target organ, which is the colon (van Vliet and Ketley, 2001).

Invasion, which results in cellular inflammation, has been reported to result from the production of cytotoxins, which compromises the absorptive capacity of the intestines (van Vliet and Ketley, 2001). The ability of *C. jejuni* or *C. coli* to reach the intestinal tract is, in part, due to resistance to gastric acids and also to bile salts. Disease severity may depend on the virulence of the strain, as well as on the host's immune condition (Silva *et al*., 2011; Galate and Bangde, 2015).

A study conducted to investigate incidence of toxin producing human *Campylobacter* strains in RSA found strains that produce heat-labile cytotonic toxin (CTON) and various cytotoxins (CTOX). The study which included 22 *Campylobacter* strains reported that CTON was the most commonly found toxin in those strains that were toxin-producing (Bok *et al*., 1991). Low virulence has been reported in animals; however, some strains of both *C. jejuni* and *C. coli* have the ability to cause serious diarrhoeal illness in humans (Jonker and Picard, 2010).

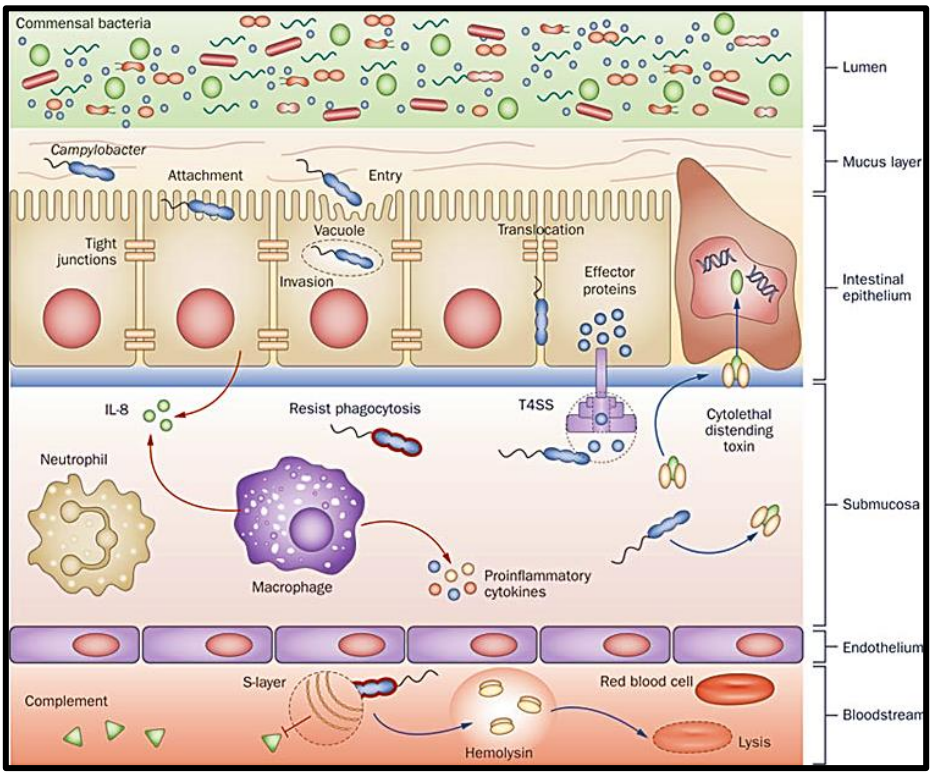


Figure 1.5: A model representation of the mechanisms of *Campylobacter* invasion of human intestinal epithelial cells and bloodstream translocation of the organism which subsequently causes haemolysis. Abbreviations: Interleukin 8 (IL8), Type IV secretion system (T4SS), and Surface layer (S-layer). Image adopted from Man, (2011).

1.6.6. Clinical characteristics of campylobacteriosis

Clinically, *Campylobacter* infections are indistinguishable from acute gastrointestinal infections caused by other bacterial pathogens (Acheson and Allos, 2001). Upon infection with *C. jejuni* and/or *C. coli*, susceptible subjects generally experience (after one to seven days) acute abdominal pain, often accompanied by fever, nausea and or vomiting, as well as general malaise (van Vliet and Ketley, 2001). Progression of symptoms is accompanied by either loose or watery profuse diarrhoea, which may contain mucous or blood (Acheson and Allos, 2001; van Vliet and Ketley, 2001; Perez-Perez and Blaser, 1996; Galate and Bangde, 2015). In developing countries, infection with *C. jejuni* and closely related organisms is less severe, without bloody diarrhoea, fever or fecal leukocytes (Perez-Perez and Blaser, 1996). The disease is usually limited to a period of five to eight days, but may continue longer and bacterial shedding often persists after clinical symptoms have ended (van Vliet and Ketley, 2001; Moore *et al*., 2005). Other clinical manifestations following campylobacteriosis are outlined in Figure 14.

1.7. Diagnosis, isolation, and identification of *Campylobacter* species

1.7.1. Phenotypic characterization of *Campylobacter*

The principle encompassing definitive diagnosis of campylobacteriosis is based on the isolation of *Campylobacter* species from a stool culture (Blaser and Reller, 1981; Perez-Perez and Blaser, 1996; UK Standards for Microbiology Investigations, 2015). However, only a small percentage of individuals suffering from campylobacteriosis consult for medical care and have their infections culture-confirmed. Alternatively, when they do consult, they submit samples for culturing after they have started antibiotic treatment, which may make it even more difficult for a laboratory to grow *Campylobacter* (Tauxe, 1992; CDC, 2010).

I. Microscopic appearance

Diagnosis can be carried out by direct examination of a stool sample using contrast microscopy or Gram's strain (Blaser, 1997; Blaser, 2000). Direct examination provides a rapid presumptive diagnosis that must still be confirmed by stool culture. Observation of darting motility in fresh fecal specimens or of vibrio forms in Gram stain provides presumptive diagnosis for *Campylobacter* (Perez-Perez and Blaser, 1996; UK Standards for Microbiology Investigations, 2015).

II. Stool culture

Sensitivity of *Campylobacter* species to oxygen and oxidizing radicals has been exploited to develop selective media for isolating *Campylobacter* from clinical specimens (Galate and Bangde, 2015). A number of selective agars have been developed, Preston, charchoal cefoperazone deoxycholate (CCDA) is one of the selective agars found to be effective because it permitted high yields of isolated *Campylobacter* strains at 42°C microaerophilically (Murray *et al*., 2007; Silva *et al*., 2011). Another technique used in conjunction with primary isolation of *Campylobacter* species on selective media is the filtration method. The method is based on the principle that campylobacters, particularly *C. jejuni* and *C. coli*, can pass through membrane filters (0.45 µm to 065 µm pore size) with relative ease while other stool flora are constricted during filtration onto the surface of the media (Murray *et al*., 2007; Galate and Bangde, 2015). One of the limitations of the culturing method is that *Campylobacter* species are fastidious organisms, taking up to 72 hours to obtain growth, thus isolation methods for these organisms are not commonly used in routine laboratories (Murray *et al*., 2007). Most routine laboratories also lack differentiating tests, limiting species differentiation of cultures.

III. Colonial morphology

On CCDA selective media, culture positive colonies for *Campylobacter* appear either grey/white or creamy grey in colour and moist in appearance. They may appear as a layer of growth over the surface of the selective agar. On blood agar, colonies are

translucent and moist in appearance (UK Standards for Microbiology Investigations, 2015).

IV. Serotyping schemes

In the 1980's two serotyping schemes for *Campylobacter* were developed in Canada; the Penner scheme, which is based on soluble heat-stable antigens; and the Lior scheme which detects variation in heat-labile antigens (Frost *et al*., 1998). The Penner scheme has been more widely used in the United Kingdom (McKay *et al*., 2001) and according to Coker *et al*., (2002) the Lior serotyping scheme has been used to determine subspecies of *Campylobacter*, particularly of *C. jejuni* and *C. coli* in laboratories in developing countries. Some of the limitations of the serotyping methods included nonspecific reactions as well as reproducibility problems (Frost *et al*., 1998; McKay *et al*., 2001).

V. Immunological aspects of *Campylobacter* infections

Serum immunoglobulin G (IgG), IgM, and IgA levels peak in response to infection, followed by a rapid decline after a few weeks post infection (Murray *et al*., 2007). In developing countries where individuals, both young and old, are constantly exposed to campylobacters in the environment, serum antibodies against *Campylobacter* species develop very early in life (Coker *et al*., 2002). Antibody titers observed in children in developing countries have been found to be significantly higher than children in developed countries (Coker *et al*., 2002). Serum antibody assays vary in both sensitivity and specificity for detecting *Campylobacter* infections, therefore, should be interpreted with caution in diagnosis of *Campylobacter* infections (Coker *et al*., 2002). Serologic testing is a useful technique for epidemiological studies and it is not recommended for routine diagnosis, as test performance appears to be population dependent (Murray *et al*., 2007; UK Standards for Microbiology Investigations, 2015).

VI. Hippurate hydrolysis

Hydrolysis of sodium hippurate is the test exploited to differentiate other species of *Campylobacter* from *C. jejuni*. A positive test with hippurate hydrolysis, along with a positive oxidase test from a culture positive strain grown microaerophilically at 42 °C, showing characteristic microscopic morphology of *Campylobacter*, should be reported as *C. jejuni*. For routine clinical purposes, no other tests need to be performed (Murray *et al*., 2007).

VII. Alternative phenotypic methods

Other rapid tests for the detection and confirmation of *Campylobacter* species include fluorescence *in situ* hybridization, and latex agglutination (Galate and Bangde, 2015). Several other rapid methods exploiting the phenotypic properties of *Campylobacter* species for detection are outlined in Table 1.1 (Murray *et al*., 2007).

Table 1.1: Properties of *Campylobacter* exploited for phenotypic identification of species.

Organism	Catalase	H_2 required	Ureas	H_2S (TSI)	Hippurate hydrolysis	Indoxyl acetate hydrolysis	Aryl sulfatase	Selenite reduction	Growth in 1% glycine
C. jejuni subsp. jejuni	+	-	-	-	+	+	V	V	+
C. jejuni subsp. Doylei	V	-	-	-	V	+	-	-	+
C. coli	+	-	-	V	-	+	-	+	+
C. fetus subsp. fetus	+	-	-	-	-	-	-	V	+
C. fetus subsp. venerealis	V	-	-	-	-	-	-	V	V
C. lari	+	-	V	-	-	-	-	V	+
C. upsaliensis	-	-	-	-	-	+	-	+	+
C. hyointestinalis subsp. hyointestinalis	+	V	-	+	-	-	-	+	+
C. hyointestinalis subsp. lawsonii	+	V	+	+[b]	-	-	-	+	V
C. lanienae	+	-	-	-	-	-	ND	+	V
C. sputorum[b] *bv. sputorum*	-	+	-	+	-	-	+	V	+
C. sputorum bv. faecalis	+	+	-	+	-	-	+	V	+
C. sputorum bv. paraureolyticus	-	+	+	+	-	-	+	V	+
C. helveticus	-	-	-	-	-	+	ND	-	V
C. hominis	-	+[c]	ND	-	-	-	ND	-	+
C. mucosalis	-	+	-	+	-	-	-	V	V
C. concisus	-	+	-	V	-	-	+	V	V
C. curvus	-	+	-	V	V	V	+	-	+
C. rectus	V	+	-	-	-	+	+	-	+
C. showae	+	+	-	-	-	V	+	-	V
C. gracilis[c]	V	ND	-	-	-	V	ND	-	+

Positive reaction (+); negative reaction (-); variable reaction (V); not determined (ND); triple sugar ion (TSI).

[b] Srains of *C. sputorum* and *C. hyointestinalis* subsp. *lawsonii* normally produce large amounts of H_2S in triple sugar ion.

[c] Anaerobic growth only.

Table adopted from Murray *et al*., (2007).

1.7.2. Molecular characterization of *Campylobacter*

The advent of molecular diagnostic technologies has circumvented the limitations of distinguishing between *Campylobacter* species based primarily on phenotypic properties commonly used in clinical laboratories (Klena *et al*., 2004; Carriço *et al*., 2013). Various sequence-based typing methods have been exploited to identify differences between species of *Campylobacter*, particularly *C. jejuni* strains, which are frequently implicated with campylobacteriosis (Klena *et al*., 2004; Quiñones *et al*., 2008; Taboada *et al*., 2013). Molecular diagnostic methods also contribute in the search for associations between genotypes and hosts, subsequently linking hosts to the source of infection for epidemiological studies of pathogens (Schouls, *et al*., 2003; Forbes *et al*., 2009). In almost all published sequence-based studies of *Campylobacter*, it is only a few isolates that have an identical genome, therefore *Campylobacter* isolates are reported to exibit genetic plasticity, which in essence is represented by a few clonal lineages (Pearson *et al*., 2003). The diversity of *Campylobacter* species is influenced by horizontal genetic exchange capabilities of the organisms (Cody *et al*., 2013). The diversity is thought to be primarily responsible for the different pathogenic properties of different isolates as seen in *C. jejuni* strains (Hofreuter *et al*., 2006).

Since assays exploiting phenotypic properties of *Campylobacter* species are often atypical and difficult to read, more robust techniques for diagnosis and species subtyping exploiting the genome of *Campylobacter* have been developed (Klena *et al*., 2004; Carriço *et al*., 2013; Taboada *et al*., 2013). Methods that are used in current research and epidemiological studies to distinguish between strains of *Campylobacter* can be categorized into three groups: (I) methods based on PCR amplification of genomic targets; (II) methods based on restriction enzyme digestion of genomic DNA; and (III) methods based on single nucleotide polymorphisms (SNPs) (Taboada *et al*., 2013). For each of the listed method category, the examples are further classified as either a molecular diagnostic tool or molecular subtyping tool below.

1.7.2.1. Molecular diagnostic PCR for *Campylobacter*

Quantitative real-time PCR (qPCR) has proven to be efficacious in determining the prevalence and epidemiological features of *Campylobacter* infections (Best *et al*., 2003; Lund *et al*., 2004; Botteldoorn *et al*, 2008; Mason *et al*., 2013). Multiplex PCR assays targeting genes specific to species of *Campylobacter* have been developed (Linton *et al*., 1996; Best *et al*., 2003; Wierzba *et al*., 2008; Mason *et al*., 2013) Target genes include the *mapA* gene specific for *C. jejuni* and the *ceuE* gene specific for *C. coli* (de Boer *et al*., 2013). Isolates from different bacteria can be identified to genus and often also to species level by sequence analysis of the *16 SrRNA* gene. Thus, regions of the *16 SrRNA* gene specific to all *Campylobacter* species have successfully been applied for the detection of *Campylobacter* with improved accuracy (Hasson *et al*., 2008).

1.7.2.2. Molecular subtyping used for *Campylobacter*

I. Pulsed-field gel electrophoresis (PFGE)

Molecular typing methods are in essence, developed to differentiate between isolates of the same species of bacteria, whereas, genotyping methods are used to identify the genetic relatedness between different strains of bacteria (Noormohamed and Fakhr, 2014). PFGE which is a primary method for typing foodborne pathogens was one of the first DNA based methods devised for typing *Campylobacter* species (Taboada *et al*., 2013; Noormohamed and Mohamed, 2014). PulseNet, which is concerned with the rapid detection of outbreaks, largely employs PFGE as a subtyping technique, which in 2001 incorporated *Campylobacter* to its database (Taboada *et al*., 2013). PFGE is based on gel electrophoresis separation of DNA fragments resulting from restriction enzyme digestion of genomic DNA, typically by *kpnI* or *SmaI* restriction enzymes for *C. jejuni* strains (Noormohamed and Mohamed, 2014; UK Standards for Microbiology Investigations, 2015). The high discriminatory power of PFGE was successfully exploited to track *Campylobacter* species in poultry farms (Taboada *et al*., 2013). However, majority of human *Campylobacter* cases are sporadic; therefore the use of PFGE would be laborious with limited benefits as far

as the detection of *Campylobacter* clusters in concerned (Taboada *et al*., 2013). The limitation of the technique as far as human cases are concerned is that epidemiologically-defined outbreak isolates could not entirely be distinguished from non-outbreak strains in one study. However, other studies have reported significant clustering of human isolates (Taboada *et al*., 2013). Due to the technique's time consuming-nature, and its requirement for special equipment and training, it is not widely used outside the reference laboratories (Taboada *et al*., 2013; Noormohamed and Mohamed, 2014).

II. Microarray comparative genomic hybridization (MCGH)

MCGH is a technique which enables the determination of the presence or absence of genes (Taboada *et al*., 2013). This is achieved through the use of competitive fluorescence *in situ* hybridization. The generated signals can be used to identify copy number changes and/or sequence divergence (Taboada *et al*., 2013). MCGH has been used in studies aimed to determine the genetic diversity of *C. jejuni*, which demonstrated evidence for significant genomic diversity in *C. jejuni* strains attributed to carriage of accessory genes which are not conserved across the entire species (Pearson *et al*., 2003; Klena *et al*., 2004; Quińones *et al*., 2008; Taboada *et al*., 2013). One study showed MCGH to have a higher resolution power compared to MLST in an epidemiological study suggesting transmission between cattle and human clinical cases (Taboada *et al*., 2013). Significant differences in gene content between isolates that were indistinguishable by MLST were revealed through comparative genomic analysis using MCGH and similar observations have been evident from whole-genome sequencing (WGS) data. Limitations that precluded the use of MCGH in molecular typing include low throughput and high cost (Taboada *et al*., 2013).

III. Multi-locus sequence typing (MLST)

Molecular differentiation of microbial isolates by the relative comparison of housekeeping gene sequence fragments involves MLST (Dingle *et al*., 2001; Dingle

et al., 2002; Sails *et al*., 2003; Schouls *et al*., 2003; Taboada *et al*., 2013). The technique primarily involves the sequencing of approximately 500 bp fragments of usually six and eight loci (Jolley *et al*., 2004; Dingle *et al*., 2005). The resulting unique fragment sequences are assigned an allele marker, and subsequently each allelic combination or the generated allelic profile for each isolate is then assigned a sequence type (ST) (Jolley *et al*., 2004). Isolates with related STs can be further grouped into clonal complexes, which are thought to be groups of organisms derived from a common progenitor (Dingle *et al*., 2002; Noormohamed and Fakhr, 2014). MLST allele sequences and ST profile tables are stored in curated databases at different geographical locations globally. Data from all databases is collected and made readily accessible through the Pubmlst website (Jolley *et al*., 2004; Noormohamed and Fakhr, 2014).

Following the successful implementation of MLST for *Neisseria meningitidis*, MLST has since been considered the "gold standard" of molecular typing, with schemes developed that cover the bacterial and fungal species (Larsen *et al*., 2012; Maiden *et al*., 2013). MLST has enabled the characterization of hypervariable genomes of *Campylobacter* species, (Dingle *et al*., 2001; Dingle *et al*., 2002; Dingle *et al*., 2005; Noormohamed and Fakhr, 2014; Sheppard and Maiden, 2015). Many of the studies carried out to understand the molecular epidemiology of campylobacteriosis using MLST focused primarily on seven housekeeping genes (Appendix A) of *C. jejuni*, which is the most frequently isolated bacterial pathogen in cases of human campylobacteriosis globally compared to *C. coli* (Dingle *et al*., 2002). Nevertheless, MLST scheme for *C. coli* has been developed (Dingle *et al*., 2001; Schouls *et al*., 2003; Miller *et al*., 2005).

C. jejuni species are genetically highly diverse organisms, represented by many distinct clonal complexes that vary from host generalist lineages commonly found in poultry, livestock, and human disease cases to host-adapted specialized lineages primarily associated with livestock or poultry (Sails *et al*., 2003; Morely *et al.,* 2015). MLST has been useful in distinguishing between isolates of *C. jejuni* subspecies *jejuni* and *C. jejuni* subspecies *doylei*, which were determined to be phylogenetically distinct (Parker *et al*., 2007). Predominant clonal complexes accounting for over 60%

of human campylobacteriosis attributed to *C. jejuni* are ST-21, ST-45, ST-206, ST-61, ST-48, and ST-257 (Dingle *et al*., 2002; Colles *et al*., 2003). A study conducted by Manning *et al*., (2003) comparing veterinary and human isolates of *C. jejuni* using MSLT indicated that isolates of animal and of human do overlap. MLST on a total of 32 clinical *C. jejuni* isolates from Southern Africa was previously performed to determine genetic variation among these isolates, from which 18 different STs were identified (Quiñones *et al*., 2008). Other studies reporting STs of South African human *C. jejuni* isolates have been documented (Manning *et al*., 2003; Ioannidou *et al*., 2013).

Transmission of *C. jejuni* from potential sources to hosts is less understood: it is not fully known whether only some of the *C. jejuni* present in environmental reservoirs are pathogenic to humans and whether all animals and microenvironments harbour the same strains (Colles *et al*., 2003; Manning *et al*., 2003; Schouls *et al*., 2003). Although the sources of *Campylobacter* are well described, the role that these sources play in the epidemiology of campylobacteriosis or how transmission from potential sources to human hosts would commonly occur remain incompletely addressed.

Traditional MLST involves a PCR amplification step using primers that are specific for the loci of the MLST scheme, followed by Sanger sequencing (Larsen *et al*., 2012). The procedure is both costly and time consuming. The advent of high-throughput sequencing techniques, tethered with the cost of DNA sequencing progressively depreciating has made it favourably less labour intensive to carry out whole-genome MLST (wgMLST) which is a gene-by-gene approach as well as MLST analysis of the seven housekeeping genes from WGS data (Larsen *et al*., 2012). In addition, MLST analysis from WGS data provides minimal experimental variation, enabling direct inter-laboratory sharing and comparison of results (Taboada *et al*., 2013). It is crucial that STs generated from WGS data is relatable to other typing schemes (Chan *et al*., 2001; Larsen *et al*., 2012). There are multiple websites with tools and servers to analyze WGS data and identify STs; The Center for Genomic Epidemiology (CGE) in Denmark Technical University (DTU) is one example which is accessible at www.cbs.dtu.dk/services/MLST (Larsen *et al*., 2012).

1.7.3. Whole genome sequencing (WGS)

The advent of next generation sequencing (NGS) technologies have resulted in rapid, high throughput, and cost effective means to carry out WGS (Loman and Pallen, 2008; Jolly and Maiden, 2010; Köser *et al*., 2012; Taboada *et al*., 2013). The sequencing-by-synthesis NGS technologies, which are already commercially available, are gradually precluding the sequencing-by-termination Sanger method. WGS technologies include the Illumina MiSeq sequencer (Illumina Inc), aimed for the smaller laboratories and the clinical diagnostic market (Quail *et al*., 2012). Studies have shown the remarkable impact made by the high-throughput function of NGS technologies on genomic research (Pinard *et al*., 2006; Morozova and Marra, 2008; Reis-Filho, 2009).

According to Cartwright *et al*., (2011) and Kösier *et al*., (2012), routine microbial sequencing in diagnostic laboratories will aid in delineation, tracking, control of hospital-acquired infections, monitoring antibiotic resistance rates and threats of new and emerging pathogens. It is being proposed that molecular epidemiology carried out for surveillance, outbreak cases, and genotypic antimicrobial susceptibility testing for fastidious pathogens best fit the applications of WGS (Köser *et al*., 2012). Recent studies have described WGS as an invaluable tool for understanding the epidemiology of *Campylobacter* in humans, farm animals, and the environment (Cody *et al*., 2013). WGS can resolve bacterial isolates with just a single base pair variation, displaying a high discriminatory power ideal for epidemiological subtyping of bacterial pathogens (Taboada *et al*., 2013). Advances in NGS platforms have made it possible to apply the technology in outbreak cases, and *C. jejuni* was one of the organisms investigated using these sequencing platforms (Taboada *et al*., 2013). The wgMLST approach carried out to characterise strains of *C. jejuni* and *C. coli* showed better strain to strain relationships than single-locus based methods (Cody *et al*., 2013; Taboada *et al*., 2013).

The limiting factor for WGS, which is likely to preclude the use of WGS data for routine *C. jejuni* subtyping, is that the capacity to generate data is somewhat surpassed by the ability to analyse and interpret it (Loman and Pallen, 2008; Taboada *et al*., 2013). However, the interpretation part of the limitations is being

circumvented by the Bacterial Isolate Genome Sequence Database (BIGSDB) which is a bioinformatics platform for evolutionary and functional analysis of genomic sequences (Jolley and Maiden, 2010).

1.7.3.1. Genomes of sequenced *Campylobacter* species

In a quest to best understand the genetics, physiology, and virulence of *Campylobacter*, a clinical human *C. jejuni* isolate, NCTC11168 was sequenced and completed in the year 2000 [Figure 1.6] (Parkhill *et al*., 2000; Fouts *et al*., 2005; Poly *et al*., 2005). The genome sequence of *C. jejuni* NCTC11168 has a circular chromosome of 1,641,481 base pair (bp) in length; a guanine (G) and cytosine (C) content of 30.6 %; as well as predicted 1,643 open reading frames (ORFs). The *C. jejuni* genome is predicted to encode 1,654 proteins and 54 stable ribonucleic acid (RNA) species (Parkhill *et al*., 2000). According to Fouts *et al*., (2005) the genome of *C. jejuni* NCTC11168 was insufficient to provide comprehensive aspects founding the biology of *Campylobacter*. The findings drove Fouts and colleagues to sequence and complete the genome of a second *C. jejuni* strain, RM1221, isolated from a chicken carcass (Fouts *et al*., 2005). The genome of *C. jejuni* RM1221 is a circular chromosome that is 1,777,831 bp in length, with a G+C content of 30.31% (Price, 2007). The predicted coding regions in the genome are approximately 1,884 with an estimated ORF length of 885 bp. The coding region is represented by 94% of the genome (Fouts *et al*., 2005).

Comparison made between *C. jejuni* NCTC11168 and *C. jejuni* RM1221 revealed unique characteristics not identified in *C. jejuni* NCTC11168. The unique features identified through experimental work in *C. jejuni* RM1221 include the colonization of chicken skin ceca, invasion of Caco-2 cells, unique lipooligosaccharide (LOS) and capsule loci, and other unique ORFs (Fouts *et al*., 2005). Another strain of *C. jejuni* that has been sequenced is *C. jejuni* 81-176, originally isolated from a patient during an outbreak associated with raw milk (Fouts *et al*., 2005; Hofreuter *et al*., 2006). This strain has been described to be highly virulent, comprising unique pathogenic features, expressed in monkeys as well as in two human trials (Hofreuter *et al*., 2006).

The *C. jejuni* 81-176 genome is a circular chromosome that is 1,616,554 bp in length, along with a G+C content of 30.6%, and it encodes 1779 genes (Price, 2007). Other species of *Campylobacter* that have been sequenced include *C. coli* (RM2228; multi-drug chicken isolate), *C. lari* (RM2100), and *C. upsaliensis* (RM3195) which were both clinical isolates, with *C. upsaliensis* isolated from a patient with Guillain-Barrè Syndrome (GBS). For *in vitro* and *in vivo* experiments, the *C. jejuni* 81116 (NCTC11828) genome has been suggested to be a genetically stable strain compared to previously described sequenced genomes of *C. jejuni*, rendering it more reliable for studies aimed at understanding the biology of *Campylobacter* (Pearson *et al*., 2007). The genome size of *Campylobacter* is relatively smaller than that of *Escherichia coli* (*E. coli*) and *Salmonella* and the reduced genome size pertains to the reduced metabolic capabilities of this organism (Pendleton *et al*., 2013).

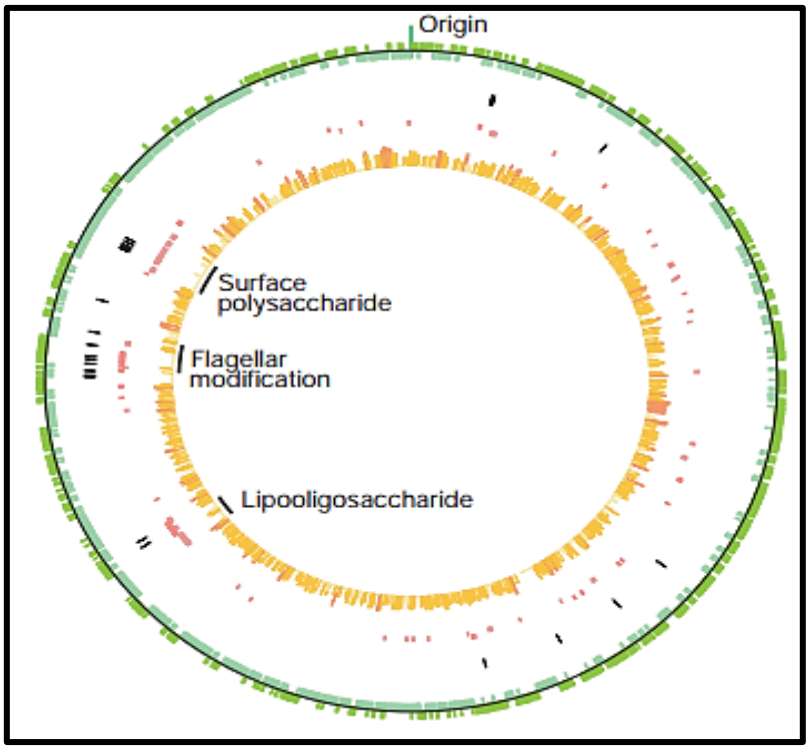


Figure 1.6: Model representation of a sequenced human *C. jejuni* NCTC11168 genome (1 641 481 bp). The outer most circle from the origin of replication represents coding sequences in both the clockwise (dark green) and anticlockwise (light green) directions. The positions of hypervariable sequences in the genome are represented in black. The genes involved in the biosynthesis of surface structures are represented in dark red (clockwise) and pale red (anticlockwise). Annotated in the innermost sphere are clusters of genes responsible for the biosynthesis of extracellular polysaccharide (EP), LOS, and flagella modification. Image adopted from Parkhill *et al*., (2000).

1.8. Treatment

Campylobacteriosis is often a self-limiting disease, with symptoms typically disappearing within one to three weeks (Schielke *et al*., 2014). For short-lived infections, fluid and electrolyte replacement are the cornerstone for treatment (Perez-Perez and Blaser, 1996; Silva *et al*., 2011). When *Campylobacter*-induced enteritis is severe and prolonged, particularly in young and immunosuppressed individuals, antimicrobial therapy is recommended for treatment of patients (Wagenaar *et al*., 2013; Galate and Bangde, 2015). The first-line choice of treatment involves macrolides (erythromycin), followed by fluoroquinolones, where ciprofloxacin is predominantly administered (Acheson and Allos, 2001; Gaudreau and Gilbert, 2003; Luber *et al*., 2003; Luangtongkum *et al*., 2007; Luangtongkum *et al*., 2009; Pollet *et al*., 2012). Other alternative drugs, depending on the severity of campylobacteriosis, include tetracylines, chloramphenicol, and serious systemic infections may be treated with aminoglycoside such as gentamicin (Acheson and Allos, 2001; Ge *et al*., 2002; Luangtongkum *et al*., 2007). All *Campylobacter* species are inherently resistant to vancomycin, rifampicin, and trimethoprim (Taylor and Courvalin, 1988; Acheson and Allos, 2001). In contrast, *Campylobacter* species are generally susceptible to aminoglycosides, chloramphenicol, clindamycin, nitrofurans, and imipenem (Acheson and Allos, 2001; Luangtongkum *et al*., 2007). In severe cases where antibiotic treatment is mandatory, appropriate and timely treatment is of importance in the light of antibiotic resistance (Moore *et al*., 2005).

1.8.1. Antimicrobial mode of action

I. Fluoroquinolones against *Campylobacter* species

Fluoroquinolones are a chemically modified form of the quinolone nalidixic acid (Ketley and Konkel, 2005). This class of antimicrobials are bactericidal agents; they impede DNA replication by targeting two bacterial enzymes [DNA gyrase (type II topoisomerase) and DNA topoisomerase IV] which play a significant role in DNA replication (Ketley and Konkel, 2005).

A few years ago fluoroquinolones were the first-line of treatment for *Campylobacter* infections requiring antimicrobial therapy (Acheson and Allos, 2001; Wimalarathna *et al*., 2013). During those years, administering these drugs empirically was advantageous because symptoms of campylobacteriosis are clinically indistinguishable from other gastrointestinal diseases caused by other organisms such as *Salmonella* or *Shigella* (Acheson and Allos, 2001; Wimalarathna *et al*., 2013), which were generally susceptible to fluoroquinolones. Resistance to quinolone treatment was first identified in the early 1990's in Asia and in the European countries such as Sweden, the Netherlands, Finland, and Spain (Acheson and Allos, 2001; Luber *et al*., 2003). The increase in quinolone resistance was tethered to licensing of the fluoroquinolone, enrofloxacin, in 1996 for veterinary use in Europe as well as the United Kingdom. As a result, the number of domestically-acquired fluoroquinolone resistant human *Campylobacter* isolates increased from 1.3% in 1992 to 10.2% in 1998 (Acheson and Allos, 2001; Ketley and Konkel, 2005).

II. Macrolides against *Campylobacter* species

Macrolides are bacteriostatic agents which inhibit protein synthesis by binding reversibly to the 50S subunit of the 70S bacterial ribosome and cause dissociation of the peptidyl-tRNA and subsequently terminating the elongation cycle of protein synthesis (Ketley and Konkel, 2005).

For campylobacteriosis that requires antimicrobial treatment, a macrolide, particularly erythromycin, has in the recent years become the first-line of treatment (Wimalarathna *et al*., 2013). The rate of erythromycin resistance has remained low and stable over the years since its introduction, particularly in developed countries (Acheson and Allos, 2001; Coker *et al*., 2002). In addition to its low and stable resistant rate properties, the drug is clinically useful in that it is of low cost, safe (children and pregnant women), allows ease of administration, and narrow spectrum activity properties. Moreover, erythromycin stearate is acid-resistant, stable, and incompletely absorbed which allows the drug to exert a contact effect throughout the bowel where most of these gastrointestinal microbes colonize (Acheson and Allos, 2001). Among many other newer macrolides, azithromycin is one of the drugs which

is effective against *C. jejuni*. However, erythromycin is chosen over these drugs because they are costly with no clinical advantage (Acheson and Allos, 2001).

III. Tetracycline against *Campylobacter* species

Tetracyclines are broad spectrum antimicrobials that also act as protein synthesis inhibitors at the bacterial ribosome level just as macrolides (Ketley and Konkel, 2005). High rates of tetracycline resistance make it a poor choice for treatment of campylobacteriosis, particularly infections caused by *C. jejuni* (Acheson and Allos, 2001; Bester and Essack, 2012).

1.9. Antimicrobial Resistance

Antimicrobial resistance is the inherent or acquired *de novo* mutation or through horizontal gene transfer (HGT)] ability of bacteria to subsist in an environment enriched with antimicrobial agent(s) (Acar and Röstel, 2001; Kumarasamy *et al*., 2010). The ability of bacteria to have mechanisms which results in higher inhibitory concentration (MIC) in comparison to the wild-type bacteria describes the definition of antimicrobial resistance in a microbiological and molecular concept (Acar and Röstel, 2001; Livermore, 2003). Mechanisms by which bacteria can achieve antimicrobial resistance include inactivation or modification of the antimicrobial, alteration of antimicrobial target site thereby reducing its binding efficacy, increased efflux or decreased influx of the antimicrobial, as well as gene duplication and amplification (Grasso *et al*., 2016) [Figure 1.7].

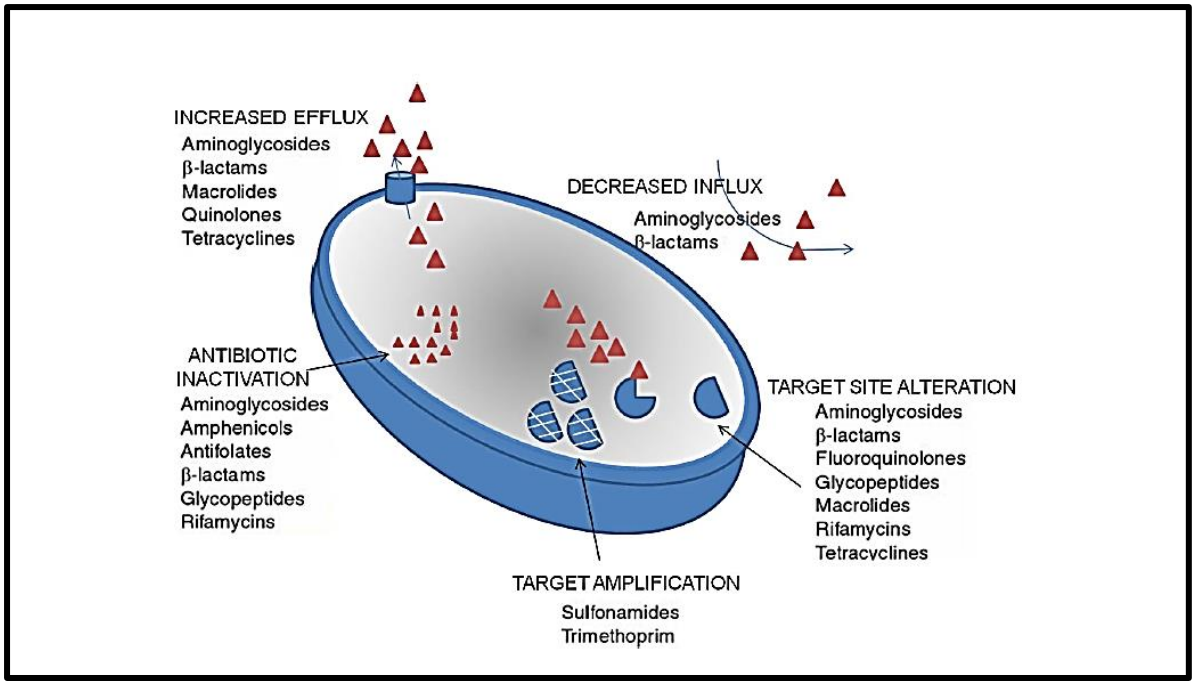


Figure 1.7: Mechanisms through which antimicrobial resistance can be achieved for the different classes of antimicrobials. Image adopted from Grasso *et al*., (2016).

1.9.1. Epidemiology of antimicrobial-resistant *Campylobacter*

Since the 1980's, there has been a growing concern of increased incidence of *Campylobacter* species being resistant to clinically used antimicrobials, particularly *C. jejuni* and *C. coli* in many European countries and other developing countries (Ge *et al*., 2002; Chokboonmongkol *et al*., 2013). The on-going use of antimicrobials in food animals has raised a concern as this could select for antimicrobial-resistant *Campylobacter* species which may ultimately reach and pose a major health-threat to humans through the food chain [Figure 1.8] (Luber *et al*., 2003; IFT, 2006; Chokboonmongkol *et al*., 2013). The fact that humans are considered to be the dead-end host necessitates the recommendation to identify the reservoirs for the acquisition of antimicrobial-resistant *Campylobacter* in poultry farms (Wimalarathna *et al*., 2013). The study of the antimicrobial resistance, as well as the resistance determinants, and their genetic composition is vital for zoonotic bacteria as this may help alleviate resistance dissemination and contribute to the understanding of the relationships that exist between bacterial species (Aarestrup *et al*., 1997; Sato *et al*., 2004; Pérez-Boto *et al*., 2014).

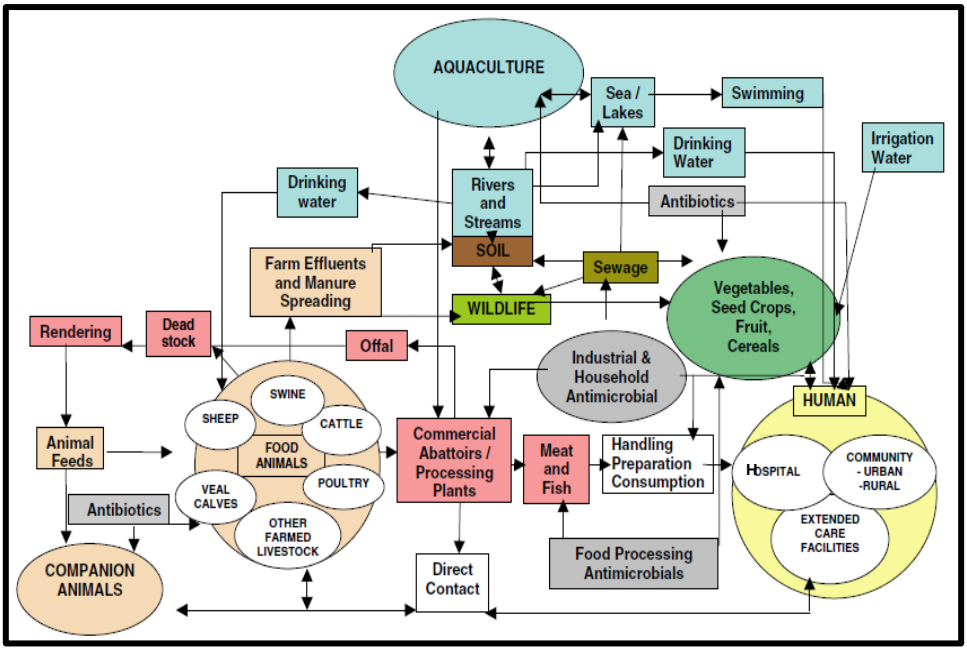


Figure 1.8: Sectors where antimicrobial use is applied and the impact it has in the ecosystem which drives the epidemiology of antimicrobial resistance and subsequently implicate human health through multiple pathways. Image adopted from IFT, (2006).

1.9.2. Prevalence of antimicrobial resistance in *Campylobacter*

As far as *Campylobacter* is concerned, resistance to erythromycin has been reported more in *C. coli* than *C. jejuni*, and coherently, many developed countries have reported low and stable rates of erythromycin resistance in *C. jejuni* strains (Aarestrup *et al*., 1997; Ge *at al*., 2002; Gaudreau and Gilbert, 2003). However, an increasing erythromycin resistance trend has been reported in developing countries (Coker *et al*., 2002). In the United States resistance to ciprofloxacin exists in almost 25% of *Campylobacter* tested and approximately 2% for azithromycin (Coker *et al*., 2002). From the year 1997 to 2011, *Campylobacter* drug resistance to fluoroquinolones has increased from 13% to 25% and as the rate of resistance increases, the clinical usefulness of recommended antimicrobials is compromised (Coker *et al*., 2002; Gouvêa *et al*., 2015). This trend is more recently seen in ciprofloxacin, where resistance to ciprofloxacin in human isolates of *C. jejuni* is increasing (Aarestrup *et al*., 1997; Acheson and Allos, 2001; Sato *et al*., 2004; Wieczorek and Osek, 2013).

A study conducted in England and Wales by Nichols *et al*., (2012) involving one million cases of campylobacteriosis from 1989 to 2009, demonstrated an increase in the percentage of *Campylobacter* isolates that were fully or intermediately resistant to ampicillin, ciprofloxacin, nalidixic acid, tetracycline and erythromycin. The percentage of strains that were resistant to ciprofloxacin was higher in people who had recently travelled abroad (1042/1601; 65%) compared with those who had not (2005/6530; 31%) and where travel status was not recorded (28646/ 90095; 32%). Isolates with a high prevalence rate of resistance to ciprofloxacin were found in people who had travelled to India (79%), Egypt (79%), Spain (78%) and Thailand (80%) (Nichols *et al*., 2012).

There is insufficient data describing antimicrobial patterns of *Campylobacter* species isolated from humans in RSA. Majority of the *Campylobacter* studies are carried out in farm and agricultural sectors. A study conducted by Jonker and Picard, (2010) which examined antimicrobial susceptibility profiles of *Campylobacter* species isolated from pigs and poultry in RSA revealed that *C. jejuni* strains from poultry origin were more resistant to fluoroquinolones, macrolides, and lincosamides. Four multi-drug resistant (MDR) *Campylobacter* strains were detected in the Western Cape region, RSA (Jonker and Picard, 2010). Another study carried out in KwaZulu-Natal, RSA in different poultry production systems showed that isolates from rurally raised chickens were significantly less resistant against ciprofloxacin (7.9%), erythromycin (0%), and tetracycline (21.6%) than isolates from industrially raised broiler and egg-laying chickens. High rate of tetracycline resistance (98.9%-100%) was detected in isolates from industrially raised chickens (Bester and Essack, 2012).

Historically, antimicrobials have been used in animal husbandries either for therapeutic use or as growth promoters, with the latter use banned in certain parts of the world. The therapeutic use of certain antimicrobials, particularly in poultry production systems has been licensed, which include, but not limited to macrolides, fluoroquinolones and tetracyclines (Wimalarathna *et al*., 2013). Variation between poultry production systems, which encompasses the use of antimicrobials, plays a role in selected antimicrobial resistance profiles of *Campylobacter* colonizing poultry (Bester and Essack, 2012).

1.9.3. Mechanisms of antimicrobial resistance in *Campylobacter*

1.9.3.1. Quinolone resistance

The quinolone resistance-determining region (QRDR) of the *gyrA* gene is the region driving quinolone resistance in *Campylobacter* species (Ketley and Konkel, 2005; Luangtonkum, *et al*., 2009; Wieczorek and Osek, 2013). The most predominant of the described mutations is the Thr88Ile which gives rise to high MIC values. Other mutations implicated with the *gyrA* gene include point mutations at the same codon: Thr86Val, Thr86lys, and Thr86Ala. Mutations in other codons include Ala87Pro, Asp90His, and Asp90Tyr which confer quinolone resistance at different MIC values (Pérez-Boto *et al*., 2014).

1.9.3.2. Macrolide resistance

Two mechanisms have been described to be the driving force of macrolide resistance in *Campylobacter* species. The most predominant mechanisms are represented by the mutations that occur in the *rrn* gene which encodes the 23S rRNA component of the large subunit (50S) of the bacterial ribosome (Ketley and Konkel, 2005; Luangtonkum, *et al*., 2009; Wieczorek and Osek, 2013). The positions of the *rrn* gene where mutations occur are 2074 and 2075. The most commonly found is the transversion A2075G in which the three copies of the *rrn* gene have mutations. This phenomenon is implicated with high MIC values against macrolides. The second mechanism through which macrolide resistance is achieved in *Campylobacter* involves mutations in specific regions of the rp/D (amino acids 55-77) and rp/V genes (amino acids 109-142). These two genes encode L4 and L22 ribosomal proteins respectively (Pérez-Boto *et al*., 2014).

1.9.3.3. Tetracycline resistance

Different mechanisms have been described for tetracycline resistance, however, for *C. jejuni* and *C. coli* in particular, the protection of the ribosomal site of the *16 SrRNA*

gene carried out by the Tet (O) protein is of importance in mediating tetracycline resistance (Ketley and Konkel, 2005; Luangtonkum, *et al*., 2009; Wieczorek and Osek, 2013). The Tet (O) protein is encoded by the *tet (o)* gene, a gene that can be carried on the chromosome and plasmids. This renders the dissemination of tetracycline resistance to be highly carried out by exchange of conjugative transmissible genetic material such as plasmids containing the *tet (o)* gene between and within species of bacteria (Ketley and Konkel, 2005; Pérez-Boto *et al*., 2014).

1.10. Postinfectious manifestations of *Campylobacter* infection

1.10.1. Guillain-Barrè Syndrome (GBS)

As with other enteropathogenic bacteria, complications may arise following campylobacteriosis. Complications that may occur following campylobacteriosis include GBS, reactive arthritis, and irritable bowel syndrome (WHO, 2013). GBS is the most severe post infectious disease and therefore of public health importance (WHO, 2013).

GBS is a reactive, self-limited, autoimmune disease triggered by a preceding bacterial or viral infection (Allos, 1997; Yuki and Hartung, 2012; Dimachkie and Barohn, 2013). For many decades the link between GBS with a preceding infection has been recognised, however, controversy lies in pointing out the primary mechanism by which GBS is elicited mainly due to the fact that a vast number of agents which include bacterial species, viruses, and strains of mycoplasma have been implicated as aetiological agents of infection preceding GBS (Aspinall *et al.,* 1994*).* With polio almost eradicated globally, GBS has now become the most common cause of acute flaccid paralysis (AFP) with an annual incidence of 0.6–4 cases per 100,000 populations (Nyati and Nyati, 2013; Kaushik *et al*., 2014). GBS which develops post *C. jejuni* infection has been investigated and reported in a number of studies rendering strains of the *C. jejuni* subject to renewed interest in their mode of pathogenesis (Aspinall *et al*., 1994; Rees *et al*., 1995; Wierzba *et al*., 2008; Nyati and Nyati, 2013).

1.10.2. *Campylobacter*-induced GBS

It was 1982 when *C. jejuni*-induced GBS was first recognized 10 days after infection with *C. jejuni* (Rhodes *et al*., 1982; Allos, 1997; Allos *et al*., 1998; Nachamkin *et al*., 1998). One of the confounding factors tempering with associating GBS antecedent *C. jejuni* enteritis is that the bacteria are usually eliminated from the body within 16 days of infection and before the onset of acute neuromuscular paralysis, which usually occurs 10 days to 3 weeks after the onset of diarrhoea (Allos, 1997; Nachamkin, 1997; Allos *et al*., 1998; Nachamkin *et al*., 1998; Acheson and Allos, 2001; Poropatich *et al*., 2010). Nevertheless, antibodies against *C. jejuni* may remain elevated for several weeks after enteritis; this has been exploited serologically to evaluate the frequency of GBS preceding *C. jejuni* infection (Allos, 1997). Many cases of *Campylobacter*-induced GBS cases go unrecognized; in part because *Campylobacter* is not routinely diagnosed in resource poor settings, as well as the possibility of bacteria having been eliminated by the time the person presents with acute neuromuscular paralysis (Allos, 1997; Poropatich *et al*., 2010). The use of antimicrobials also compromises isolating species of *Campylobacter* in culture (Nachamkin, 1997).

The spectrum of disease manifestation following *C. jejuni* infection, which may involve acute inflammatory diarrhoea and the onset of autoimmune neuropathies, could be associated with the differential expression of virulence factors in *C. jejuni* strains (Quiñones *et al*., 2008). The broad variability in the severity and spectrum of clinical symptoms in GBS patients suggests that host susceptibility may play a substantial role in disease development (Quiñones *et al*., 2008). Species of *Campylobacter*, other than *C. jejuni*, have been isolated from GBS patients (Molnar *et al*., 1982); one study reported *C. upsaliensis* isolated from a GBS patient (Nachamkin, 1997; Price, 2007).

C. jejuni-induced GBS is a severe disease which is characterized by acute or subacute symmetrical ascending motor weakness, areflexia, and mild-to-moderate sensory abnormalities (Acheson and Allos, 2001; Coker *et al*., 2002; Moore *et al*., 2005; Nyati and Nyati, 2013). The sequelae are attributed to sialylated surface polysaccharide structures and flagella of some *C. jejuni* strains, which are thought to

be responsible for the ganglioside mimicry leading to GBS (Misawa *et al*., 1998; van Vliet and Ketley, 2001). Upon infection, immune responses directed towards the infecting organism are involved in the pathogenesis of GBS by cross-reaction with neural tissues (Acheson and Allos, 2001; Mcarthy and Gieseck, 2001). The adaptive immune response comprising the humoral and cellular immune responses is triggered upon infection and because of the sharing of homologous epitopes, cross-reaction with ganglioside surface components of peripheral nerves occurs causing axonal degeneration resulting in paralysis [Figure 1.9] (Quińones *et al*., 2008; van den Berg *et al*., 2014).

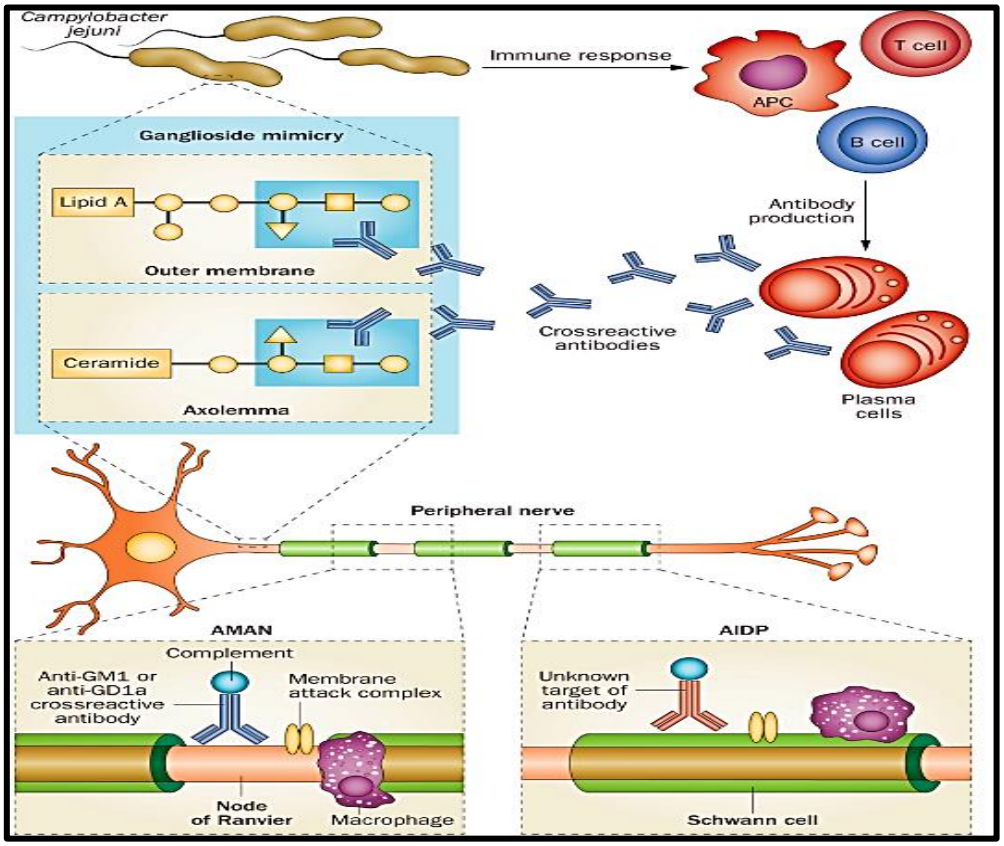


Figure 1.9: A model representation of the mechanisms facilitating the pathogenesis of *C. jejuni*-induced GBS. Abbreviations: Alpha C protein (APC); acute motor axonal neuropathy (AMAN); acute inflammatory demyelinating polyneuropathy (AIDP). Image adopted from van den Berg *et al*., (2014).

1.10.3. Clinical and electrophysiological subtypes of GBS

For a number of years GBS has been described as a single homogenous clinical entity (Dimachkie and Barohn, 2013). With insightful studies carried out, GBS has been categorized into heterogeneous clinical and electrophysiological subtypes: acute inflammatory demyelinating polyneuropathy (AIDP), acute motor axonal neuropathy (AMAN) and in a case where sensory fibres are affected, this axonal subtype is called acute motor and sensory axonal neuropathy (AMSAN) (Nachamkin *et al*., 1998; Dimachkie and Barohn, 2013; van den Berg *et al*., 2014).

1.10.4. Mechanism by which *C. jejuni* induces GBS

The mechanism by which autoimmune processes result in neuropathy is by affecting different structures (myelin or axons), at different locations (nerve root, nerve cell bodies or peripheral nerve) (Dimachkie and Barohn, 2013). Infections with *C. jejuni* are significantly associated with AMAN form of GBS (children and young adults) and not with AIDP (older population in Western countries) (Nachamkin *et al*., 1998; Kuwabara, 2007). Immune responses against *C. jejuni* have been implicated to cause AMAN by the induction of antibodies that cross-react with ganglioside structures that are expressed on the peripheral axons. Individuals who have AMAN subsequent to *C. jejuni* infection have been found to have high titers of antibodies against gangliosides GM1, GM1b, GD1a or GalNAc-GD1a (Nachamkin *et al*., 1998; Kuwabara, 2007; van den Berg *et al*., 2014).

1.10.5. Prevalence of *Campylobacter*-induced GBS

In spite of the plethora of studies aimed at understanding and elucidating *C. jejuni*-induced diarrhoea with subsequent inception of GBS, the specific clinical features and epidemiology remains a black box (Rees *et al*., 1995; Nyati and Nyati, 2013). The prevalence of *Campylobacter* in stool specimens of AFP patients and successively the description of the proportion of *C. jejuni*-induced GBS in RSA has not yet been profoundly investigated upon.

In Cape Town, RSA, one study sought to determine the frequency of *Campylobacter*-induced GBS from isolates of patients admitted to the Red Cross War Memorial Children's Hospital during the years 1994-1995 (Quiñones *et al*., 2008). *Campylobacter* species were recovered from three adults and six paediatric patients from a total of 14 patients whom submitted stool specimens (Goddard *et al*., 1997; Lastovica *et al*., 1997). A total of six of the nine isolates were identified as *C. jejuni* biotype 2, serotype O:41 (Goddard *et al*., 1997; Lastovica *et al*., 1997). The other three positive isolates were identified as *C. concisus*, *C. upsaliensis*, and *C. jejuni* biotype 1. *C. jejuni* biotype 1, serotype O:41 was newly associated with GBS, the severe form, reflecting a novel isolation of this strain confined to the Cape Town region (Goddard *et al*., 1997; Lastovica *et al*., 1997; Wassenaar *et al*., 2000; Quiñones *et al*., 2008).

The risk of developing GBS post *C. jejuni* infection is seldom, with reported cases of less than 1% (1:3000 cases) (Acheson and Allos, 2001; Moore *et al*., 2005; Nyati and Nyati, 2013). The isolation rate of *C. jejuni* from stool cultures of GBS patients range from 8% to 50% (Misawa *et al*., 1998; Nyati and Nyati, 2013). The Penner heat-stable (HS) serotypes of *C. jejuni* strains attributed to GBS include serotype HS:19 which is reported to occur globally, and serotype HS:41 reported to be restricted to Cape Town, RSA (Lastovica *et al*., 1997; Nachamkin *et al*., 2001; Coker *et al*., 2002; Moore *et al*., 2005; Poly *et al*., 2005; Quińones *et al*., 2008). A study carried out by Wassenaar *et al*., (2000), to genetically characterize *C. jejuni* 0:41 isolates in relation to GBS in Cape Town, RSA, indicated that *C. jejuni* 0:41 isolates represented a genetically stable clone for a long time.

Due to the scarcity of the development of the sequelae following *C. jejuni* enteritis, the mortality rate ranges from 2% to about 8% (Moore *et al*., 2005; Wierzba *et al*., 2008; Nyati and Nyati, 2013). Mortality from GBS has been linked with autonomic nervous system (ANS) malfunction, respiratory failure, and subsequently compromised vital functions (Newswanger and Warren, 2004; Dimachkie and Barohn, 2013). The majority of GBS patients experience partial or total recovery which can take over a period of weeks to months (Nachamkin *et al*., 1998; Dimachkie and Barohn, 2013). Approximately 20% of patients are left with severe

disability with men being more frequently affected than women (Nachamkin *et al*., 1998; Nyati and Nyati, 2013). The incidence of GBS increases with age, but some studies have shown an early peak among the 15 to 30 years old suggesting a possible bimodal distribution of cases by age (Nachamkin *et al*., 1998). Most GBS cases are sporadic; however, a seasonal trend has been reported with a surge in the summer months (Dowling *et al*., 1977; Nachamkin *et al*., 1998; Dimachkie and Barohn, 2013).

1.10.6. General clinical presentation of GBS

The disease is frequently observed one to three weeks after onset of *Campylobacter*-induced diarrhoea, and it is characterized by ascending paralysis, with patients presenting with AFP (Misawa *et al*., 1998; Acheson and Allos, 2001; Dimachkie and Barohn, 2013). Progression of the disease can lead to respiratory muscle compromise and subsequently death (Misawa *et al*., 1998).

1.10.7. Management and treatment of GBS

With the majority of GBS patients (30%) undergoing respiratory muscle compromise, good supportive care is the most fundamental element of management of the disease. Patients are admitted to the neurological intensive care unit or an intermediary care telemetry unit to allow for close and frequent monitoring of respiratory, bulbar and automatic function (Dimachkie and Barohn, 2013). Other indicated treatments for GBS include plasma exchange (PE) and intravenous immunoglobulin (IVIg). PE treatment directly facilitates the removal of humoral factors such as autoantibodies, immune complexes, complement, cytokines, and other nonspecific inflammatory mediators (Dimachkie and Barohn, 2013). IVIg treatment aids in the initiation of an anti-inflamatory cascade, the end result being alleviating autoantibody-initiated inflammation (Dimachkie and Barohn, 2013).

1.11. Prevention and control measures of *Campylobacter* infections

Diarrhoeal diseases remain a leading cause of preventable death, especially among children of less than five years of age in developing countries (Keusch *et al*., 2006). Human campylobacteriosis is primarily associated with the consumption or handling of poultry. Strategies that aim to eliminate and prevent *Campylobacter* infections consider the multiple pathways through which the pathogen reaches the human host from the source (Wagenaar *et al*., 2013). Mitigation strategies from a public health point of view facilitate effective control of food safety, including food production, processing, distribution, storage, and food preparation (Mangni and Arvntikis, 2010). Poultry contamination with *Campylobacter* takes place in the slaughterhouse as a result of high concentration of *Campylobacter* in the intestines. During automated slaughtering processes, chicken carcasses are almost universally contaminated on the surface (Acheson and Allos, 2001). Food and safety authorities sought cost-effective ways to intervene in the poultry production chain. One of the postulated strategies that aims to target the food and environmental pathway for infection is reducing the prevalence of *Campylobacter* colonization in living farm animals, decreasing the introduction of high numbers of *Campylobacter* into the slaughterhouse (Acheson and Allos, 2001; Wagenaar *et al*., 2013). This may either reduce or eliminate *Campylobacter* in the final product, resulting in a better public health impact rather than implementing site specific interventions later in the production chain (Wagenaar *et al*., 2013). According to Wagenaar *et al*., (2013), the major issue at hand concerning the risk factors in the production line is the lack of biosecurity. In theory, the implementation of biosecurity at animal husbandries should prevent the introduction of *Campylobacter* into flocks. However, the ubiquitous nature of *Campylobacter* in the environment renders biosecurity a non-eliminating approach. Flies have been found to carry *Campylobacter* in and out from broiler houses (Nylen *et al*., 2002; Ekdahl *et al*., 2005).

Control measures to combat human *Campylobacter* infections place emphasis on good hygiene practices throughout production and processing of poultry, not only in the slaughterhouse but handling of the final product by the consumers. Failure in good food-handling practices is the major pathway for transmission (Acheson and Allos, 2001; Wagenaar *et al*., 2013). Undercooked chicken is one of the underlying

causes of infection, but of minor importance because interior contamination of meat with *Campylobacter* is very minimal because campylobacters are unable to multiply outside the intestines of warm-blooded animals. By the time the final product reaches the consumer, a considerable fraction of *Campylobacter* would have died, and only a small fraction will reach the consumer (Acheson and Allos, 2001).

Cutting boards and utensils used during meat preparation orchestrate cross-contamination in the kitchen from contaminated meat to foods that are eaten raw such as salads. This route is considered the major pathway for human campylobacteriosis (Wagenaar *et al*., 2013). Consumers are required to accept some level of risk and to take appropriate precautions (Wagenaar *et al*., 2013).Transmission can be avoided by thorough washing of utensils used during meat preparation with soap and water. Unpasteurized milk has been linked to *C. jejuni* outbreak cases, and should not be consumed (Acheson and Allos, 2001; Fouts *et al*., 2005; Hofreuter *et al*., 2006). Other prevention approaches include persons with acute diarrhoea avoiding the preparation and handling of food until their illness resolves (Acheson and Allos, 2001). Good hygiene dictates that all persons should wash their hands after using the bathroom, especially if they have enteritis (Acheson and Allos, 2001). Tourists should be cautious against taking antibiotics as prophylaxis to prevent campylobacteriosis, and also when travelling to developing countries, not to drink untreated water (Acheson and Allos, 2001).

1.11.1. Vaccination

Despite the burden estimates of human campylobacteriosis globally, a vaccine as a preventative approach is still not yet available (Wagenaar *et al*., 2013). Genetic diversity and incomplete understanding of pathogenesis, along with post infectious manifestations associated with *C. jejuni*, which accounts for over 80% of all human campylobacteriosis, are some of the factors that impede current approaches aimed at developing a vaccine against these species of *Campylobacter* (WHO PD-VAC, 2014). Nevertheless, development of vaccines against *C. jejuni* for human clinical cases and in poultry production systems is still underway.

1.12. Study objectives

To date, there are limited numbers of studies comprehensively describing the molecular epidemiology of *Campylobacter* in South Africa. The current study proposed to improve the understanding of the molecular epidemiology of campylobacteriosis in South Africa by exploiting molecular diagnostic and subtyping tools.

Objectives of the study:

- To investigate the prevalence of *Campylobacter* infections in South Africa through screening of clinical isolates and stool specimens from diarrhoeagenic patients.

    1. GERMS-SA *Campylobacter* Surveillance Program
    2. Rotavirus Surveillance Program (stool specimens of children <5 years of age with sever acute diarrhoea)

- Furthermore, to investigate the prevalence of *Campylobacter* in polio-negative stool specimens of patients with symptoms of acute flaccid paralysis (AFP) to define the proportion of AFP which may be attributed to GBS post *Campylobacter* infection.

    3. AFP Surveillance Program

- To identify associated risk factors and risk groups associated with campylobacteriosis
- To investigate antimicrobial susceptibility profiles of *Campylobacter* isolates associated with diarrhoeal diseases
- To achieve this molecular tools included in our current study were real-time PCR, WGS, and MLST; these tools were used to characterize *Campylobacter* species and subtype prevalent strains associated with illnesses to better under the molecular epidemiology of *Campylobacter* in RSA.

Chapter 2: Materials and Methods

2.1. Human bacterial isolates and stool specimens

2.1.1. Bacterial isolates for *Campylobacter* surveillance

The reference centre in South Africa for enteric infections is the Centre for Enteric Diseases (CED), National Institute for Communicable Diseases (NICD). The CED now also includes surveillance for the enteric pathogen *Campylobacter*, to better understand the epidemiology of this organism in South Africa. Isolates were voluntarily sent from approximately 200 clinical microbiology laboratories across South Africa as part of the Group for Enteric, Respiratory and Meningeal disease in South Africa (GERMS-SA) laboratory-based surveillance program. The study focused on all laboratory-confirmed *Campylobacter* cases from respective laboratories sent to the CED-NICD in the years January 2014 to December 2015. Bacterial isolates were sent on either Dorset Egg slope transport medium or on CCDA selective media (plates or Transwabs) and Trypticase Soy Agar (TSA) + 5% sheep blood media. A total of 848 bacterial isolates were submitted during the January 2014-December 2015 study period from various South African provinces participating in the GERM-SA *Campylobacter* Surveillance Program.

2.1.2. Stool specimens from the Rotavirus Surveillance Program

Stool specimens sent to CED (virology division) as part of the Rotavirus Surveillance Program (implemented in 2009), which is concerned with identification of the aetiology of diarrhoeal disease in children (<5 years of age), were screened for the presence of *Campylobacter*. The Rotavirus Surveillance Program enrolled children aged <5 years with sever acute diarrhoea (three or more loose stools within a 24 hour period) who were admitted to selected sentinel hospitals in RSA. Nucleic acid extracted from stool specimens during February 2014 and December 2015 were screened for the presence of *Campylobacter*, where a total of 981 samples formed the basis of the study.

2.1.3. Stool specimens from the AFP Surveillance Program

Polio-negative stool specimens from the AFP Surveillance Program in South Africa were screened for the presence of *Campylobacter*. The AFP Surveillance Program is based at the NICD and it is concerned with the screening of all AFP cases in South Africa for polio. The case definition of a suspected polio case is any case of AFP, including GBS, in a person under 15 years of age for any reason other than severe trauma, or paralytic illness in a person of any age in which polio is suspected. Raw stool samples are collected in universal sample containers and transported to the World Health Organization (WHO) accredited Regional Polio Reference Laboratory (National Institute for Communicable Diseases) for processing and testing. For optimal isolation, two stool samples, 24 – 48 hours apart and within 14 days of onset of paralysis must be collected from each case and transported between 2-8°C. The first stool specimen for each non-polio case in RSA was screened for the presence of *Campylobacter* to investigate the proportion of AFP attributed to GBS post *Campylobacter* infection. A total of 512 polio-negative stool specimens were submitted and processed for *Campylobacter* screening in the years October 2014 to December 2015.

2.2. Bacterial isolates of animal origin

Veterinary institutes in RSA were requested to send *Campylobacter* bacterial cultures upon isolation from animals from January 2014-December 2015. Animal isolates were received from the Western Cape Veterinary institute (*n*=6) and from the University of Pretoria in Gauteng (*n*=12) between January 2014 to December 2015. The Western Cape animal isolates were from chicken (*n*=2), sheep (*n*=2), and bovine (*n*=2). All the animal isolates from Gauteng were of chicken origin.

2.3. Detection of *Campylobacter* from bacterial isolates

2.3.1. Phenotypic identification of *Campylobacter*

All received bacterial isolates were streaked onto selective media; CCDA agar plates [Diagnostic Media Products (DMP), National Health Laboratory Service (NHLS), Johannesburg, RSA] and TSA + 5% blood agar plates (DMP, NHLS, RSA), followed by incubation at 42°C microaerophilically [GENBox microaer, (BioMèrieux, Marcy-L'Etoile, France)] for 48 hours. Colonial morphology, microscopy, Hippurate hydrolysis, catalase, and an oxidase test were performed on all culturable isolates as rapid presumptive identification of positive or negative *Campylobacter* isolates. Definite identification of *Campylobacter* from culturable and non-culturable isolates was carried out using a molecular characterization tool (multiplex real-time PCR) according to Best *et al*., (2003).

2.3.2. Genomic DNA extraction for the detection of *Campylobacter*

a. Crude genomic DNA preparation from bacterial cultures

A total of 400 microliters (µL) autoclaved Tris-Ethylenediaminetetraacetic acid (TE) buffer (Appendix B) was aliquoted into 1.5 mL eppendorf tubes. A sterile loop (1 µL) was used to collect and transfer a half loopfull of bacterial culture from agar plates into 1.5 mL eppendorf tubes containing 400 µL TE buffer. The bacterial culture suspension in each tube was vortexed for 5 seconds and the tubes were placed on a heating block set at 95°C for 25 minutes (min). After incubation the tubes were vortexed for 10 seconds and centrifuged [Eppendorf MiniSpin; (Eppendorf, Hamburg, Germany)] at 13400 revolutions per minute (rpm) for 3 min at 4°C to pellet the cellular debris and obtain the DNA in the supernatant. A 20 µL of supernatant was diluted into new 1.5 mL eppendorf tubes containing 80 µL TE buffer and mixed by vortexing for 5 seconds, followed by pulse centrifugation to collect the contents at the bottom of the tube. The crude DNA was stored at -20°C for later use.

b. Crude genomic DNA preparation from non-culturable bacterial isolates

In situations where bacterial isolates submitted [Dorset Egg slope transport media (DMP, NHLS, Johannesburg, RSA)/CCDA plate or transwab/TSA + 5% sheep blood plate] were non-culturable, a loopfull of bacterial culture was collected from the dorset slope or agar plate using a 1 µL sterile loop and suspended into a 1.5 mL eppendorf tube containing 200 µL autoclaved TE buffer and processed as described in section a. Non-culturable bacterial isolates sent on CCDA transport swabs were extracted by suspending the swab into a 1.5 mL eppendorf tube containing 200 µL autoclaved TE buffer and processed as described in section a.

c. Genomic DNA preparation from stool specimens

QIAamp Fast DNA Stool Mini Kit (06/2012) [QIAGEN®, Hilden, Germany] was used to extract DNA from stool specimens received as part of the AFP Surveillance Program. The preliminary preparations of the reagents in the kit and the validation of extraction are outlined in Appendix C. DNA extraction was performed according to the manufacturer's instructions. The initial step involved suspending a pea sized stool sample (~200 mg) in a 2 mL microcentrifuge tube with 1 mL InhibitEX Buffer. The tubes were vortexed until the stool sample was thoroughly homogenized. The suspension was incubated at 70°C for 5 min on a heating block, followed by vortexing for 15 seconds. The Eppendorf 5415R centrifuge (DJB Labcare, UK) was used at 13200 rpm to pellet the stool suspension and 200 µL of the supernatant was transferred into a new 1.5 mL centrifuge tube containing 15 µL of proteinase K. Two hundred microliters of lysis buffer (Buffer AL) was added into the proteinase K-supernatant mixture. The tubes were vortexed for 15 seconds and incubated at 70°C for 10 min. Furthermore 200 µL of absolute ethanol (Merck Chemicals Ltd., Nottingham, England and Wales) was added to the lysate which was vortexed and spun down.

Six-hundred microliters of the lysate was transferred into a QIAamp spin column in a 2 mL collection tube and centrifuged at 13200 rpm for 2 min. The QIAamp spin column was transferred into a new 2 mL collection tube and 500 µL of wash buffer (Buffer AW1) was added into the QIAamp spin column and centrifuged at 13200 rpm

for 2 min. The QIAamp spin column was again placed into a new 2 mL collection tube and 500 µL of a second wash buffer (Buffer AW2) was added into the QIAamp spin column and centrifuged at 13200 rpm for 3 min. To eliminate the chance of possible Buffer AW2 carryover, the QIAamp spin column was transferred into a new 2 mL collection tube and centrifuged at 13200 rpm for 3 min. The QIAamp spin column was transferred into a new, labelled 1.5 mL eppendorf tube and 200 µL of elution buffer (Buffer ATE) was added and incubated for 5 min at room temperature (RT). Subsequently, the DNA was eluted into the 1.5 mL microcentrifuge tube by centrifuging at 13200 rpm for 3 min and stored at -20°C.

2.3.3. Genotypic detection of *Campylobacter* using multiplex real-time PCR

A previously described multiplex real-time PCR assay (Best *et al*., 2003; Lund *et al*., 2004; Botteldoorn *et al*., 2008) was performed to identify *Campylobacter* at species level from genomic DNA and nucleic acid prepared from bacterial isolates and stool specimens. The assay detected the presence of *C. jejuni* and *C. coli* by targeting genes that are specific for each species. The targeted genes were *mapA* gene specific for *C. jejuni* and the *ceuE* gene specific for *C. coli*. A *16SrRNA* gene unique to all *Campylobacter* species was also targeted which served as both an internal amplification control and for the identification of non-*C. jejuni*/*C. coli* species of *Campylobacter*. A non-template control (NTC) was also incorporated to rule out possible false positive tests as a result of contamination. Target gene sequences, primers, and probes used for *Campylobacter* multiplex real-time PCR are indicated in Appendix D.

A multiplex real-time PCR was performed in an Optical 96-well Reaction Plate (Applied Biosystems® by Life Technologies, Shanghai, China). For each sample the following reagents were added: 25 µL TaqMan Gene Expression Master Mix (Applied Biosystems®, Foster City, USA); 3 µL *Campylobacter* primer/probe mix (Appendix D); 12 µL internal positive control (IPC) mix (Applied Biosystems®, Foster City, USA); 1-7 µL genomic DNA (Appendix D); and autoclaved deionized water to make up to a final PCR reaction volume of 50 µL. The control strains (WHO) used for each run are outlined in Appendix D.

After adding all the reagents the wells were capped with Optical caps (Applied Biosystems® by Life Technologies, Shanghai, China) and the plate was briefly centrifuged using the Allegra™ X-22R centrifuge (Beckman Coulter™, Fullerton, USA) at 13000 rpm for 5 seconds to settle the fluid at the bottom of the reaction wells. The PCR reaction was carried out on the Applied Biosystems (AB) 7500 real-time PCR machine (Applied Biosystems®, Foster City, USA) and the cycling conditions for a 50 µL reaction were set as follow: initial heating stage at 50°C for 2 min (1 cycle); second stage invovled two denaturation steps at 95°C for 10 min (1 cycle), followed by 95°C for 15 seconds. The third stage was conducted at 60°C for 1 min (40 cycles) and the results were viewed and analyzed using the 7500 System SDS Software after 90 min. Interpretations of real-time PCR results for *Campylobacter* are outlined in Appendix D.

2.4. Antimicrobial susceptibility testing of *Campylobacter* isolates

Antimicrobial susceptibility profiles of viable bacterial cultures that were positive for *Campylobacter* were examined and a total of 229 *Campylobacter* isolates [*C. jejuni* (*n*=185), *C. coli* (*n*=40), and other non-*C. jejuni*/*C. coli* species (*n*=4)] were tested against erythromycin, azithromycin, ciprofloxacin, and tetracycline (BioMèrieux, Marcy-L'Etoile, France). Antimicrobial susceptibility profiles of the isolates were determined using the Epsilometer test (Etest) method and analyzed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines issued for the year 2014 and 2015 (CLSI, 2014 suppl 24; CLSI, 2015 suppl 25).

Upon initial use of a new Etest batch, the Etest strips were validated by using control strains (*E. coli* ATCC 25922 and *K. Pneumonia* ATCC700608). The controls strains ought to be susceptible to all tested antimicrobials in order to deem the Etest and antimicrobial susceptibility test valid. *Campylobacter* isolates were sub-cultured on TSA + 5% Sheep blood for 48-72 hours at 42°C microaerophilically. When growth was obtained after 48-72 hours on TSA + 5% Sheep blood plates, a 0.5 MacFarland bacterial suspension was prepared and measured using a Microscan Turbidity Meter (Dade Behring, Deerfield, USA) for each isolate in sterile saline (DMP, NHLS, Johannesburg, RSA). A sterile swab was immersed in the bacterial suspension with

the subsequent release of excess suspension on the inside wall of the tube. By using a rotating plate holder (BioMérieux, Askim, Sweden), the swab was uniformly swabbed on the surface of a pre-incubated (RT) 10% blood agar plate (DMP, NHLS, Johannesburg, RSA).

Subsequently, two different Etest strips [erythromycin and azithromycin; ciprofloxacin and tetracycline (BioMèrieux, Marcy-L'Etoile, France) were placed in each plate, with the Etest strips facing in opposite directions. The plates were then incubated for 48 hours at 42°C microaerophilically. The bactericidal or bacteriostatic nature of each antimicrobial dictated how the minimum inhibitory concentration (MIC) was read. Bactericidal antimicrobial MIC was read at the point of complete (100%) inhibition of growth and bacteriostatic antimicrobial MIC was read at 80% growth inhibition. The results were reported as being susceptible, intermediate or resistant according to CLSI guidelines for each antimicrobial (CLSI, 2014 suppl 24; CLSI, 2015 suppl 25).

2.5. Molecular subtyping of *C. jejuni* isolates using MLST

2.5.1. Genomic DNA extraction

Extraction of genomic DNA was carried out using the QIAamp DNA Mini Kit (QIAGEN®, Hilden, Germany) as outlined by the manufacturer. Preparation of reagents was carried out as indicated in Appendix E. Using a sterile 1 µL inoculating loop, bacterial cultures of pure *C. jejuni* isolates cultured on TSA + 5% Sheep blood agar microaerophilically were collected (loop full) and resuspended in 180 µL tissue lysis buffer (Buffer ATL) and vortexed. Twenty microliters of proteinase K was added into the culture suspension and mixed by vortexing, followed by incubation at 56°C for 90 min. Following the incubation step, 200 µL of lysis buffer (Buffer AL) was added to the suspension, mixed by a pulse vortex step and incubated at 70°C for 10 min. A total volume of 200 µL absolute ethanol (Merck Chemicals Ltd., Nottingham, England and Wales) was added to the lysate, and mixed by vortexing.

The lysate was transferred to a QIAamp Mini spin column and centrifuged at 8000 rpm for 2 min. The QIAamp Mini spin column was transferred to a clean 2 mL collection tube, where 500 µL of wash buffer (Buffer AW1) was added into the spin column and centrifuged at 8000 rpm for 2 min. Subsequently, the QIAamp Mini spin column was transferred once again to a clean 2 mL collection tube, where 500 µL of a second wash buffer (Buffer AW2) was added into the spin column and centrifuged at 13400 rpm for 3 min. An additional centrifugation step was performed to remove excess AW2 buffer. The QIAamp Mini spin column was then transferred into a newly labelled 1.5 mL microcentrifuge tube and 200 µL of elution buffer (Buffer AE) was added into the QIAamp Mini spin column and incubated for 5 min at RT. DNA was eluted into the 1.5 mL microcentrifuge tube by centrifuging at 13200 rpm for 3 min and stored at -20°C.

2.5.2. Quantification of extracted genomic DNA

Extracted genomic DNA for MLST was quantified using the Qubit™ dsDNA BR Assay kit (Life Technologies Corporation, Carlsbad, USA). Once the reagents had reached RT, the Qubit™ Working Solution was prepared (Appendix E) for each standard and sample and the quantification assay was carried out in 0.5 mL tubes. Following the preparation of the assay tubes, the tubes were mixed by pulse vortexing and incubated for 2 min at RT. The standards (Standard #1 and Standard #2) were initially inserted in the Qubit® 2.0 Fluorometer (Life Technologies Corporation, Carlsbad, USA) respectively to calibrate the fluorometer, followed by reading the DNA samples. The dilution feature of the Qubit® 2.0 Fluorometer was used to determine the stock concentration of each sample in nanogram per mole (ng/mol). The accepted minimum genomic DNA concentration for WGS was 10 ng/mol.

2.5.3. WGS and analysis of WGS data

Purified and quantified genomic DNA of 111 *C. jejuni* isolates [human hosts (*n*=99), and animal hosts (*n*=12)] were sequenced by the technicians at the NICD Sequencing Core Facility (NSCF) using the Illumina MiSeq genome analyzer (StarSeq, Mainz, Germany). Following DNA extraction, Nextera XT DNA sample

preparation kit (Illumina San Diego, CA, US) was used to generate multiplexed paired-end libraries which required 1 ng of extracted DNA as starting material (48 samples per run). During the library preparation input DNA was tagmented (tagged and fragmented) by Nextera XT transposome. The Nextera XT transposome simultaneously fragmented the input DNA and added adaptor sequences to the end, allowing amplification by PCR in subsequent steps. The resulting DNA was amplified using Nextera XT Indexing tags, to generate multiplexed paired-end libraries which allowed pooling of samples on the MiSeq. Genome sequencing was carried out using an Illumina Miseq instrument to generate 300 bp paired-end reads with 100x genome coverage. The resultant reads were checked for quality, trimmed and de novo assembled using the CLC Genomics Workbench software version 7.5.2 (CLC Bio-Qiagen, Aarhus, Denmark) at the CED.

2.5.4. MLST from WGS data of *C. jejuni* isolates

The resultant genomic assemblies of *C. jejuni* isolates were analyzed on the basis of molecular subtyping using MLST targeting the seven housekeeping genes from generated WGS data. MLST pipelines developed at CGE in DTU based on the seven housekeeping genes for *C. jejuni* (Appendix A) were used to define allele sequences and STs for the *C. jejuni* isolates in our current study (accessible at https://cge.cbs.dtu.dk//services/MLST/). Clonal complex assignment for the isolates was carried out using tools available on the Bacterial Isolate Genome Sequence Database (BIGSDB) accessible at http://pubmlst.org/software/database/bigsdb/.

2.6. Data analysis

Results (phenotypic, real-time PCR, and antimicrobial susceptibility testing) of all bacterial isolates and stool specimens included in the study were illustrated by using tables on Microsoft Excel 2010 and statistical analysis was performed using Epi Info 7 (CDC). Demographic data for case-patients from the various surveillance programs included in the study were obtained from the Access Database at the NICD. Testing for statistical significance was performed using Chi-squared and Fisher's Exact tests from which a p-value of <0.05 was taken to be statistically significant.

The analysis of subtyped *C. jejuni* isolates by MLST from WGS data was carried out by collecting generated MLST profile data in BioNumerics Software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). Phylogenetic relationships between strains of *C. jejuni* were calculated and defined on the basis of STs by using unweighted pair group method with arithmetic mean (UPGMA) cluster analysis and Minimum Spanning Trees (MSTs) were generated on the basis of the allele sequence profiles of the seven housekeeping genes of *C. jejuni*. The generated dendrogram was performed using the categorical coefficient with a zero (0) tolerance and the MSTs were constructed using the MST categorical coefficient on Bionumerics. The Simpson's index of diversity was calculated for all typable *C. jejuni isolates* with defined STs to statistically determine the extent of diversity that exist within the *C. jejuni* isolates in the study. The diversity calculator was accessed at http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl.

Chapter 3: Results

3.1. *Campylobacter* Surveillance Program

3.1.1. Submission of bacterial isolates during 2014 and 2015

During the course of the study, which commenced from January 2014 to December 2015, a total of 848 bacterial cultures were submitted from various GERMS-SA participating laboratories in South Africa [Table 3.1]. The results obtained were stratified by year (2014 and 2015) of submission, during which 320 isolates were submitted in 2014 and 528 were submitted in 2015 [Table 3.1]. Testing for statistical significance by year, demographic characteristics, and specimen type respectively was performed using Chi-squared and Fisher's Exact tests. The difference in the number of received isolates based on age categories, sex, and specimen type during the two study years were not statistically significant (p-value >0.05) [Table 3.1]. However, annual occurrences of infections, and submission of isolates by province, as well as culture viability of isolates was significantly different between 2014 and 2015 (p-value <0.05) [Table 3.1].

The age range of individuals from whom bacterial cultures were submitted in this study was less than one years to 90 years [Table 3.1]. Majority of these case patients were male (53%; 452/848) children of less than five years of age (35% (297/848). The provinces that highly participated in the *Campylobacter* Surveillance Program during 2014 and 2015 were the Gauteng and Western Cape Provinces, with a prominent year to year increase (p-value <0.01) in the number of isolates submitted. These provinces accounted for an overall of 47% and 45% respectively, while 8% was accounted for by the remaining provinces, with the exception of the Eastern Cape. Majority of the submitted bacterial isolates were from stool cultures (99%; 840/848), with only 1% (8/848) representing those bacterial isolates from blood cultures [Table 3.1]. Overall, 52% of the submitted bacterial isolates were non-viable following submission, 45% were viable, and the remaining 3% was accounted for by contaminated isolates and those lost in transit [Table 3.1].

Table 3.1: Demographic information of the study population collected during the years 2014 and 2015.

Characteristic	Overall (*n*=848) (%)	2014 (*n*=320) (%)	2015 (*n*=528) (%)	£p-value
Age (years)				
Median (€IQR)	20 (2-45)	20 (2-40)	19 (2-47)	0.99
Range	<1-90	<1-90	<1-90	
Age category				
<1-4	297 (35)	108 (34)	189 (36)	0.57
5-14	94 (11)	38 (12)	56 (11)	
15-24	71 (8)	30 (9)	41 (8)	
25-34	96 (11)	39 (12)	57 (11)	
35-44	72 (8)	35 (11)	37 (7)	
45-54	65 (8)	19 (6)	46 (9)	
55-64	57 (7)	17 (5)	40 (8)	
≥65	93 (11)	33 (10)	60 (11)	
*Unk	3 (<1)	1 (<1)	2 (<1)	
Sex				
Female	393 (46)	149 (46)	244 (46)	0.88
Male	452 (53)	169 (53)	283 (53)	
Unk	3 (<1)	2 (<1)	1 (<1)	
ØProvince				
Free State	3 (<1)	3 (<1)	0	<0.0001
Gauteng	405 (47)	189 (59)	216 (41)	
KwaZulu-Natal	26 (3)	17 (5)	9 (2)	
Limpopo	28 (3)	4 (1)	14 (3)	
Mpumalanga	6 (<1)	3 (<1)	3 (<1)	
North West	2 (<1)	1 (<1)	1 (<1)	
Northern Cape	7 (<1)	5 (2)	2 (<1)	
Western Cape	381 (45)	98 (30)	283 (53)	
ΦAnnual quarter				
Jan-Mar (Q 1)	257 (30)	56 (18)	201 (38)	<0.0001
Apr-Jun (Q 2)	151 (18)	20 (6)	131 (25)	
Jul-Sep (Q 3)	166 (20)	71 (22)	95 (18)	
Oct-Dec (Q 4)	274 (32)	173 (54)	101 (19)	
Specimen type				
Stool	840 (99)	318 (99)	522 (99)	0.72
Blood culture	8 (1)	2 (<1)	6 (1)	
Culture viability				
Contaminated	22 (2)	9 (3)	13 (2)	0.04
Missing	1 (<1)	0	1 (<1)	
Non-viable	438 (52)	145 (45)	293 (55)	
Viable	387 (45)	166 (52)	221 (42)	

Number of isolates (*n*); Percentage (%); €Interquartile range (IQR); ΦAnnual Quarter (Q); *Unknown (Unk); ØEastern Cape *n*=0; £p-value <0.05 statistically significant.
Footnote: Findings were stratified by year of study, therefore p-value represents whether or not findings in year 2014 were significantly different from findings in year 2015. For all statistical analysis unknowns were omitted.

3.1.2. Proportion of real-time PCR positive isolates for *Campylobacter*

Ninety-four per cent (801/848) of all submitted bacterial isolates during January 2014 to December 2015 were positive for *Campylobacter* by a multiplex real-time PCR assay [Table 3.2]. Of the positive isolates, 38% were those isolates submitted in the year 2014, and 62% were those submitted in the year 2015. Collectively, 6% (47/848) represented those isolates that were negative for *Campylobacter* species in both study years. A total of 94% of the isolates submitted were accurately identified by GERMS-SA participating laboratories in the *Campylobacter* Surveillance Program.

3.1.3. Detected species of *Campylobacter* by real-time PCR

Of the total real-time PCR positive *Campylobacter* isolates (801/848), *C. jejuni* was the most predominant species detected in this study, accounting for an overall positivity rate of 83% (665/801) [Table 3.2]. *C. coli* was infrequently detected, accounting for only 12% (97/801) of the total positive cases. Coinfection with both *C. jejuni* and *C. coli* was identified 16 times throughout the study period, accounting for 2% (16/801). Several cases of *Campylobacter* species other than *C. jejuni* or *C. coli* were detected, which accounted for 3% (23/801). The detection of *Campylobacter* species by year of isolation was slightly higher in the year 2014 (86%; 260/303) for *C. jejuni* as compared to the year 2015 (81%; 405/81). *C. coli* was slightly more detected in the year 2015 (14%; 69/498) as compared to the year 2014 (9%; 28/303), whereas the detection of coinfections and non-*C. jejuni*/*C. coli* cases was the same in both respective study years (3%). However the differences seen by year were not statistically significant (p-value 0.28).

3.1.4. Sex distribution of characterized species of *Campylobacter*

Males were slightly more frequently associated with all detected species of *Campylobacter* infections compared to females, with an overall positivity rate of 53.6% (429/801) for males, and 46% (369/801) for females in both study years [Table 3.3]. Patients of unknown sex with isolates positive for *Campylobacter* were <1% (3/801).

Table 3.2: Distribution of real-time PCR confirmed *Campylobacter* isolates during the 2014-2015 study period.

Characteristic	*(Jan 2014-Dec 2015) Overall positive (%) *n*=801	2014 Total positive (%) *n*= 303	2015 Total positive (%) *n*=498	£p-value
Specimen type				
Stool	796 (99)	302 (99.7)	494 (99)	0.41
Blood culture	5 (<1)	1(<1)	4 (1)	
Age group (years)				
<1-4	281 (35)	104 (34)	177 (36)	0.40
5-14	90 (11)	35 (12)	55 (11)	
15-24	67 (8.4)	29 (10)	38 (8)	
25-34	95 (12)	39 (13)	56 (11)	
35-44	68 (8.5)	33 (11)	35 (7)	
45-54	62 (7.7)	18 (6)	44 (9)	
55-64	52 (6.5)	16 (5)	36 (7)	
≥65	84 (10)	29 (10)	55 (11)	
Unk	2 (<1)	0 (0)	2 (<1)	
Province				
Free State	3 (<1)	3 (1)	0 (0)	<0.001
Gauteng	379 (47)	180 (59)	199 (40)	
KwaZulu-Natal	25 (3)	16 (5)	9 (2)	
Limpopo	17 (2)	4 (1)	13 (3)	
Mpumalanga	4 (<1)	3 (1)	1 (<1)	
North West	6 (<1)	5 (2)	2 (<1)	
Northern Cape	2 (<1)	1 (<1)	1 (<1)	
Western Cape	364 (45)	91 (30)	273 (55)	
Annual quarter				
Jan-Mar (Q 1)	233 (28)	52 (17)	181 (36)	<0.001
Apr-Jun (Q 2)	146 (18)	19 (6)	127 (26)	
Jul-Sep (Q 3)	161 (20)	68 (22)	93 (19)	
Oct-Dec (Q 4)	261 (33)	164 (54)	97 (19)	
£***Campylobacter* spp.**				
C. jejuni	665 (83)	260 (86)	405 (81)	0.28
C. coli	97 (12)	28 (9)	69 (14)	
Mixed	16 (2)	7 (2)	9 (2)	
Other	23 (3)	8 (3)	15 (3)	

*****January (Jan) and December (Dec). £*Campylobacter* spp.: mixed (positive for both *C. jejuni* and *C. coli*); Other (positive for non-*C. jejuni*/*C. coli*). £p-value <0.05 statistically significant.

3.1.5. Detection of *Campylobacter* by age groups

Case patients in this study displayed a median age of 20 years and an Interquartile Range (IQR) of 2-40 in the year 2014 and a median age of 19 years (IQR=2-47) in the year 2015; of which the difference between 2014 and 2015 was not significant (p-value 0.99) [Table 3.1]. The difference in the number of submitted bacterial isolates based on age groups in year 2014 and 2015 respectively were not statistically significant (p-value 0.57) [Table 3.1] as well as the detection of *Campylobacter* in those respective years (p-value 0.40) [Table 3.2].The age categories chosen for the study population displayed three peaks for both study years combined [Table 3.2]. The peaks were represented by individuals in the 0-4 age group, 25-34 age group, and those aged ≥65 in years. In this study, children in the 0-4 age group were more implicated with campylobacteriosis and they accounted for an overall positivity rate of 35% (281/801). The second most implicated group were young adults aged 25-34 in years, accounting for 12% (95/801) of the total positive cases. The elderly (≥65 years) were another group of individuals who showed a *Campylobacter* infection fraction of note following the young adults, accounting for 10% (84/801) of the total *Campylobacter* positive cases.

Table 3.3: The overall distribution of real-time PCR positive *Campylobacter* isolates submitted in the years 2014-2015 by age-group and sex.

Age group	**Sex**			**Overall Positive (%)**	**£p-value**
	Female	Male	Unk		
0-4	113	166	2	281 (35)	0.02
5-14	37	53	0	90 (11)	
15-24	29	38	0	67 (8.4)	
25-34	54	41	0	95 (12)	
35-44	39	29	0	68 (8.5)	
45-54	30	31	1	63 (7.9)	
55-64	22	30	0	52 (6.5)	
≥65	44	40	0	84 (10)	
Unk	1	1	0	2 (<1)	
Total positive (%)	369 (46)	429 (53.5)	3 (<1)	801 (94)	

£The total number of positive isolates for females and males per age category were evaluated for any variation; the overall differences for sex per age-group was of statistical significance (p-value of 0.02).

3.1.6. Detection of *Campylobacter* by both sex and age groups

Overall males were slightly more associated with the positive cases of *Campylobacter* in this study, however, instances where females were slightly more associated with campylobacteriosis than males were seen in age groups 25-34, 35-44, and those aged ≥65 years [Table 3.3]. Overall there was a difference in sex distribution which was significant (p-value 0.02).

3.1.7. Invasive cases of *Campylobacter*

During January 2014 to December 2015, a total of eight (1%; 8/848) invasive isolates attributed to *Campylobacter* infection were submitted [Table 3.1]. In the year 2014, two invasive isolates (1%; 2/320) were submitted, of which only one of the two cases (0.3%; 1/320) were positive for *Campylobacter* species, which was other than *C. jejuni*/*C. coli* by real-time PCR. In the year 2015 six invasive isolates (1%; 6/528) of *Campylobacter* infection were submitted, and less than 1% (4/528) of these isolates were positive for *Campylobacter* [*C. jejuni (n*=3*);* and non-*C. jejuni/C. coli (n*=1*)*] based on real-time PCR results. Collectively, less than 1% (5/848) of the submitted isolates was invasive cases that were positive for *Campylobacter* by real-time PCR in this study.

3.1.8. Annual occurrences of *Campylobacter* infections

In the years 2014 and 2015 [Table 3.1] the total number of isolates submitted during the first annual quarter were represented by 18% and 38% for the year 2014 and 2015 respectively. This was followed by a decrease in the number of submitted isolates during the second annual quarter (6% in 2014 and 25% in 2015). In 2014 the total number of submitted isolates increased from 6% (quarter two) to 22% (quarter three) and then to 54% (quarter four). In 2015 the submitted isolates further decreased from 25% (quarter two) to 18% (quarter three) and then increased to 19% (quarter four). The difference in annual occurrences of *Campylobacter* by year were significant (p-value <0.01).

This trend by year held true for the number of detected *Campylobacter* species in those respective annual quarters using real-time PCR [Table 3.2]. Collectively, 28% (233/801) of the positive cases of *Campylobacter* by real-time PCR were submitted during the first annual quarter, followed by a decrease in the number of positive *Campylobacter* cases in the second quarter, represented by 18% (146/801) of the positive cases. The number of positive *Campylobacter* cases increased from the third quarter, represented by 20% (161/801), to 33% (261/801) in the fourth quarter. Notably, the majority of the *Campylobacter* positive isolates were submitted during the fourth quarter of 2014 and the first quarter of 2015, represented by 20% (164/801) and 23% (181/801) respectively [Table 3.2].

3.1.9. Antimicrobial susceptibility profiles of *Campylobacter*

During January 2014 to December 2015, only 59% (229/387) of the viable isolates represented in Table 3.1 remained viable and therefore were tested against the antimicrobials: erythromycin, azithromycin, ciprofloxacin, and tetracycline. Isolates showed the highest sensitivity to erythromycin, with 90% (207/229) of the isolates being sensitive against the antimicrobial and only 10% (22/229) representing those isolates that were resistant [Figure 3.1]. Eighty-six per cent (198/229) of the isolates were sensitive against azithromycin and only 14% (31/229) were resistant. The isolates showed high resistance against ciprofloxacin, represented by 53.3% (122/229) and only 42.4% (97/229) accounted for isolates sensitive against ciprofloxacin, while intermediate resistance was seen in eight isolates, which accounted for 4.3% (10/229) of the total isolates. The isolates displayed a similar resistance pattern against tetracycline as for ciprofloxacin; 53% (121/229) were resistant against tetracycline, while only 43% (99/229) were sensitive. A total of 4% (9/229) of the isolates were intermediately resistant against tetracycline. Of the total isolates, 3.1% (7/229) were resistant against all antimicrobials tested in the study.

I. Species-specific antimicrobial susceptibility profile: *C. jejuni*

Of the total isolates tested, 81% (185/229) were *C. jejuni* [Table 3.4]. *C. jejuni* isolates were sensitive against the tested antimicrobials as follows: erythromycin

(92%; 170/185), azithromycin (88%; 163/185), ciprofloxacin (42%; 77/185), and tetracycline (44%; 81/185). Resistance against antimicrobials were lower for macrolides [erythromycin (8%; 15/185); and azithromycin (12%; 22/185)], however, higher for ciprofloxacin (54%; 100/185) and tetracycline (52%; 97/185). A few cases of intermediate resistance against both ciprofloxacin and tetracycline were seen, which accounted for 4% (8/185) and 4% (7/185) respectively.

II. Species-specific antimicrobial susceptibility profile: *C. coli*

Seventeen per cent (40/229) of the tested isolates were *C. coli* [Table 3.4]. *C. coli* isolates were sensitive to the tested antimicrobials as follows: erythromycin (82.5%; 33/40), azithromycin (77.5%; 31/40), ciprofloxacin (47.5%; 19/40), and tetracycline (45%; 18/40). Resistance to antimicrobials was lower for macrolides [erythromycin (18%; 7/40); and azithromycin (23%; 9/40)]. *C. coli* isolates that were resistant against ciprofloxacin (48%; 19/40) were equal to susceptible *C. coli* isolates against the antimicrobial, with only two cases (5%; 2/40) of intermediate resistance. Fifty per cent (20/40) of the isolates were resistant against tetracycline, and only 5% (2/40) were intermediately resistant against the antimicrobial.

III. Antimicrobial susceptibility profile of non-*C. jejuni*/*C. coli* isolates

Only 2% (4/229) of the total isolates tested against antimicrobials were species of *Campylobacter* other than *C. jejuni*/*C. coli* [Table 3.4]. These species of *Campylobacter* showed 100% (4/4) sensitivity against both the macrolides (erythromycin and azithromycin). Only one (25%; 1/4) isolate was susceptible to ciprofloxacin, and the remaining 75% (3/4) were resistant. One hundred per cent (4/4) resistance against tetracycline was seen in these isolates and intermediate resistance against both ciprofloxacin and tetracycline was not observed for these isolates.

Table 3.4: Species-specific antimicrobial susceptibility profiles of detected *Campylobacter* in the years January 2014 to December 2015 (*n*=229).

Class of ATB	**ATB**	***C. jejuni*** (*n*=185)			***C. coli*** (*n*=40)			**Other species** (*n*=4)		
		(%)								
		S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Macrolide	Erythromycin	170 (92)	-	15 (8)	33 (82.5)	-	7 (17.5)	4 (100)	-	0
	Azithromycin	163 (88)	-	22 (12)	31 (77.5)	-	9 (22.5)	4 (100)	-	0
Fluoroquinolone	Ciprofloxacin	77 (42)	8 (4)	100 (54)	19 (47.5)	2 (5)	19 (47.5)	1 (25)	0	3 (75)
Tetracycline	Tetracycline	81 (44)	7 (4)	97 (52)	18 (45)	2 (5)	20 (50)	0	0	4 (100)

Abbreviations: Antibiotic (ATB), Susceptible (S), Intermediate (I), and Resistance (R).
Footnote: Etest Break points (µg/mL): Erythromycin [S≤16; R≥16], Azithromycin [S≤16; R≥16], Ciprofloxacin [S≤1; I=2; R≥4], Tetracycline [S≤4; I=8; R≥16]. Antimicrobials interpreted according to CLSI guidelines (CLSI, 2014 and CLSI, 2015).

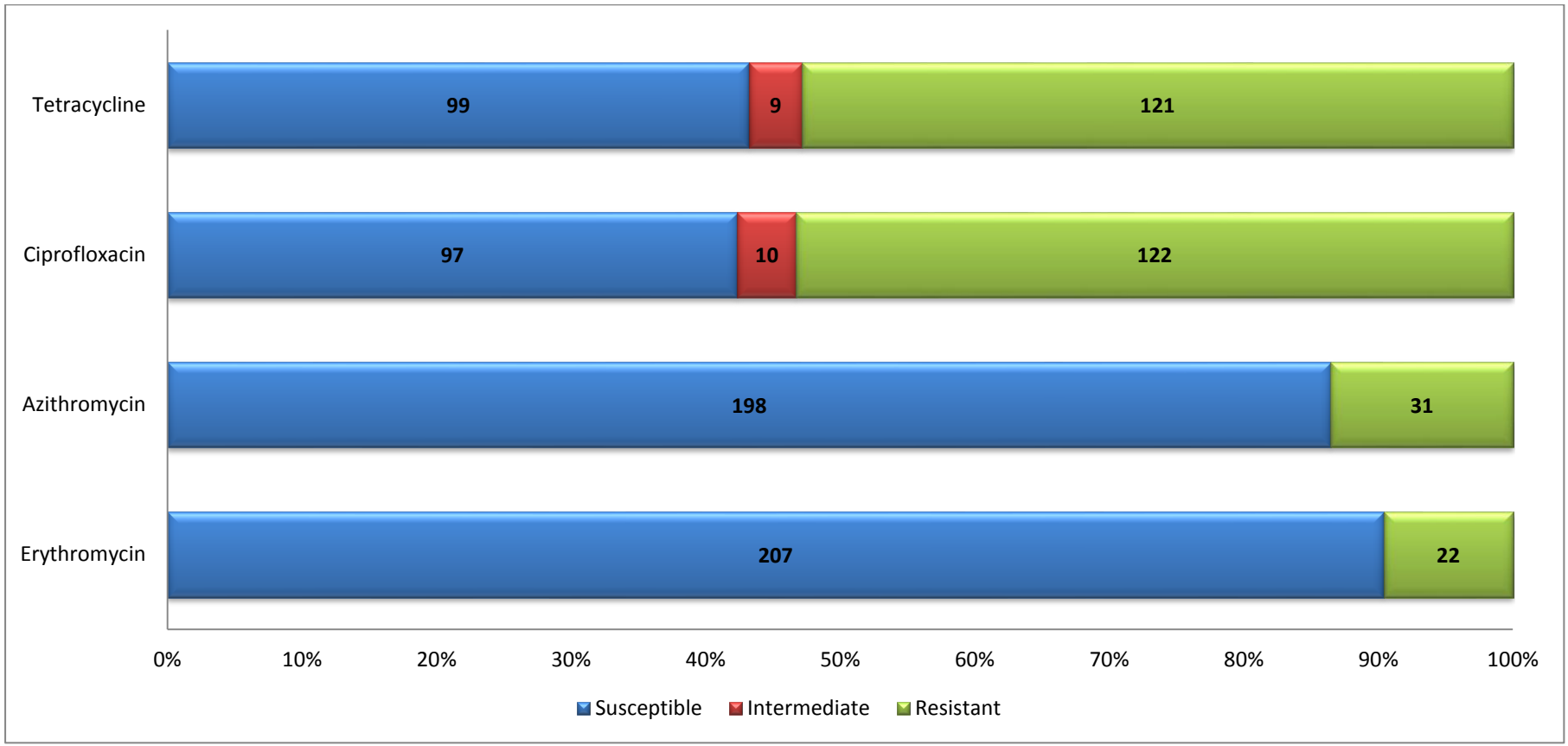


Figure 3.1: Antibiogram of the total viable *Campylobacter* species (*n*=229) tested against various antimicrobial agents during the years 2014-2015. Depicted on the vertical axis are the antimicrobial agents (erythromycin, azithromycin, ciprofloxacin, and tetracycline) tested against the *Campylobacter* isolates. The horizontal axis shows the generated (susceptible/intermediate/resistance) antimicrobial susceptibility profiles in percentage (%).

3.2. Animal isolates

3.2.1. Detected *Campylobacter* species

During January 2014-December 2015 *Campylobacter* species from animal isolates recovered in Gauteng [*n*=12 chicken isolates] and Western Cape [*n*=2 chicken isolates; *n*=2 sheep isolates; *n*=2 bovine isolates] were characterized using real-time PCR. Of the 12 isolates received from Gauteng, 58% (7/12) were *C. jejuni* and 42% (5/12) were *C. coli*. Five of the Western Cape isolates were *C. jejuni* (83%; 5/6) with one isolate positive for *C. coli* (17%; 1/6).

3.2.2. Antimicrobial susceptibility profiles of *Campylobacter*

All isolates from Gauteng and Western Cape were susceptible against erythromycin and azithromycin. Two chicken isolates from Gauteng (17%; 2/12) were susceptible against ciprofloxacin with the remaining 83% (10/12) being resistant. Four of the Western Cape isolates were susceptible to ciprofloxacin (67%; 4/6), with one intermediately resistant isolate recovered from chicken, as well as one isolate with full resistance against ciprofloxacin recovered from sheep. Eleven of the Gauteng chicken isolates were resistant against tetracycline (92%; 11/12) with only one susceptible isolate. Four of the Western Cape isolates were resistant against tetracycline while the remaining two isolates were susceptible (chicken isolate) and intermediately resistant (bovine isolate) against tetracycline respectively.

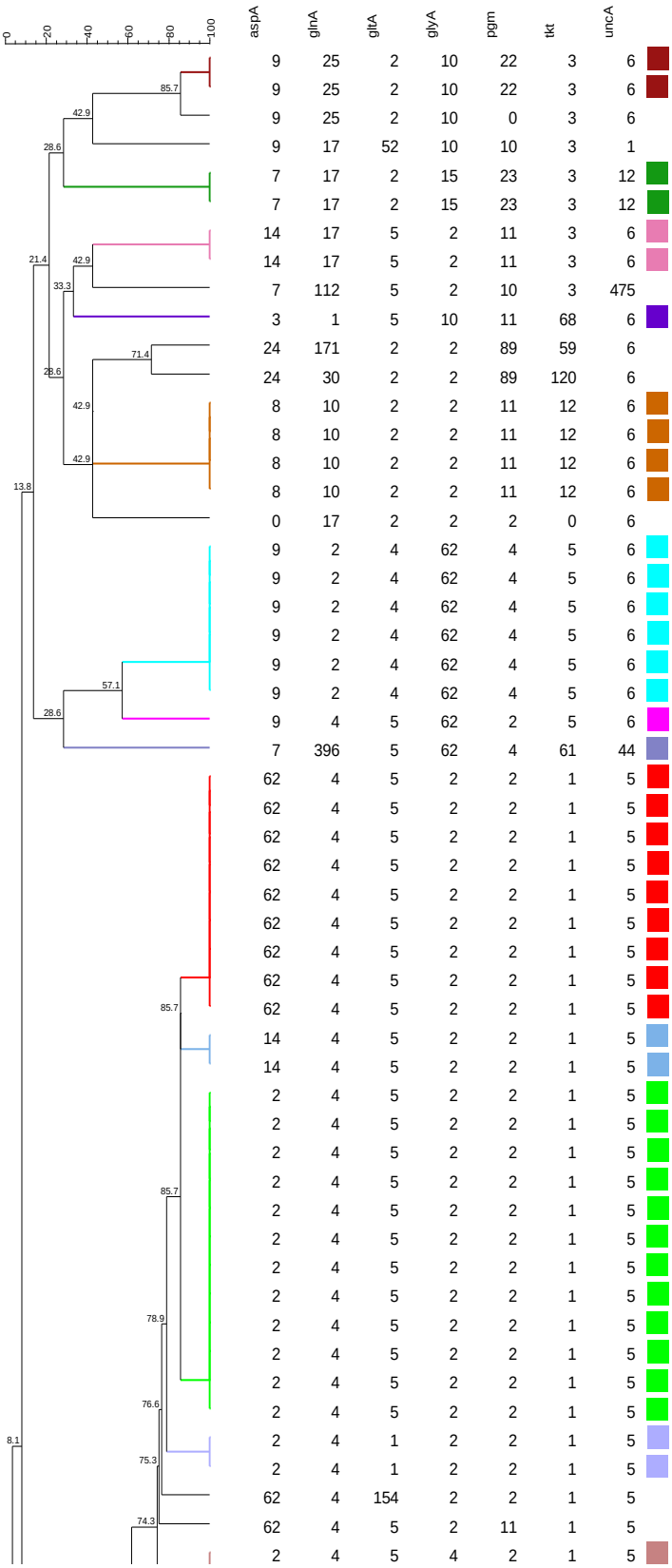
Table 3.5: *Campylobacter* species of animal origin recovered in Western Cape and Gauteng Provinces during January 2014 to December 2015.

[ϕ]Province	Source	[€]Additional information	*Campylobacter* spp.
WC	Sheep	Aborted fetus; stomach content	*C. jejuni*
	Chicken	Carcass ; organ pool	*C. jejuni*
	Chicken	Organic farm layer chicken; caecal content	*C. coli*
	Bovine	Dairy farm; calf ; diarrhoea; small intestine	*C. jejuni*
	Sheep	Neonatal; liver presented with a multifocal necrotic hepatitis	*C. jejuni*
	Bovine	Aborted fetus; commercial dairy; cotyledon	*C. jejuni*
GP	Chicken	NS	*C. jejuni*
	Chicken	NS	*C. jejuni*
	Chicken	NS	*C. jejuni*
	Chicken	NS	*C. jejuni*
	Chicken	NS	*C. jejuni*
	Chicken	NS	*C. jejuni*
	Chicken	NS	*C. coli*
	Chicken	NS	*C. coli*
	Chicken	NS	*C. coli*
	Chicken	NS	*C. coli*
	Chicken	NS	*C. jejuni*
	Chicken	NS	*C. coli*

[ϕ]Province: Western Cape (WC), Gauteng (GP). [€]Additional information: Not supplied (NS).

3.3. MLST characterization of *C. jejuni* isolates in South Africa

Human *C. jejuni* isolates from the *Campylobacter* Surveillance Program which were viable and pure were then further characterized using MLST to examine the degree of clonality that exists within species of *C. jejuni*. Veterinary *C. jejuni* isolates [Table 3.7] were also characterized using MLST for comparison to human *C. jejuni* isolates to determine whether human and animal isolates show similar MLST profiles, which may be indicative of transmission from animal to human. In total, the genetic relationships of 111 *C. jejuni* isolates from human hosts (*n*=99) and animal hosts (*n*=12) were examined [Figure 3.2]. Of the 111 *C. jejuni* isolates only 93 isolates [human (*n*=84); and animals (*n*=9)] could be assigned a ST previously represented in the *Campylobacter* MLST database accessible at http://pubmlst.org/campylobacter/. Fifty per cent (9/18) of the *C. jejuni* isolates with unassigned STs represented new STs (NST) of novel combinations of previously known alleles (8/9), with a single case (1/9) of novel alleles not previously represented in the PubMLST database. The remaining 50% represented isolates that were non-typable (NT) due to incomplete allele sequences [the allele length did not equal the high-scoring segment pair (HSP) length]. These isolates were given a zero digit in Figure 3.2 under allele profiles, which as a result impeded sequence comparison with previously assigned alleles.



Allele profiles

aspA	glnA	gltA	glyA	pgm	tkt	uncA	ST	Clonal complex	ϕ	Province	Source	Year
9	25	2	10	22	3	6	52	ST-52 complex		GP	Human	2015
9	25	2	10	22	3	6	52	ST-52 complex		GP	Chicken	2015
9	25	2	10	0	3	6	NT.5			WC	Human	2015
9	17	52	10	10	3	1	881	Not assigned		KZ	Human	2014
7	17	2	15	23	3	12	51	ST-443 complex		GP	Human	2014
7	17	2	15	23	3	12	51	ST-443 complex		GP	Human	2014
14	17	5	2	11	3	6	356	ST-353 complex		WC	Human	2014
14	17	5	2	11	3	6	356	ST-353 complex		GP	Human	2014
7	112	5	2	10	3	475	NST.1			GP	Human	2014
3	1	5	10	11	68	6	4624	ST-49 complex		GP	Human	2014
24	171	2	2	89	59	6	1471	ST-460 complex		GP	Human	2015
24	30	2	2	89	120	6	1932	ST-460 complex		GP	Chicken	2015
8	10	2	2	11	12	6	354	ST-354 complex		WC	Human	2015
8	10	2	2	11	12	6	354	ST-354 complex		WC	Human	2015
8	10	2	2	11	12	6	354	ST-354 complex		WC	Human	2015
8	10	2	2	11	12	6	354	ST-354 complex		WC	Human	2015
0	17	2	2	2	0	6	NT.7			GP	Human	2015
9	2	4	62	4	5	6	257	ST-257 complex		GP	Human	2014
9	2	4	62	4	5	6	257	ST-257 complex		GP	Human	2014
9	2	4	62	4	5	6	257	ST-257 complex		WC	Human	2015
9	2	4	62	4	5	6	257	ST-257 complex		GP	Human	2015
9	2	4	62	4	5	6	257	ST-257 complex		GP	Human	2015
9	2	4	62	4	5	6	257	ST-257 complex		GP	Chicken	2015
9	4	5	62	2	5	6	NST.7			GP	Human	2015
7	396	5	62	4	61	44	NST.9			WC	Sheep	2014
62	4	5	2	2	1	5	572	ST-206 complex		GP	Human	2014
62	4	5	2	2	1	5	572	ST-206 complex		GP	Human	2014
62	4	5	2	2	1	5	572	ST-206 complex		GP	Human	2014
62	4	5	2	2	1	5	572	ST-206 complex		WC	Human	2014
62	4	5	2	2	1	5	572	ST-206 complex		WC	Human	2015
62	4	5	2	2	1	5	572	ST-206 complex		WC	Human	2015
62	4	5	2	2	1	5	572	ST-206 complex		GP	Human	2015
62	4	5	2	2	1	5	572	ST-206 complex		GP	Human	2015
62	4	5	2	2	1	5	572	ST-206 complex		GP	Human	2015
14	4	5	2	2	1	5	1737	ST-206 complex		GP	Human	2014
14	4	5	2	2	1	5	1737	ST-206 complex		WC	Human	2014
2	4	5	2	2	1	5	227	ST-206 complex		GP	Human	2014
2	4	5	2	2	1	5	227	ST-206 complex		GP	Human	2014
2	4	5	2	2	1	5	227	ST-206 complex		NW	Human	2014
2	4	5	2	2	1	5	227	ST-206 complex		GP	Human	2014
2	4	5	2	2	1	5	227	ST-206 complex		MP	Human	2014
2	4	5	2	2	1	5	227	ST-206 complex		GP	Human	2014
2	4	5	2	2	1	5	227	ST-206 complex		GP	Human	2014
2	4	5	2	2	1	5	227	ST-206 complex		GP	Human	2015
2	4	5	2	2	1	5	227	ST-206 complex		GP	Human	2015
2	4	5	2	2	1	5	227	ST-206 complex		GP	Human	2015
2	4	5	2	2	1	5	227	ST-206 complex		GP	Chicken	2015
2	4	5	2	2	1	5	227	ST-206 complex		GP	Chicken	2015
2	4	1	2	2	1	5	474	ST-48 complex		GP	Human	2014
2	4	1	2	2	1	5	474	ST-48 complex		GP	Human	2014
62	4	154	2	2	1	5	NST.4			WC	Human	2014
62	4	5	2	11	1	5	613	ST-48 complex		GP	Chicken	2015
2	4	5	4	2	1	5	5809	ST-206 complex		GP	Human	2014

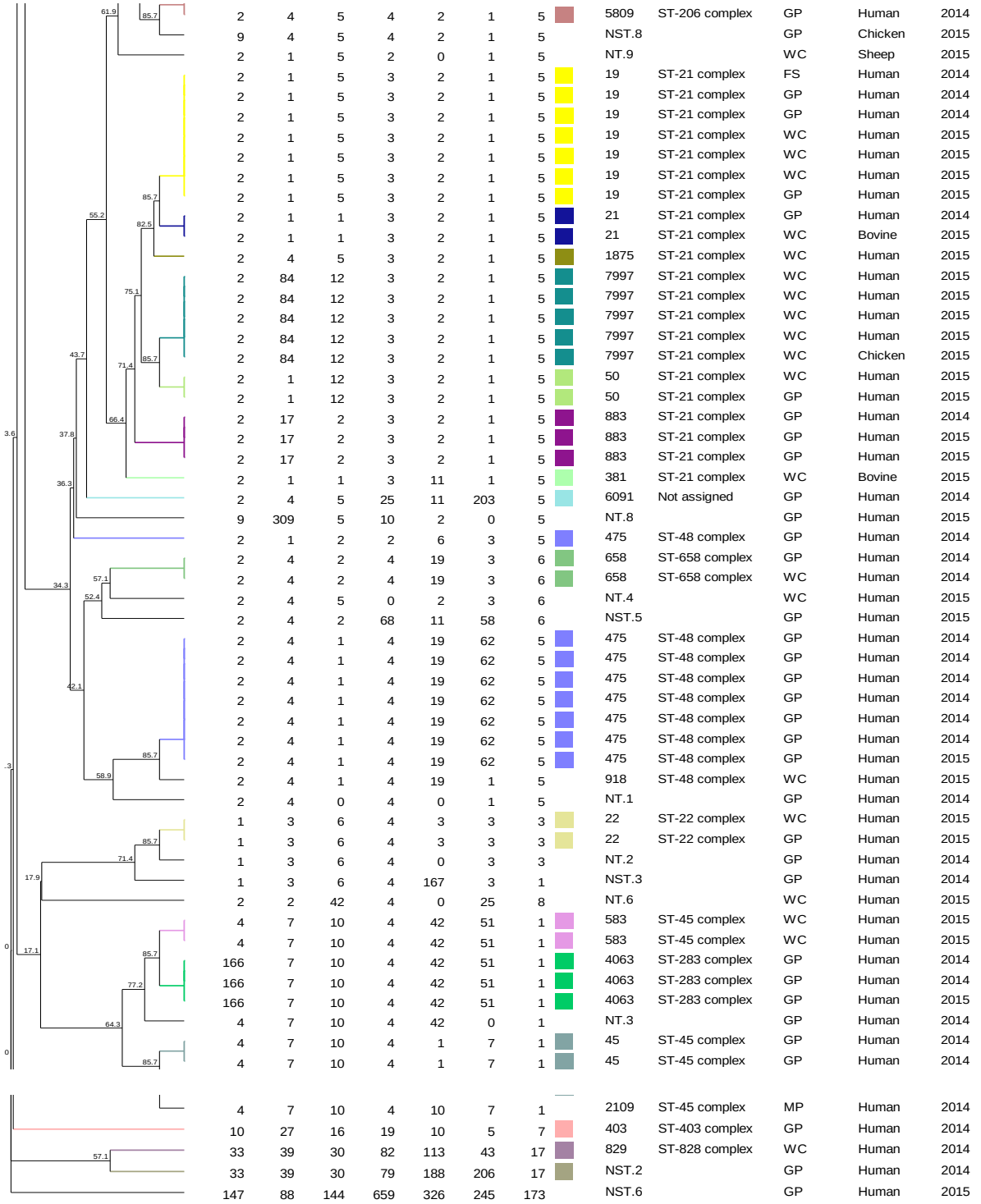


Figure 3.2: Dendrogram demonstrating phylogenetic relationships of 111 *C. jejuni* isolates on the basis of MLST profiles which were grouped by UPGMA clustering. The *C. jejuni* isolates were from human hosts and animal hosts (chicken, sheep, and bovine). For each isolate an allele profile, Sequence type (ST), clonal complex, province, source of isolate, and year of isolation was presented. Non-typable (NT) isolates and isolates with new combinations of previously known alleles (NST) were presented. An allele digit of zero (0) represented an incomplete allele sequence. ϕProvinces: Gauteng (GP), Western Cape (WC), KwaZulu-Natal (KZN), North West (NW), Free-State (FS), and Mpumalanga (MP).

3.3.1. Distribution of STs and clonal complexes of *C. jejuni* isolates

The 93 typable *C. jejuni* isolates (for which MLST STs could be assigned) were represented by a total of 33 different STs which were illustrated on the MST in Figure 3.3. The STs that were represented by ≥5 *C. jejuni* isolates were ST-7997, ST-257, ST-19, ST-475, ST-572, and ST-227 in the order of increasing number of isolates found in each ST respectively [Figure 3.3]. These STs accounted for 51% (47/93) of the total *C. jejuni* isolates. ST-227 which accounted for 13% (12/93) of the total *C. jejuni* isolates was the most common ST, followed by ST-572 which accounted for 10% (9/93) of the isolates. The ST distribution of the isolates was highly diverse with the remaining 27 STs representing *C. jejuni* isolates that were ≤4 in each ST; this diversity was also evident from the dendrogram [Figure 3.2] with many isolates represented by a single branch. Isolates with new combinations of previously known alleles as well as the single case of novel allele combinations were submitted to the PubMLST database to be assigned new allele numbers, STs, and clonal complexes.

Sixteen different clonal complexes grouped all the typable *C. jejuni* isolates in the study, with the exception of the two human isolates represented by ST-6091 and ST-881, which were not previously assigned to any clonal complex in the PubMLST database. The typable *C. jejuni* isolates were predominantly grouped into ST-45 complex; ST-257 complex; ST-48 complex; ST-21 complex; and ST-206 complex in the order of increasing number of isolates into each respective clonal complex. These five predominant clonal complexes were represented by ≥5 *C. jejuni* isolates, while the remaining 11 clonal complexes were represented by *C. jejuni* isolates that were ≤4 in each respective clonal complex.

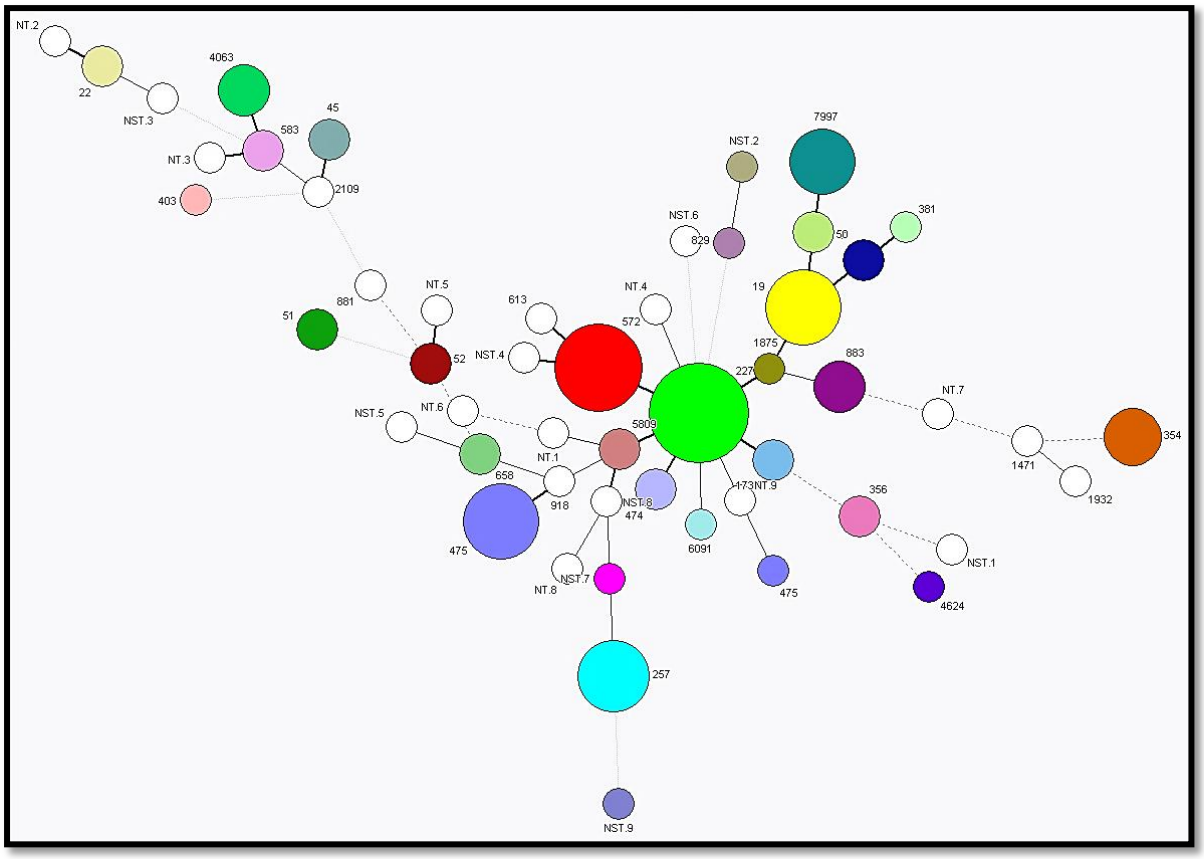


Figure 3.3: Phylogenetic analysis (minimal spanning tree) of South African *C. jejuni* isolates from humans and of veterinary origin (chicken, sheep, and bovine). Each coloured node (circle) represents one ST along with the ST number. STs representing one strain appear small in diameter, with or without the nodes being shaded in colour. The diameter of the node represents the number of strains within one ST; the bigger the node, the higher the number of strains found within the ST. The connecting lines between the different STs depict the number of allelic differences between them; one allele difference (black bold lines); two allele difference (grey bold lines or thin lines); three alleles difference (grey hatched lines); more than three alleles difference (grey dotted lines).

3.3.2. Province distribution of STs

Majority of the typable *C. jejuni* isolates [Figure 3.4] were from Gauteng [human (*n*=54), and animal (*n*=6)] followed by the Western Cape Province [human (*n*=25), and animal (*n*=3)]. Mpumalanga, KwaZulu-Natal, Free-State, and the North West Provinces were represented by ≤2 typable *C. jejuni* isolates. The majority of the STs were represented by *C. jejuni* isolates from Gauteng; where they branched as singletons, clustered together, as well as clustered with other isolates from other provinces as seen in ST-572 (Gauteng and Western Cape), ST-19 (Gauteng,

Western Cape, and Free-State) and ST-227 (Gauteng, Mpumalanga, and North West). STs only representing *C. jejuni* isolates (≥3) from a specific province were identified; such as ST-475, ST-4063, and ST-4063 for Gauteng isolates. STs representing *C. jejuni* isolates (≥3) from Western Cape only were ST-354 and ST-7997. The single isolate from KwaZulu-Natal had three allele differences from ST-52 (Gauteng) and more than three allele differences from ST-2109 (Mpumalanga). For the typable Gauteng isolates (*n*=60), a total of 24 different STs were identified which were further grouped into 14 clonal complexes. The most predominant ST and clonal complex for the Gauteng isolates was ST-227 and ST-206 complex. The typable Western Cape isolates (*n*=28) were represented by 16 different STs and 10 clonal complexes of which ST-7997 and ST-21 complex were predominant.

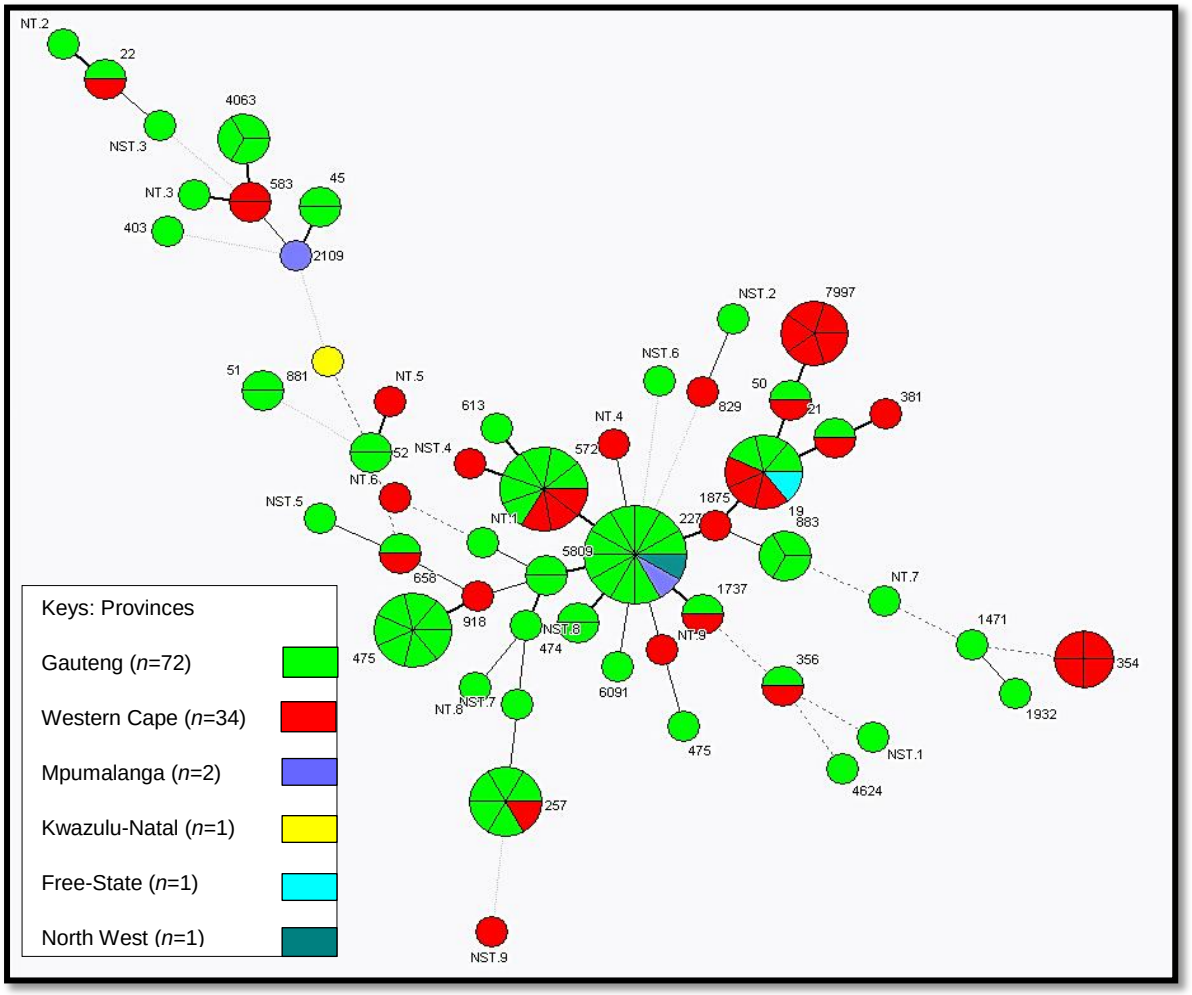


Figure 3.4: Minimal spanning tree of 111 *C. jejuni* isolates based on STs and the province distribution thereof. Each node represents a ST and the segments found within some of the nodes represent ≥2 *C. jejuni* strains belonging to the same ST, while un-segmented nodes represents a single *C. jejuni* strain. The shade of colour found in each node represents the province of the isolate(s) as stipulated in the keys.

3.3.3. STs of animal isolates

A total of eight different STs were identified for *C. jejuni* isolates of animal origin (*n*=9) which were further grouped into six different clonal complexes. The six Gauteng isolates which were all from chicken were represented by five different STs and five clonal complexes. ST-227 which falls under ST-206 clonal complex was represented by two chicken isolates. The three Western Cape isolates [chicken (*n*=1), and bovine (*n*=2)] had different STs, however, they all belonged to the same ST-21 complex.

Human and animal *C. jejuni* isolates that shared homologous MLST profiles were represented by ST-227, ST-257, ST-7997, ST-21, and ST-52. ST-227 was represented by 10 isolates from human hosts and two chicken isolates [Figure 3.5]. ST-21 represented a human *C. jejuni* isolate and a bovine *C. jejuni* isolate, whereas the previous shared STs represented human and chicken isolates. The three STs found in animal isolates but not in human isolates were ST-381, ST-613, and ST-1932. However, all the six clonal complexes of animal isolates overlapped with clonal complexes of human isolates. Allele similarities between isolates of human and animal origin were identified, although they respectively represented different STs; this was noted particularly between human and chicken isolates. ST-381 represented an isolate of bovine origin which had six homogenous alleles with ST-21 representing an isolate from a human and another bovine isolate.

3.3.4. Diversity of *C. jejuni* isolates

The Simpson's index of diversity for *C. jejuni* isolates on the basis of MLST loci was calculated [Table 3.8]. The diversity index at each locus for the set of samples ranged from 0.692 [confidence interval (CI), 0.569 - 0.815] to 0.828 [CI, 0.745 - 0.911]. Loci associated with the highest number of different repeats were *aspA*, *glnA*, and *tkt*, with a K-value of 13, while the locus with lowest number of different repeats was *uncA* with a max (pi) value of 0.485. The MLST loci for the sample set showed substantial genomic diversity of *C. jejuni* isolates.

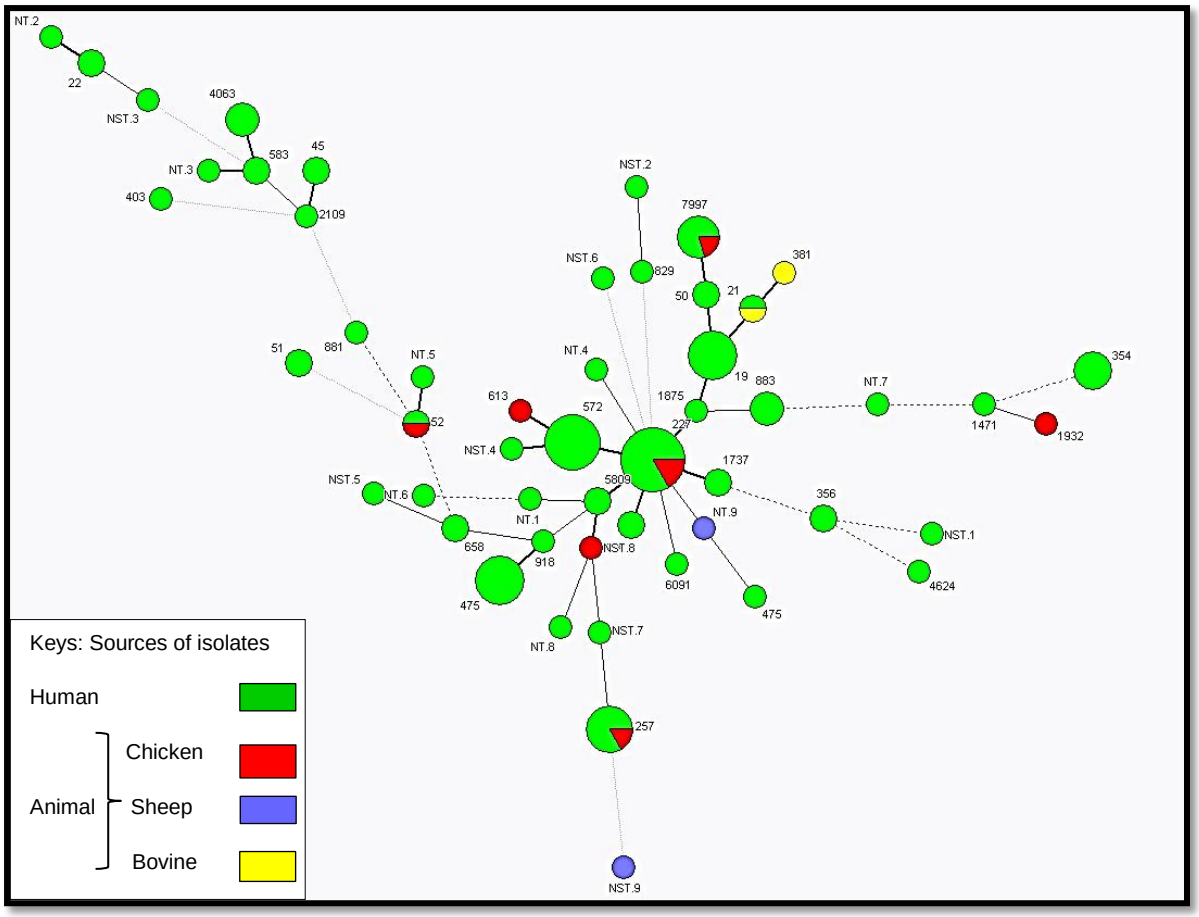


Figure 3.5: Minimal spanning tree of 111 *C. jejuni* strains illustrating shared STs between human hosts and animal hosts. Each node represents a ST and the shade of colour within each node represent the sources, depicted in the keys, from which *C. jejuni* strains belonging to a specific ST were isolated.

Table 3.6: Diversity indices of South African *C. jejuni* isolates.

		95% ^Confidence Interval (CI)			
Locus	***Diversity index**	**Lower**	**Upper**	**£K**	**Φmax(pi)**
aspA	0.786	0.662	0.91	13	0.424
glnA	0.828	0.745	0.911	13	0.333
glytA	0.817	0.75	0.884	10	0.303
glyA	0.826	0.764	0.889	12	0.242
pgm	0.826	0.743	0.91	12	0.333
tkt	0.771	0.652	0.891	13	0.424
uncA	0.692	0.569	0.815	8	0.485

***Diversity index**: measure of the variation of the number of repeats at each locus. Ranges from 0.0 (no diversity) to 1.0 (complete diversity).

^Confidence interval: Precision of Diversity Index at 95% upper and lower boundaries.

£K: Number of different repeats found at a locus in the sample set.

ΦMax (pi): proportion of isolates with commonly repeated number in each locus (range 0.0 to 1.0).

(Simpson, 1949; Hunter and Gaston, 1988).

3.4. Rotavirus Surveillance Program

3.4.1. Demographic information

A total of 981 nucleic acid samples extracted from stool specimens submitted for the Rotavirus Surveillance Program were screened for the presence of *Campylobacter* from February 2014 to December 2015 [Table 3.5]. These samples were from children of less than five years of age (range <1-60 months), with a median age of 10 months (IQR=5-16). Fourty-four per cent (436/981) of these stool samples were from females, and 55% (535/981) were from males, with the remaining 1% (10/981) representing specimens with unspecified sex. Eight different study sites formed the basis of the study, each site representing a hospital(s) in a specific province in South Africa (Appendix F). Majority of the specimens were submitted during the first three quarters of the year, with a few specimens submitted during the fourth quarter [Table 3.5].

3.4.2. Detection of *Campylobacter* from children <5 years of age with diarrhoea

Of the total submitted and screened nucleic acid samples from stool specimens, *Campylobacter* species were detected in 7% (69/981) of the samples using real-time PCR [Table 3.5]. *C. jejuni* was the most commonly detected species of *Campylobacter*, accounting for 81% (56/69), while *C. coli* was detected in nine samples, accounting for 13% (9/69). Four cases of coinfection with both *C. jejuni* and *C. coli* were detected, which accounted for 6% (4/69). Other species of *Campylobacter* were not detected from these samples. The positive samples were more frequently detected from children belonging to the <1-12 age group (months), represented by 62% (43/69), followed by those aged 13-24 months, accounting for 23% (16/69). Case patients from the remaining age groups were represented by ≤6% detection of *Campylobacter*. Majority of the positive cases were slightly more from males (54%; 37/69) than females (46%; 32/69) but the difference was not statistically significant (p-value 0.22). However, *C. coli* was detected more in females (6/9) in comparison to males (3/9) in this study. The *Campylobacter* positive samples

were mostly from Chris Hani Baragwanath Hospital (45%; 31/69), Red Cross Childrens Hospital (14%; 10/69), Edendale Hospital (13%; 9/69), and Mapulaneng Hospital (10%; 7/69). Annual occurrences of detected *Campylobacter* were as follows; quarter 1 (26%; 19/69), quarter 2 (20%; 14/69), quarter 3 (38%; 26/69), and quarter 4 (13%; 9/69). Quarter 3 had the highest number of submitted stool specimens and subsequently the highest detection of *Campylobacter* throughout the study.

Table 3.7: Stool specimens of children under five years of age with diarrhoea screened for the presence of *Campylobacter* in the years February 2014 to December 2015.

Characteristic	**Overall submitted (%)**	**Total positive (%)**	***Campylobacter* spp. (%)**		
			C. jejuni	*C. coli*	Mixed
Specimen type					
Stool	981	69 (7)	56 (81)	9 (13)	4 (6)
Age (months)					
Median (IQR)	10 (5-16)				
Range	<1-60				
Age category (months)					
<1-12	615 (63)	43 (62)	32 (57)	8 (89)	3 (75)
13-24	239 (24)	16 (23)	14 (25)	1 (11)	1 (25)
25-36	83 (8)	4 (6)	4 (7)	0	0
37-48	28 (3)	4 (6)	4 (7)	0	0
≥49	12 (1)	1 (1)	1 (2)	0	0
Unk	4 (<1)	1 (1)	1 (2)	0	0
Sex					
Female	436 (44)	32 (46)	25 (45)	6 (67)	1 (25)
Male	535 (55)	37 (54)	31 (55)	3 (33)	3 (75)
Unk	10 (1)	0	0	0	0
*p-value		0.22			
^Study sites					
Site 1	493 (50)	31 (45)	26 (46)	4 (44)	1 (25)
Site 2	78 (8)	7 (10)	5 (9)	2 (22)	0
Site 3	63 (6)	4 (6)	2 (4)	0	2 (50)
Site 5	81 (8)	9 (13)	6 (11)	3 (33)	0
Site 6	108 (11)	10 (14)	9 (16)	0	1 (25)
Site 8	48 (5)	4 (6)	4 (7)	0	0
Site 9	27 (3)	1 (1)	1 (2)	0	0
Site10	83 (8)	3 (4)	3 (5)	0	0
£Annual quarter					
Feb-Mar	257 (26)	19 (26)	17 (30)	2 (22)	0
Apr-Jun	285 (29)	14 (20)	12 (21)	1 (11)	1 (25)
Jul-Sep	292 (30)	26 (38)	18 (32)	6 (67)	2 (50)
Oct-Dec	145 (15)	9 (13)	8 (14)	0	1 (25)
Unk	2 (<1)	1 (1)	1 (2)	0	0

^Study sites (refer to Appendix F)

£Annual quarter: February (Feb), March (Mar), April (Apr), June (Jun), July (Jul), September (Sept), October (Oct), December (Dec).

*****p-value <0.05 statistically significant

3.5. Acute Flaccid Paralysis Surveillance Program

3.5.1. Polio-negative stool specimens of AFP patients

A total of 512 stool specimens were submitted from October 2014 to December 2015 from all South African provinces participating in the AFP Surveillance Program [Table 3.6]. Specimens from AFP patients with polio negative results were screened for the presence of *Campylobacter* species, particularly *C. jejuni* which has been reported to elicit GBS. The age range of AFP patients in this study was <1-67 years, with a median age of four years (IQR=2-12). Fifty-three per cent (271/512) of the specimens were from males, 47% (239/512) from females, and less than 1% (2/512) from patients with unspecified sex. These isolates were submitted from all South African provinces, particularly Gauteng (20%), Eastern Cape (18%), KwaZulu-Natal (18%), Limpopo (13%), and Mpumalanga (12%), while the remainder (19%) were distributed across the remaining provinces [Table 3.6].

3.5.2. *Campylobacter* detected from AFP patients

Campylobacter species were detected in 12% (62/512) of the submitted stool specimens using real-time PCR, of which *C. jejuni* accounted for majority of detected species with 77.4% (48/62), *C. coli* accounted for 19.4% (12/62), and mixed infections accounted for 3.2% (2/62) [Table 3.6]. The detection of *Campylobacter* species was notable in the 0-2 age group (37%; 23/62), 3-5 age group (32%; 20/62), and the 6-9 age group (18%; 11/62) [Table 3.13]. Majority of the detected species of *Campylobacter* in these age groups were *C. jejuni*, accounting for ≥19%. Other age groups from which *Campylobacter* was detected (≤6%) were those aged 10-19 years (6%; 4/62), 40-49 years (2%; 1/62), and those with unknown age groups (5%; 3/62).

Campylobacter species were slightly more frequently detected in females than males, accounting for 55% (34/62) and 45% (28/62) respectively (p=value 0.22). Females accounted for 54% (26/48) of the detected *C. jejuni* and males accounted for 46% (22/48). *C. coli* was detected equally in both females and males, accounting

for 50% (6/12) respectively. Coinfection with both *C. jejuni* and *C. coli* were detected in females only. The Eastern Cape, Gauteng, Limpopo, and KwaZulu-Natal Provinces had the highest number of detected *Campylobacter* species in this study, each province accounting for 32 % (20/62), 24% (15/62), 15% (9/62), and 13% (8/62) respectively [Table 3.6]. In the Eastern Cape Province, 85% (17/20) of the detected *Campylobacter* species were *C. jejuni*, as well as 73% (11/15) of the detected *Campylobacter* species in Gauteng. Overall, *C. jejuni* was the most common species of *Campylobacter* detected from each province with the exception of the Northern Cape, from which *Campylobacter* was not detected. Specimens positive for both *C. jejuni* and *C. coli* were from the North West and Gauteng Province. The majority of the *Campylobacter* positive specimens were submitted during the fourth quarter of the year (34%; 21/62), followed by the first quarter (29%; 18/62), and lastly the third quarter (24%; 15/62). *Campylobacter* species were detected the least during the second quarter of the year (8%; 5/62).

Table 3.8: Stool specimens from polio-negative AFP case patients screened for the presence of *Campylobacter* species during October 2014 to December 2015.

Characteristic	Total submitted (%)	Total positive (%)	*Campylobacter* spp. (%)		
			C. jejuni	*C. coli*	Mixed
Specimen type					
Stool	512	62 (12)	48 (77.4)	12 (19.4)	2 (3.2)
Age (years)					
Median (IQR)	4(2-12)				
Range	<1-67				
Age category (years)					
0-2	140 (27)	23 (37)	20 (42)	3 (25)	0
3-5	188 (37)	20 (32)	13 (27)	5 (42)	2 (100)
6-9	85 (17)	11 (18)	9 (19)	2 (17)	0
10-19	68 (13)	4 (6)	3 (6)	1 (8)	0
20-29	6 (1)	0	0	0	0
30-39	4 (<1)	0	0	0	0
40-49	3 (<1)	1 (2)	1 (2)	0	0
50-59	2 (<1)	0	0	0	0
≥60	1 (<1)	0	0	0	0
Unk	15 (3)	3 (5)	2 (4)	1 (8)	0
Sex					
Female	239 (47)	34 (55)	26 (54)	6 (50)	2 (100)
Male	271 (53)	28 (45)	22 (46)	6 (50)	0
Unk	2 (<1)	0	0	0	0
[£]p-value		0.22			
Province					
Eastern Cape	94 (18)	20 (32)	17 (35)	3 (25)	0
Free State	21 (4)	3(5)	3 (6)	0	0
Gauteng	104 (20)	15 (24)	11 (23)	3 (25)	1 (50)
KwaZulu-Natal	93 (18)	8 (13)	7 (15)	1 (8)	0
Limpopo	64 (13)	9 (15)	5 (10)	4 (33)	0
Mpumalanga	63 (12)	1 (2)	1 (2)	0	0
North West	24 (5)	1 (2)	0	0	1 (50)
Northern Cape	11 (2)	0	0	0	0
Western Cape	36 (7)	4 (6)	4 (8)	0	0
Unk	2 (<1)	1 (2)	0	1 (8)	0
Annual quarter					
Jan-Mar	107 (21)	18 (29)	13 (27)	3 (25)	2 (100)
Apr-Jun	106 (21)	5 (8)	3 (6)	2 (17)	0
Jul-Sep	122 (24)	15 (24)	11 (23)	4 (33)	0
Oct-Dec	164 (32)	21 (34)	19 (40)	2 (17)	0
Unk	13 (3)	3 (5)	2 (4)	1 (8)	0

[£]p-value <0.05 statistically significant.

Chapter 4: Discussion

4.1. *Campylobacter* Surveillance Program

4.1.1. Submission of bacterial isolates

From when the *Campylobacter* Surveillance Program began in January 2014, laboratories participating in the GERMS-SA surveillance program were continuously motivated to submit laboratory confirmed *Campylobacter* species at CED. This progressive participation was seen from the total number of submitted bacterial isolates in 2015 (62%; 528/848), which was greater (p-value 0.04) than the total number of isolates submitted in 2014 (38%; 320/848). However, the quality of submitted bacterial isolates in terms of viability, upon arrival at CED, did not improve; in the year 2014, 45% of the submitted isolates were non-viable with only 52% viable isolates, whereas in the year 2015 55% were non-viable and only 42% were viable. The remaining 3% in the year 2014 was constituted by both missing and contaminated isolates, and in the year 2015, the remaining 3% represented contaminated isolates only. *Campylobacter* species are fastidious organisms that can enter a VNC state (Cappelier *et al*., 1997; van Vliet and Katley, 2001), which may explain the frequency of non-viable cultures even under optimal growth conditions in this study. The difference seen in the number of isolates submitted in 2014 and 2015 was only a reflection of improved participation of laboratories in the GERMS-SA *Campylobacter* Surveillance Program. This improvement was facilitated by continuous requests that laboratories send isolates to the CED.

Due to the short study years, seasonality of *Campylobacter* infections could not be extrapolated in this study, however, annual occurrences of submitted cases showed an overall (2014-2015) suggestive trend of more *Campylobacter* cases occurring in the warmer months (quarter 1), followed by a decrease during the cooler months (quarter 2), with a subsequent increase from the third quarter, which progresses all the way to the fourth quarter, characterized with warmer months. The seasonal

suggestive trend is inconsistent with previously 'unrecognized' seasonal patterns for *Campylobacter* infections in developing countries, nevertheless, the trend is consistent to what has been reported in temperate countries, where the number of *Campylobacter* cases reported increase with increasing temperature (Nylen *et al*., 2002; Ekdahl and Andersson, 2004; Louis *et al*., 2005; Galate and Bangde, 2013). However, longer systematic studies would need to be carried out to deem what was observed in this study true. The weather in Africa can largely be classified as tropical; the lack of extreme temperature fluctuations, as well as the lack of surveillance for epidemics may be a contributing factor for the unrecognized *Campylobacter* seasonal peaks (Nylen *et al*., 2002; Ekdahl and Andersson, 2004; Galate and Bangde, 2013). In temperate regions, risk factors that are associated with campylobacteriosis in the summer months include animal contact, eating barbequed meals, drinking untreated water from streams and other natural sources, and most recently, flies have been postulated to be a risk factor, functioning as a mechanical vector (Nylen *et al*., 2002; Ekdahl *et al*., 2005). Studies have shown transfer of *Campylobacter* from the environment to flies with relative ease; in one such study, *Campylobacter* was isolated from 8% (4/49) of flies caught outside a broiler house in Denmark (Ekdahl *et al*., 2005).

4.1.2. *Campylobacter* positive isolates and the detected species

Overall, majority of the bacterial isolates submitted from the various laboratories were identified correctly as *Campylobacter*, with only 6% representing those isolates that could not be characterized as *Campylobacter* using real-time PCR. The most predominant species of *Campylobacter* detected in this study was *C. jejuni*, represented by over 80% of the tested isolates in 2014 and 2015 respectively, and *C. coli* was the second most frequently detected species of *Campylobacter*, accounting for 9% of the isolates submitted in 2014, and 14% of the isolates submitted in 2015. The detection of coinfection with both *C. jejuni* and *C. coli*, as well as the detection of non-*C. jejuni*/*C. coli* was the same in both study years, accounting for 2% and 3% respectively. The findings coincided with previous reports of *C. jejuni* accounting for over 80% of human infections and *C. coli* accounting for

most of the remainder (Acheson and Allos, 2001; Sheppard *et al*., 2009; Wagenaar *et al*., 2013; Schielke *et al*., 2014; Vidal *et al*., 2016). Various studies from developing countries have reported on high rates of mixed or polymicrobial infections involving *Campylobacter* (Glass *et al*., 1983; Rajendran *et al*., 2012; Mason *et al*., 2013): this occurrence was also seen in our study, with a prevalence of 2% among the isolates. The detection of non-*C. jejuni*/*C. coli* suggests that there are species of *Campylobacter* other than *C. jejuni*/*C. coli* in circulation in South Africa, which may also cause infection, but these are present in very low numbers. Reports of species of *Campylobacter* other than *C. jejuni*/*C. coli* recovered from patients with diarrhoea in developing countries have been documented, such as *C. concisus, C. fetus*, and *C. upsaliensies (*Mason *et al*., 2013; Wagenaar *et al*., 2013; Platts-Mills and Kosek, 2014). The isolation of non-*C. jejuni*/*C. coli* species has been facilitated primarily by the use of less-selective isolation techniques and culture-independent diagnostic tests (Platts-Mills and Kosek, 2014). In South Africa, efficient isolation of non-*C. jejuni*/*C. coli* species of *Campylobacter* has been facilitated by the adoption of non-selective isolation techniques, where *C. concisus* and *C. upsaliensies* were recovered with a frequency close to that of *C. jejuni* from diarrhoeal stools of children (Lastovica and Rouxe, 2000; Platts-Mills and Kosek, 2014). According to Platts-Mills and Kosek, (2014) the incidence and pathogenicity of these species of *Campylobacter* recovered in developing countries stands subject for investigation, because they may well be of public health importance. The 3% representing non-*C. jejuni*/*C. coli* isolates could only be identified to genus level for the purpose of this study which only looked at predominant species associated with high incidence rates of campylobacteriosis.

In South Africa, reports of the recovery of *Campylobacter* species from pigs and chickens from various poultry production systems as well as retailers have been documented (van Nierop *et al*., 2005; Suzuki and Yamamoto, 2009; Jonker and Picard, 2010; Bester and Essack, 2012). *C. jejuni* is commonly recovered from poultry and poultry by-products, whereas *C. coli* isolation is associated with pigs (Jonker and Picard, 2010; Wagenaar *et al*., 2013). It is conceivable that the high incidence of campylobacteriosis associated with *C. jejuni* may reflect on poultry and possibly other ruminants (e.g. bovine intestine) being a cheaper affordable food

source for South Africans than porcine, which may explain why *C. coli* was less frequently recovered from patients. However, due to the less understood epidemiology of *Campylobacter*, and the lack of literature in South Africa, there are still uncertainties how each possible pathway contributes to human infections, because all known risk factors only explain less than 50% of human cases (Nylen *et al*., 2002; Müllner *et al*., 2010; MacRitchie *et al*., 2013). One of the few studies in Soweto, South Africa, showed the relative ease of *C. jejuni* acquisition by families as a result of eating chicken, which is the cheapest meat available for these communities, and their custom of keeping flocks in their backyards (Richardson *et al*., 1983). In the same study by Richardson *et al*., (1983) *C. jejuni* was recovered in 86% of the tested fowl faeces, some samples of bovine intestine, which is another cheap popular food source, as well as from pet dog faeces, which are usually fed poultry scraps (Richardson *et al*., 1983).

4.1.3. Sex-specific occurrences of *Campylobacter* infections

Of the total submitted isolates, it was males from whom more *Campylobacter* species were detected in both study years, accounting for 53.6% (429/801) as compared to females (46%; 369/801), and individuals of unknown gender with *Campylobacter* positive results (<1%; 3/801). This observation of male infections being slightly more than females is consistent to previously documented findings, with sex-specific differences in food handling practices being a risk factor (Acheson and Allos, 2001; Strachan *et al*., 2008; Cody *et al*., 2012). According to Samuel *et al*., (2004) the prevalence of males amongst infected individuals is explained by unsafe food handling, preparation, and consumption practices than females. High risk foods for foodborne diseases have been reported to be frequently consumed by males more than females, including foods that may be risky for *Campylobacter* contamination (Samuel *et al*., 2004). Sex-specific immunity described by Olivier and William, (2010) could in part explain the slightly high incidence of *Campylobacter* infections associated with males.

4.1.4. Age-related occurrences of *Campylobacter* infections

The age-related data from this study showed campylobacteriosis to commonly occur amongst those aged 0-4 years, whom accounted for 35% (281/801) of the total infections, which was a common observation from previous reports (Miller *et al*., 2005; Mshana *et al*., 2009). The high incidence rate in this group of individuals is attributed to *Campylobacter* risk factors such as a lack of a developed immune system amongst the young, living in close proximity to animals, particularly in developing countries where children can easily come into contact with and ingest bacteria that have been shed by animals into the environment, as well as drinking contaminated milk (Richardson *et al*., 1983; Sibbald and Sharp, 1985; Fernandes *et al*., 2015). According to Lund and O'Brien, (2011) for children of less than five years of age to be implicated, smaller infective doses are required for infection, as a result of lack of developed immune system.

Another factor taken into account for these individuals is overreporting, where the young are more likely to receive medical care and have stools cultured compared to adults who are most likely to have short lived, and less severe diarrhoeal episodes (Miller *et al*., 2005; Weinberger *et al*., 2013). The precise infection pathway implicated with the high incidence rate of campylobacteriosis among children of less than five years of age has not yet been identified (Miller *et al*., 2005; MacRitchie *et al*., 2013), this was inferred from studies that showed that greater exposure to risk factors does not particularly coincide with greater incidence for age groups.

A study in Australia investigating risk factors for sporadic *Campylobacter* infection in infants and young children aged 0-2 years proposed that children of less than three years of age are at risk of *Campylobacter* infection if residing in a household which has puppies or chicken as pets (Tenkate and Stafford, 2001). These findings were similar to the findings of Richardson *et al*., (1983) conducted in Soweto, South Africa, for children under two years with *C. jejuni* infections. According to Tauxe *et al*., (1988) sources of *C. jejuni* infection among infants have not been examined systematically. Consumption of unpasteurized/incorrectly pasteurized raw milk from dairy (Acheson and Allos, 2001), particularly in developing countries, could be a

possible risk factor, which may in part explain the high rates of *C. jejuni* seen amongst the young in South Africa (O'Ferrall-Berndta, 2003).

Individuals aged 5-14 and 15-24 in years showed a marked reduction of *Campylobacter* infections, accounting for 11% (90/801) and 8.4% (67/801) respectively. According to previous reports, the reduction is attributed to a change in behavioural patterns, such as improved hygiene which subsequently reduces the risk of environmental exposure and infection (Miller *et al*., 2005). A second peak ensued in the 25-34 age group, accounting for 12 % (95/801). This trend then reversed with a decline for those individuals falling within the 35-44; 45-54; and 55-64 age groups. The observed second peak in the young adults (25-34 age group) has been previously recognized (Miller *et al*., 2005; Schielke *et al*., 2014), however, it was reported to be more pronounced in the urban than rural populations (Sibbald and Sharp, 1985). The risk factors associated with young adults include international and national travel, where individuals harbour different strains of *Campylobacter* species from visited geographical location. According to Tauxe *et al*., (1988) campylobacteriosis associated with young adults may be related to their food handling practices following leaving their childhood homes and beginning to cook for themselves. Diseases, stress, pregnancy, eating habits and hygiene practices are some of the life experiences that impact individuals entering young adulthood. All these factors increase the risk of foodborne infections or severity of the disease (Lund and O'Brien, 2011). Stress which leads to changes in metabolism results in reduced resistance to infections such as campylobacteriosis. Pregnancy generally alters the mother's immune system, making pregnant women susceptible to foodborne illness. Consuming fatty foods (Lund and O'Brien, 2011), undercooked meats (Acheson and Allos, 2001), and unhygienic habits of preparing raw foods, like salads (Acheson and Allos, 2001; Wagenaar *et al*., 2013) may in part explain the incidence peak observed in young adults.

The global pandemic of Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) which result in weakened immunity, particularly affecting young female adults, could also account for the peak observed. Gastrointestinal tract infections are common among HIV/AIDS individuals; enteric pathogens recovered

from stool specimens of HIV/AIDS patients include *Campylobacter*, amongst many other pathogens (Prasad *et al*., 2000; Lund and O'Brien, 2011). *C. jejuni* has been recovered more frequently from HIV/AIDS patients than in the healthy population (Snijders *et al*., 1997; Altekruse *et al*., 1999). A study carried out by Snijders *et al*., (1997) indicated that species of *Campylobacter*, other than *C. jejuni*, are frequently recovered from HIV infected individuals than previously recognized. However, the HIV status for the case patients in this study was not provided, thus HIV/AIDS being a contributing factor amongst the young adults could only be speculated.

According to Sibbald and Sharp (1985), the lower incidence rate observed in adults from developing settings reflects on widespread immunity which results from post *Campylobacter* exposure during infancy. Immunity may confer protection against *Campylobacter* infections or symptomatic infections all together (Miller *et al*., 2005). The latter further substantiates the report from Acheson and Allos, (2001) that in developing countries asymptomatic infections occur in both the young and adults. This might in part explain the decrease observed from those adults aged ≥35≤64 in years. According to Miller *et al*., (2005) reduced incidence of campylobacteriosis might be explained by reduced exposure to the infection pathways more common in younger age groups, pathways which still stand subject for investigation (Weinberger *et al*., 2013). Following this decrease seen in adults in this study, there was an increase in the number of infections for those aged 65 and older in years.

The elderly with positive *Campylobacter* results accounted for 10% (84/801) in this study. A study conducted in England and Wales (Gillespie *et al*., 2009a; Gillespie *et al*., 2009b), where age-related changes in the incidence of campylobacteriosis was investigated, showed a shift, with individuals aged 60 years and older found to be the emerging at-risk group for campylobacteriosis as compared to children who displayed a reduced incidence rate. The increase in incidence rate for those aged 60 and older in years was in part attributed to increased life expectancy in both England and Wales, from which it was hypothesized that the incidence of campylobacteriosis in the elderly will continue to increase, as with other chronic illnesses affecting the elderly, thus weakening their immune system. Risk factors for campylobacteriosis

associated with this group of individuals still needs to be investigated (Gillespie *et al*., 2009a; Gillespie *et al*., 2009b).

South Africa has the lowest average life expectancy in the world; however, recently, the South African Medical Research Council (SAMRC) and Statistics South Africa have released latest reports which show a steady improvement in life expectancy in the country which has increased by 8.5 years since 2005 to an average of 62 years in 2013 (SAMRC, 2015; Statistics South Africa, 2015). As life expectancy increases in South Africa, as seen in England and Wales, the number of individuals living with chronic conditions, and subsequently weakened immunity, are likely to increase as reflected by the *Campylobacter* peak amongst the elderly (Gillespie *et al*., 2009a; Gillespie *et al*., 2009b). The phenomenon of acquired immunity in developing countries as a result of exposure during infancy is not reflected in the elderly age group (≥60 years) with an increased number of infections. This could be as a result of a decrease in serum antibodies against *Campylobacter* species previously exposed to or new species exposed to as an individual ages. According to Lund and O'Brien (2011), age-related deterioration of the immune system and comorbidity result in increased susceptibility to infections such as *Campylobacter*. The incidence rate of campylobacteriosis three peaks, resulting from the three identified age risk groups in this study (0-4; 24-34; and >65 age groups). The resulting curve coincided with the previously reported U-shaped curved by Miller *et al*., (2005) and Weinberger *et al*., (2013) observed in developing countries for children and young adults, furthermore, our results showed the elderly to be the third emerging at risk group for campylobacteriosis.

4.1.5. Gender and age distribution of positive *Campylobacter* cases

The distribution of positive *Campylobacter* cases was examined by both sex and age to investigate any possible variation. The number of males with *Campylobacter* positive isolates was greater in all age groups, with the exception of those aged 25-35 years, 35-44 years, and individuals ≥65 years of age, where the number of positive *Campylobacter* cases in females were higher than males (p-value 0.02). A similar trend of females being slightly more infected than males in the 25-35 age

group (young adults) has been reported in England, Wales, the Netherlands, and Germany (Louis *et al*., 2005; Schielke *et al*., 2014).

Possible risk factors associated with women of this age group may include: (1) weakened immunity due to diseases such as HIV/AIDS which is prevalent in young female adults in South Africa, (2) exposure as a result of human-to-human transmission from young children they take care of, (3) or they handle/prepare and eat chicken more frequently than males in the same age group, which could lead to campylobacteriosis if chicken is undercooked, or from possible cross contamination in the kitchen (Schielke *et al*., 2014). What was observed for females in the elderly (≥65 years) age group associated with more campylobacteriosis cases than males conflicted with the report from California, United States of America (USA), by Samuel and colleagues (2004) where sex-related incidence was highest among males across all age groups, even the elderly. More studies would need to be carried out to deem this current observation for South African *Campylobacter* cases true.

4.1.6. Province distribution of detected *Campylobacter*

The distribution of positive *Campylobacter* cases throughout the study period was not interpreted as a true reflection of which province had the highest burden of disease or where the detected species of *Campylobacter* were most prevalent. The results were perceived as a reflection of which province participated the most in the *Campylobacter* Surveillance Program. Throughout the study period, bacterial isolates were submitted the most from the Gauteng Province followed by Western Cape Province; as a result the majority of positive *Campylobacter* cases were seen from these two provinces. In order to mitigate the existing research gap as far as *Campylobacter* infections are concerned in South Africa, all provinces would need to show participation which will enhance the *Campylobacter* Surveillance Program and subsequently contribute to strategies aimed at controlling and preventing human infections and thus lessening the burden on healthcare systems.

4.1.7. Detection of invasive *Campylobacter* species

Overall the positive invasive cases of *Campylobacter* remained as low as 1% (5/848) throughout both the study years, an observation seen globally (Acheson and Allos, 2001; Moore *et al*., 2005; Wagenaar *et al*., 2013). Five invasive isolates of *Campylobacter* were identified, 60% (3/5) were *C. jejuni*, while 40% (2/5) were species other than *C. jejuni*/*C. coli*. In this study bacteraemia was associated more with children of less than five years of age (80%; 4/5) compared to young adults (20%; 1/5), an observation that is consistent with previous reports (Acheson and Allos, 2001). Age-related lack of immunity may be the underlying cause of bacteraemia for children in this study, whereas the young adult case may be due to weakened immune system as a result of other underlying medical conditions, or an encounter with a very virulent strain of *Campylobacter* (Acheson and Allos, 2001; Moore *et al*., 2005).

4.1.8. Antimicrobial susceptibility profiles of detected *Campylobacter* species

Bacterial cultures submitted as part of the *Campylobacter* Surveillance Program that were positive for *Campylobacter* species as well as viable during January 2014-December 2015 were 387 of a total of 848 submitted isolates. However, only 59% (229/387) of the bacterial cultures remained viable following sub-culturing for antimicrobial testing. The remainder (41%) was accounted for by non-culturable isolates and the few cases of mixed *Campylobacter* isolates. The ability of *Campylobacter* to enter a VNC state (Cappelier *et al*., 1997; van Vliet and Katley, 2001), may partly explain the high number of isolates that lose viability even under optimal growth conditions as well as the inherent fastidious nature of the genus *Campylobacter* in culture. Mixed *Campylobacter* isolates were omitted in this analysis as a result of difficulty encountered in separating these two species in culture due to their spread out growth form. The overall results of the 229 isolates combined (*C. jejuni*, *C. coli*, and other species of *Campylobacter*) showed considerable sensitivity to the macrolides (erythromycin and azithromycin) and considerable resistance to ciprofloxacin and tetracycline. These findings coincide with previous reports (Coker *et al*., 2002; Moore *et al*., 2005; Galate and Bangde, 2013).

In developing countries increase in resistance to both erythromycin and ciprofloxacin have been documented (Coker *et al*., 2002; Moore *et al*., 2005; Galate and Bangde, 2013), which is largely influenced by the use of antimicrobials for infections other than gastrointestinal diseases and the pressing issue of self-medication (Coker *et al*., 2002; Tafa *et al*., 2014; Rastyani *et al*., 2015). Conversely the increase in resistance seen in developed countries is associated with the use of the antimicrobials in food animals and travel to developing countries (Coker *et al*., 2002; Galate and Bangde, 2013). Resistance to azithromycin was found in 7% to 15% of *Campylobacter* isolates in Thailand (Coker *et al*., 2002) which is similar to the findings of our study, where resistance to azithromycin was accounted for by 14% (31/229) of all the *Campylobacter* isolates combined.

In 2009 *Campylobacter* species isolated from the Vhembe district, South Africa, from diarrhoeic and non-diarrhoeic patients showed significant prevalence of resistance against erythromycin; with susceptibility of less than 30% (Samie *et al*., 2009). Over 90% of the isolates showed susceptibility to ciprofloxacin, and 34% of the isolates were resistant to tetracycline (Samie *et al*., 2009). Susceptibility data in our present study were not comparable to the study reported from the Vhembe district; in our study, isolates were least susceptible to ciprofloxacin and tetracycline, with an overall susceptibility of less than 43%. Moreover, isolates showed the highest susceptibility to erythromycin, with an overall susceptibility of 90%, followed by azithromycin, with an overall susceptibility of 86%.

4.1.8.1. Antimicrobial susceptibility patterns of *C. jejuni*

Of the total 229 *Campylobacter* isolates tested against the different antimicrobials in the study, 185 were *C. jejuni*. These isolates showed the highest susceptibility to erythromycin (92%) and azithromycin (88%). Over 50% of the *C. jejuni* isolates were resistant to both ciprofloxacin and tetracycline with only a small fraction of these isolates intermediately resistant to both the antimicrobials. Susceptibility against ciprofloxacin and tetracycline was accounted for by 41% and 44% of the *C. jejuni* isolates respectively. This renders tetracycline the third treatment option for *C. jejuni*

infections over ciprofloxacin, while erythromycin may be the first treatment option against *C. jejuni* infections in South Africa, followed by azithromycin.

4.1.8.2. Antimicrobial susceptibility patterns of *C. coli*

According to Best *et al*., (2003) antimicrobial susceptibility profiles of *C. jejuni* and *C. coli* differ, particularly for erythromycin and ciprofloxacin. Species-specific association of erythromycin resistance has been linked to *C. coli*, whereas tetracycline and quinolone resistance showed no affinity for either species of *Campylobacter* (Wimalarathna *et al*., 2013). In our current study, resistance against erythromycin and azithromycin for the 40 *C. coli* isolates was slightly higher than seen for *C. jejuni* isolates; a similar observation has been reported by Jonker and Picard, (2010) for animal isolates in the Western Cape region. A total of 18% of the *C. coli* isolates were resistant to erythromycin and 23% were resistant to azithromycin. Nonetheless, majority of the *C. coli* isolates were sensitive to both the macrolides, accounting for 82.5% and 77.5% for erythromycin and azithromycin respectively. Susceptibility, intermediate resistance, and complete resistance against ciprofloxacin and tetracycline were similar for the *C. coli* isolates; a binomial pattern of antimicrobial resistance previously reported by Jonker and Picard, (2010) for macrolides was seen for ciprofloxacin and tetracycline in our current study. Distribution of these isolates between being susceptible and resistant against ciprofloxacin and tetracycline was virtually equally distributed. Macrolides are more clinically useful to combat *C. coli* infections as compared to ciprofloxacin and tetracycline whose clinical usefulness has been compromised for isolates in our current study with almost 50% of the isolates being resistant.

4.1.8.3. Antimicrobial susceptibility patterns of non-*C. jejuni*/*C. coli*

Complete susceptibility against erythromycin and azithromycin was observed for all four non-*C. jejuni*/*C. coli* isolates. One of these isolates was susceptible against ciprofloxacin, while the remaining showed complete resistance to both ciprofloxacin and tetracycline. As seen for infections caused by *C. jejuni* and *C. coli*, macrolides prove to be a better treatment option; while antimicrobial treatment of *Campylobacter*

infections is infrequent, as majority of human infections are self-limiting, macrolides are recommended for the treatment of laboratory confirmed *Campylobacter* cases (Wimalarathna *et al*., 2013).

4.1.8.4. MDR *Campylobacter* species

MDR *Campylobacter* was accounted for by 3.1% of the 229 isolates tested against antimicrobials included in the study. Four of these isolates were *C. jejuni* and three were *C. coli*. Only one of these isolates was from a child of less than five years of age, while the remaining six were individuals aged >19 years. It is plausible that this may be an underreporting of the actual number of MDR *Campylobacter* cases in South Africa because over 40% of the *Campylobacter* isolates in the study were non-culturable for antimicrobial testing. Nevertheless, studies reporting the emergence and mechanisms of MDR *Campylobacter* have been documented (Wieczorek and Osek, 2013). The global resistance to fluoroquinolones together with the increase in macrolide resistance, particularly in developing countries, poses a great threat for human health (Aarestrup and Engberg, 2001; Wieczorek and Osek, 2013; Wang *et al*., 2014). Resistance stemming from inappropriate use or failure to complete the prescribed course of antimicrobials does not sufficiently explain the increasing burden of antimicrobial-resistant *Campylobacter* because many of these infections resolve on their own. In addition, human-to-human transmissions are uncommon, providing minimal opportunity for resistant strains of *Campylobacter* to propagate (Wimalarathna *et al*., 2013). The epidemiology of antimicrobial-resistant *Campylobacter* in South Africa still stands subject for investigation.

Globally, studies conducted to compare antimicrobial resistance patterns of human *Campylobacter* clinical isolates with that recovered from chicken meats have found patterns of human isolates and chicken meats to be comparable (Jonker and Picard, 2010; Silva *et al*., 2011; Bester and Essack, 2012; Wimalarathna *et al*., 2013). Robust molecular characterizing techniques such as MLST have shown association between clinical isolates of human *Campylobacter* with that recovered from poultry, which suggests that is it plausible that antimicrobial resistance patterns from poultry will therefore also be prevalent in human clinical isolates (Gormley *et al*., 2010; Vidal *et al*., 2016) but the precise pathways stands subject for investigations. Events that

result in favour of resistant strains occur potentially on poultry farms and HGT has been described to have a major role in these events (Wimalarathna *et al*., 2013).

4.2. Animal isolates

As seen from the surveillance programs concerned with human health included in our study, *C. jejuni* was the most commonly detected species of *Campylobacter* from animal isolates submitted from the Gauteng and the Western Cape Provinces, with the remaining few isolates accounted for by *C. coli*. Animal isolates were not submitted in a systematic manner and therefore the sample size was too small to make substantial comparisons with isolates from human hosts as far as transmission from animal to human is concerned.

Animal *Campylobacter* isolates from Gauteng (*n*=12) and Western Cape (*n*=6) tested against azithromycin and erythromycin showed complete susceptibility against these macrolides. However, the number of received animal isolates was small to reflect plausible antimicrobial patterns of animal isolates in South Africa. Studies reporting macrolide resistance have been documented in different poultry and pig production systems in South Africa where they are the recommended drugs in veterinary medicine to treat infections of Mycoplasma and Spirochaete (Jonker and Picard, 2010).

Fluoroquinolone resistance in animal husbandries has been associated with bird treatment of resistant *E. coli* infections, which may possibly be the treatment method used in South Africa, reflected by the high resistance patterns (61%; 11/18) noted for the animal isolates. However, it is worth noting that the sample size was small in our current study. Only two of the Gauteng chicken isolates were susceptible to ciprofloxacin while four of the Western Cape isolates were susceptible, with one intermediately resistant isolate and one isolate with complete resistance against ciprofloxacin. This may reflect different antimicrobial therapies administered in different provinces (Jonker and Picard, 2010). In South Africa tetracyclines are extensively used both in the poultry and pig farms (Jonker and Picard, 2010; Bester

and Essack, 2012), which may explain the high level of resistance (83%) obtained for this class of antimicrobial agents against the tested animal isolates in our study. A similar case of isolates from commercially raised chickens showing high rates of resistance against tetracycline was reported in KwaZulu-Natal (Bester and Essack, 2012). In Gauteng and Western Cape similar resistance patterns have been reported for tetracycline resistance (Jonker and Picard, 2010).

4.3. Population structure of *C. jejuni* isolates defined by MLST

MLST was performed on viable pure cultures of South African *C. jejuni* isolates from human hosts (*n*=84) and animal hosts (*n*=9). These isolates (*n*=93) proved to be highly diverse with a total of 33 different sequence types belonging to 16 different clonal complexes. The identified STs for these isolates were comparable to frequently identified *C. jejuni* STs associated with human infections (Colles *et al*., 2003; Manning *et al*., 2003; Dingle *et al*., 2005; Sheppard *et al*., 2009; Nielsen *et al*., 2010; Vidal *et al*., 2016). The most predominant STs identified in this study were ST-7997, ST-257, ST-19, ST-475, ST-572, and ST-227 in the order of increasing number of isolates found in each ST respectively. ST-227 and ST-572 were the most frequently identified STs for the *C. jejuni* isolates in the study accounting for 13% and 10% respectively for all typable *C. jejuni* isolates with defined STs. These STs have been reported in many European countries, and in the USA in association with human disease (Colles *et al*., 2003; Manning *et al*., 2003; Sails *et al*., 2003; Kinana *et al*., 2006; Sheppard *et al*., 2009). Two human isolates represented by ST-474 were identified in the study; ST-474 has been reported to be rare in other countries, but highly prevalent in New Zealand, associated with isolates of human and poultry origin (Biggs *et al*., 2011).

All typable isolates could be grouped into clonal complexes with the exception of isolates belonging to ST-6091 and ST-881 which were not assigned to any clonal complex in the PubMLST database. Five clonal complexes frequently associated with the typable isolates in the study were ST-45 complex, ST-257 complex, ST-48 complex, ST-21 complex, and ST-206 complex in the order of increasing number of

isolates in each clonal complex. ST-206 complex was represented by 25 isolates, accounting for 27% (25/91) of all the typable isolates with defined clonal complexes. ST-21 complex was the second common clonal complex, followed by ST-48 complex, accounting for 23% (21/91) and 13% (18/91) respectively. These clonal complexes fall amongst previously described clonal complexes associated with over 60% of human disease isolates (Dingle *et al*., 2002; Colles *et al*., 2003; Best *et al*., 2005). Many studies report a predominance of ST-21 complex (Colles *et al*., 2003;Manning *et al*., 2003; Nielsen *et al*., 2010), an observation not seen in this study; with ST-206 complex taking precedence. Nevertheless, it could be that the isolates that were non-viable (>40%) represent some of the clonal complexes reported to be common among human disease isolates.

Province distributions of STs between Gauteng and Western Cape varied, with a few shared STs and clonal complexes. ST-227 complex and ST-206 complex represented the most common strains of *C. jejuni* in Gauteng, and ST-7997 complex and ST-21 complex represented the most common strains of *C. jejuni* in Western Cape. However, diversity within isolates of the respective provinces was still substantial.

Shared STs and clonal complexes between isolates of human hosts and animal hosts was observed, they were represented by ST-227, ST-257, ST-7997, ST-21, and ST-52. All the STs were common in human isolates and isolates of chicken origin, with the exception of ST-21 which was common between a human isolate and an isolate of bovine origin. These findings were consistent with previous reports of human isolates overlapping with veterinary isolates which may signify potential transmission from animal to human through the food chain (Colles *et al*., 2003; Sails *et al*., 2003; Kinana *et al*., 2006; Sheppard *et al*., 2009; Cody *et al*., 2015; Fernandes *et al*., 2015; Vidal *et al*., 2016). ST-21 complex, which was the most common clonal complex for the animal isolates, and the second most common clonal complex amongst human isolates, has been reported to be the most stable cluster of isolates colonizing a wide spectrum of hosts (Colles *et al*., 2003).

Although three STs (ST-381, ST-613, and ST-1932) found in animal isolates [bovine (ST-381); and chicken (ST-613 and ST-1932)] were not found in human isolates in our current study, these isolates had common clonal complexes with human isolates (ST-21 complex, ST-48 complex, and ST-460 complex). Studies carried out to subtype *Campylobacter* have reported that isolates from human hosts and animal hosts are not totally congruent; suggesting that some isolates colonizing chickens do not infect humans, and conversely, some isolates infecting humans do not colonize chickens (Manning *et al*., 2003). The reported observation may in part explain the three STs of animal origin that do not overlap with the human isolates in our current study. Moreover the association of poultry samples with human *Campylobacter* infections have not been consistent, and of ruminants are less well defined (French *et al*., 2005). The genetic diversity observed in human disease isolates has also been reported in veterinary isolates; particularly in poultry samples (French *et al*., 2005; Vidal *et al*., 2016). This reported observation held true for isolates in our current study, which was represented by 25 different STs for human isolates (*n*=84) and 8 different STs for animal isolates (*n*=9). However, in our current study the comparison (STs of animal isolates and STs of human isolates) was done to see if there are STs that were identified from our animal isolates (small sample number) that were not present in our human *Campylobacter* isolates (large sample number), not necessarily to emphasize that there are unique STs found in animals and not in humans, as our animal sample number was small to draw such conclusions.

Although the sample size of animal isolates in the study was small to extrapolate substantial conclusions, potential existing diversity between the isolates could be seen by the number of sequence types and clonal complexes defining these isolates. Nevertheless, distinguished STs and clonal complexes appeared to be predominant between isolates of human origin and animal origin regardless of the small number of *C. jejuni* isolates. Other STs and clonal complexes of *C. jejuni* may still be present in South Africa. Manning *et al*., (2003) reported on three human South African *C. jejuni* isolates which had STs not represented by isolates in this study; these STs were ST-497, ST-631, and ST-442. This may reflect that isolates in the study have not covered all the possible *C. jejuni* STs in circulation in South Africa, which can only be unravelled through more molecular epidemiological studies of the genus *Campylobacter*. One ST identified in the study which was previously documented in

the PubMLST database originating from a South African (Cape Town) human *C. jejuni* isolate in 1994 (sporadic case) was ST-1471 belonging to ST-406 complex; the case patient was from Gauteng Province in our current study. Majority of the South African *Campylobacter* isolates documented in the PubMLST database are represented by ST-362 complex, a complex which was not identified for isolates in this study. *C. jejuni* isolates that could not be subtyped due to confounding factors such as difficulty obtaining growth and purity of bacterial cultures could represent other STs that were not represented presently in our current study.

Non-typable isolates and isolates with new combinations of previously represented alleles, as well as the single case of novel alleles were included in the phylogenetic analysis to determine how they relate to typable isolates. Isolates with novel combinations of previously known alleles (NST) had one to three allele differences with typable isolates in the study, with the exception of NST.3 and NST.9, which had more than three allele differences with isolates in the study. The human isolate (NST.6) with novel allele combinations had more than three allele differences (*glnA*, *gltA*, *tkt*, and *uncA*) to ST-227. Majority of the NT *C. jejuni* isolates had one to three allele differences with the typable isolates in the study; e.g. NT.9 isolate from sheep had two allele differences (*glnA* and *pgm*) to ST-227. These isolates may not necessarily represent new clonal complexes as they do not have significant allele differences from typable isolates in this study.

The Simpson's diversity index of *C. jejuni* showed a significant degree of genetic diversity and a weakly clonal lineage ranging from 0.692 to 0.828 with a 95% confidence interval. The *uncA* locus was found to be the less diverse allele associated with less different repeats as compared to *aspA*, *glnA*, and *tkt*, associated with significant number of different repeats. These findings coincided with a study by Kinana *et al*., (2006) and conversely opposed the findings by Colles *et al*., (2003) describing high diversity in the *uncA* locus. The population structure of isolates in the study resembled that of high genetic diversity and weak clonality, as with *C. jejuni* isolates from previous studies (Meinersmann *et al*., 2002; Colles *et al*., 2003; Manning *et al*., 2003; Sails *et al*., 2003; Kinana *et al*., 2006; Vidal *et al*., 2016). The driving force influencing the evolution of *C. jejuni* is primarily intraspecific

recombination (Suerbaum *et al*., 2001; Biggs *et al*., 2011; Sheppard *et al*., 2011; Sheppard and Maiden, 2015).

4.4. Rotavirus Surveillance Program

The detection rate of *Campylobacter* was 7% (69/981) for children of less than five years of age with diarrhoea in South Africa. Majority of the positivity was accounted for by children aged less than two years, represented by 85% (59/69) of the positive samples. The results coincided with previous reports showing the prevalence of campylobacteriosis to be hyperendemic among the young in developing countries, particularly those aged less than two years of age (Acheson and Allos, 2001; Mshana *et al*., 2009). However, the detection of *Campylobacter* could not confidently be associated with the resultant diarrhoea as this could be asymptomatic carriage; other untargeted enteropathogenic bacteria could be the underlying cause of diarrhoea. For both the young and adults residing in developing countries, asymptomatic infections with *Campylobacter* occur (Acheson and Allos, 2001) due to recurrent *Campylobacter* infections as residents are repeatedly exposed (Wierzba *et al*., 2008).

The most predominant species of *Campylobacter* identified was *C. jejuni*, accounting for 81% of the positive samples for *Campylobacter*. The high detection of *C. jejuni* was similar to what was observed from the *Campylobacter* Surveillance Program, which coincided with previous reports of *C. jejuni* being the most ubiquitous species of *Campylobacter* associated with human infections, followed by *C. coli*, which in this study accounted for 13% of the positive samples (Moore *et al*., 2005; Persson and Olsen, 2005; Chokboonmongko *et al*., 2013; Ioannidou *et al*., 2013; Mason *et al*., 2013; Wagenaar *et al*., 2013; Schielke *et al*., 2014; Vidal *et al*., 2016). In 1990, Konkel and Joens postulated a possible synergistic relationship between *C. jejuni* and other enteric pathogens to exist in developing countries. Other enteric pathogens, particularly viruses, which mediate virus-induced alterations in cell membrane receptors, may contribute to *Campylobacter* colonization and penetration; this possibility was considered because these samples were from a Rotavirus Surveillance Program aimed at identifying aetiological agents associated with

childhood diarrhoea, primarily viruses. Children coinfected with both *C. jejuni* and *C. coli* were identified (6%), while other species of *Campylobacter* (non-*C. jejuni*/*C. coli*) were not detected from this study group, as seen from the 0-4 age group from the *Campylobacter* Surveillance Program.

Majority of the positive samples were slightly more from males (54%) than females (46%), proportions which were similar to what was observed from the *Campylobacter* Surveillance Program.

Campylobacter was detected throughout February 2014 to December 2015, during which the third annual quarter had the highest number of positive cases (38%), followed by the first and second quarter accounting for 26% and 20% respectively. The fourth annual quarter had the lowest number of screened samples, and subsequently the lowest number of detected *Campylobacter*, accounting for 13%. There was synergy between the number of screened samples and the frequency of detected *Campylobacter*; the higher the number of screened samples, the higher the likelihood of detecting *Campylobacter*.

Majority of the detected *Campylobacter* were from Chris Hani Baragwanath Hospital (45%), followed by Red Cross Childrens Hospital (14%), Edendale Hospital (13%), and lastly Mapulaneng Hospital (10%). Provinces represented by these study sites were Gauteng, Western Cape, KwaZulu-Natal, and Mpumalanga respectively. As with isolates from the *Campylobacter* Surveillance Program, detected *Campylobacter* species from the respective provinces is only a reflection of which hospitals sent in more specimens for the Rotavirus Surveillance Program, and not the burden of disease for respective provinces.

4.5. Acute Flaccid Paralysis Surveillance Program

4.5.1. Detection of *Campylobacter* from AFP patients

The most predominant species of the 12% (62/512) detected *Campylobacter* from the AFP Surveillance Program was *C. jejuni*; accounting for 77.4% and *C. coli* was the second most frequently detected species of *Campylobacter*, accounting for 19.4%, while coinfection with both *C. jejuni* and *C. coli* was the least detected, accounting for 3.2%. *C. jejuni* is a well-documented trigger of GBS; however, an interesting finding was the detection of *C. coli* from stool specimens of AFP patients. According to Nachamkin (1977), it is unknown whether *C. coli* is also an elicitor of GBS. *C. coli* was also detected as a coinfecting pathogen with *C. jejuni*. In developing countries, asymptomatic infections with *Campylobacter* occur (Acheson and Allos, 2001) due to recurrent *Campylobacter* infections as residents are repeatedly exposed (Wierzba *et al*., 2008). This could in part explain the detection of species of *Campylobacter* from AFP patients which have not yet been documented as elicitors of the sequelae. If *C. coli* remains overruled as a trigger of GBS, pathogens documented as triggers of GBS (Aspinall *et al*., 1994), other than *Campylobacter*, may be the underlying cause of AFP in those patients with positive *C. coli* results and those from which *Campylobacter* was not detected, which remains considerably high (88%; 452/512).

The detection of *C. jejuni* from specimens of AFP patients can only be interpreted as possible elicitors of the resultant AFP. Not all serotypes of *C. jejuni* trigger GBS; specific serotypes of *C. jejuni* found globally and in Cape Town, RSA, have been documented (Goddard *et al*., 1997; Lastovica *et al*., 1997; Wassenaar *et al*., 2000; Quiñones *et al*., 2008). To ascertain that the detected *C. jejuni* were a trigger of GBS, a PCR-based serotyping technique targeting serotypes frequently associated with GBS would need to be designed to screen all the *C. jejuni* positive samples from the AFP Surveillance Program.

4.5.2. Annual occurrences of detected *Campylobacter*

Specimens from which *Campylobacter* was frequently detected were submitted during quarter one, three, and four which are characterized by warmer months compared to quarter two (with low number of detected *Campylobacter*) characterized by cooler months. Most GBS cases are reported to be sporadic; however, a seasonal trend has been reported with a surge in the summer months according to Nachamkin *et al*., (1998) as well as Dimachkie and Barohn (2013). Studies describing the precise seasonal distribution of GBS are still lacking, however, summer peaks of GBS have been reported in China, Mexico, and Spain (Nachamkin *et al*., 1998; Nyati and Nyati, 2013). In 2008, Wierzba and colleagues reported GBS to lack a marked seasonal variation in Egypt, a developing country. Seasonal trends of GBS in South Africa have not yet been documented and the study period was short to define seasonal trends for positive cases in this study.

4.5.3. Distribution of detected *Campylobacter* by sex

Sex distribution has in almost all reports shown males to be more frequently associated with *Campylobacter*-induced GBS in comparison to females (Nachamkin *et al*., 1998; Desai *et al*., 2010; Islam *et al*., 2012; Nyati and Nyati, 2013). Most of these studies investigating *Campylobacter*-induced GBS are conducted in developed countries. One study conducted in Egypt, a developing country, showed the frequent association of GBS cases with males which coincides with what is observed in developed countries (Wierzba *et al*., 2008). However, in our study, although not statistically significant (p-value <0.22), it was specimens from females from whom more *Campylobacter* was detected than males, accounting for 55% and 45 % respectively. This observation was the inverse of what was observed for isolates from the *Campylobacter* Surveillance Program and samples from the Rotavirus Surveillance Program where there were more males among infected persons.

To further substantiate the findings, polio negative specimens screened for the presence of *Campylobacter* during the October 2014-December 2015 were more frequently from males (53%; 271/512) than females (47%; 239/512). According to Lastovica *et al*., (1997) the nine GBS patients from which *Campylobacter* species

were recovered from Groote Schuur and Red Cross hospital (Cape Town, RSA), five were males and four were females. Therefore, sex distribution of *Campylobacter* positive specimens of GBS patients in this study was not comparable with previous reports of GBS being slightly more common in males than females (Lastovica *et al*., 1997; Nachamkin *et al*., 1998; Nyati and Nyati, 2013).

4.5.4. Distribution of detected *Campylobacter* by age category

AFP patients from whom *Campylobacter* was frequently detected were those aged 0-2 years (37%), 3-5 years (32%), and those aged 6-9 years (18%). *Campylobacter* species were not detected in all age groups, other age groups with relatively few specimens from which *Campylobacter* was detected were those aged 10-19 years (6%), and the single adult case (40-49 age group) accounting for 2%, as well as the 5% represented by patients of unspecified age groups. Age-specific trends in this surveillance program were similar to what was observed previously (*Campylobacter* and Rotavirus Surveillance Programs) and the risk factors associated with these age groups described previously were applicable for the AFP case patients.

According to Khuzwayo *et al*., (2013) a case definition of AFP in South Africa is defined as any child of less than 15 years of age presenting with acute onset of focal weakness or paralysis which is characterized as flaccid, including GBS, without any other obvious cause. This explains why majority of the *Campylobacter* positive cases in this study were accounted for by children and teenagers, which are individuals forming part of the AFP case definition. A study conducted in Egypt by Wierzba *et al*., (2008) showed the incidence of *C. jejuni*-induced GBS to be higher in children from developing countries; an observation which contrasts reports from developed countries where the prevalence of GBS is commonest among adults (Nachamkin *et al*., 1998; Desai *et al*., 2010; Islam *et al*., 2012). According to Nyati and Nyati, (2013) all age groups are implicated with GBS, however, reports have shown incidence of GBS to increase with age, and according to Islam *et al*., (2012) *C. jejuni*-induced GBS incidence is lower in children. Nevertheless, results from our study coincided with that of Wierzba *et al*, (2008).

Given that gastroenteritis due to *C. jejuni* infections are hyperendemic among young children (<5 years) in tropical resource-poor settings, it is expected that the prevalence of *C. jejuni*-induced GBS would be prevalent among individuals of less than five years of age. Reports of bimodal distribution of GBS cases by age, with more identified cases in young adults (15-30) and the elderly (significantly higher) have been document (Nachamkin *et al*., 1998; Desai *et al*., 2010; Islam *et al*., 2012; Nyati and Nyati, 2013). However, an increase in the detection of *Campylobacter* with age, as well as the bimodal distribution (AFP case definition: <15 years) of positive cases by age in this AFP study group was not observed.

4.5.5. Province distribution of detected *Campylobacter*

The majority of detected *C. jejuni* was from stool specimens submitted from the Eastern Cape and Gauteng Province respectively. The difference in the total number of screened stool specimens submitted from Eastern Cape and KwaZulu-Natal, as well as between Limpopo and Mpumalanga was one; however, the number of detected *C. jejuni* did not correlate between the compared provinces. The Eastern Cape Province had ten more detected *C. jejuni* than KwaZulu-Natal, and Limpopo had four more detected *C. jejuni* than Mpumalanga. The synergy observed from the Rotavirus Surveillance Program where the more samples screened the higher the likelihood of detecting *Campylobacter* did not hold true for AFP stool specimens. For example, stool specimens were submitted the most from the Gauteng Province, however, only 11/104 were positive for *C. jejuni*, whereas in the Eastern Cape Province 17/94 were positive for *C. jejuni*. The detection of *C. jejuni* from the respective provinces may be largely compromised by the fact that many *Campylobacter*-induced GBS cases go unreported because by the time patients present with AFP the bacteria has already been eliminated from the body (Allos, 1997; Nachamkin, 1997; Poropatich *et al*., 2010). According to Nechamkin, (1997) history of antimicrobial use from patients with GBS should be clarified, as their use will have a marked challenge when isolating the underlying pathogen triggering GBS.

4.6. Limitations

The GERM-SA surveillance program is laboratory-based which means that bacterial cultures were submitted only for patients from whom stool specimens were collected. Laboratory-based surveillance may not best represent the burden of *Campylobacter* in South Africa, especially because this was the first study ever to investigate the prevalence of *Campylobacter* at a national level. Furthermore, only the Western Cape and Gauteng Provinces showed participation in the *Campylobacter* Surveillance Program, therefore data in this study are not representative of South Africa as far as *Campylobacter* infections are concerned, as many laboratories from the rest of the provinces did not show substantial submission of isolates. Nevertheless, the focus of this study was not to describe the incidence rate of *Campylobacter* in South Africa, but rather to characterize *Campylobacter* isolates using molecular tools utilized in the our current study. The fastidious nature of *Campylobacter* species made it challenging to maintain culture viability; the majority of isolates lost viability during transportation to CED, and some of the isolates lost viability upon storage. This compromised obtaining antimicrobial susceptibility profiles as well as subtyping some of the isolates using MLST. The possibility of *Campylobacter* asymptomatic carriage rendered the detection of *Campylobacter* from samples from the Rotavirus Surveillance Program and the AFP Surveillance Program inconclusive with respect to the fact that we could not definitely link disease (diarrhoea or AFP) to the *Campylobacter* organism, i.e., we could not say for sure that *Campylobacter* was causing the disease. However the findings are highly suggestive that *Campylobacter* might be associated with the resulting diarrhoeal diseases and AFP cases. In addition, information on history of diarrhoea for AFP patients was not available and the detected *C. jejuni* were not further characterized to determine their serotypes, therefore could not be confidently associated with AFP because only certain serotypes of *C. jejuni* elicit AFP. The AFP analysis was also limited by the absence of non-AFP control group, which makes it challenging to interpret these finding without knowing the background prevalence of *Campylobacter* in the population.

There was insufficient published literature describing the epidemiology of *Campylobacter* infections in South Africa or developing countries, therefore findings in this study were more concluded upon based on what has been reported in developed countries, a situation which may disqualify unique *Campylobacter* patterns in South Africa.

The multiplex real-time PCR assay used in this study only targeted *C. jejuni* and *C. coli*, which compromised the species detection other campylobacters in the study. The use of PCR to detected *Campylobacter* directly from specimens of AFP patients compromised obtaining the detected species in culture, where more phenotypic tests could be conducted to further characterize the isolates, which could help reveal if there was an association of *Campylobacter* to GBS for AFP patients in our study. Majority of the patients enrolled in the AFP Surveillance Program were aged <15 years, which narrowed down other potential at risk age groups for AFP, which could be caused by *Campylobacter*. As part of the objectives, the identification of potential transmission routes of *Campylobacter* from non-human isolates (food sources from animals) to humans was investigated; however, despite many appeals, veterinary sectors did not send isolates to the CED systematically for such investigations to be conducted. Thus potential sources of *Campylobacter* infections in South Africa could not be identified in our current study.

4.7. Future prospects

This study produced preliminary data on *Campylobacter* infections in South Africa. Therefore, surveillance of *Campylobacter* needs to be expanded in order to establish the incidence of *Campylobacter* infections in South Africa. This will aid in the monitoring of emerging *Campylobacter* strains and MDR *Campylobacter* since macrolides were more clinically effective against the isolates as compared to ciprofloxacin and tetracycline with high resistance profiles. An established surveillance which is representative of South African *Campylobacter* isolates will help encourage molecular epidemiological studies, which will help identify patterns of important strains, as well as motivate vaccine developments. Subsequently, such efforts will help fill the current existing research gaps for *Campylobacter* through new published literature in South Africa. Participation of more veterinary laboratories is

necessary in order to investigate possible transference between animals and humans. The use of MLST in such studies will enable us to identify common strains of *Campylobacter* between animals and humans.

Chapter 5: Conclusions

This study represented the first comprehensive study looking at the molecular epidemiology of *Campylobacter* in South Africa. The baseline data provided by this study included: prevalent species of *Campylobacter* characterized by real-time PCR, possible risk factors and risk groups of infection, antimicrobial susceptibility profiles, and the genetic population structure of predominant species of *Campylobacter* in circulation in South Africa using MLST. The burden estimate of *Campylobacter* was further investigated by screening specimens from various surveillance programs; which included specimens from the Rotavirus Surveillance Program, which is concerned with aetiology of childhood diarrhoea (<5 years) as well as polio negative stool specimens from the AFP Surveillance Program to determine the proportion of AFP possibly associated with species of *Campylobacter*, particularly *C. jejuni*.

The burden of *Campylobacter* infection in South Africa may have been underestimated as a result of lack of *Campylobacter* surveillance; 94% (801/848) of the isolates submitted for the *Campylobacter* Surveillance Program were positive for species of *Campylobacter* and real-time PCR proved useful characterizing these isolates. The most predominant species of *Campylobacter* identified from all surveillance programs included in this study was *C. jejuni,* which collectively accounted for over 80% of all *Campylobacter* positive cases and *C. coli* was the second most detected species of *Campylobacter.* Coinfection with both *C. jejuni* and *C. coli* was identified in some case patients in the respective surveillance programs. Non-*C. jejuni*/*C. coli* species were detected only from the *Campylobacter* Surveillance Program, which signifies that other species of *Campylobacter*, although presently low in numbers, are causing disease and should be monitored. *Campylobacter* positive samples from the Rotavirus and AFP Surveillance Programs could not, with utmost certainty, be associated with campylobacteriosis; due to the high level of asymptomatic cases of *Campylobacter* reported in developing countries, as a result of repeated exposure which stems from living in close proximity with animals. There is a pressing need for the public to be educated about the ramifications associated with living in close proximity with animals and to raise more

awareness of *Campylobacter* infections, which are currently still under reported in South Africa as a common disease which affects many people. Emphasis on good hygiene practices should be motivated, which includes good food handling practices, particularly after being in contact with animals, handling raw meats, and after using the toilet.

C. jejuni detected from the AFP Surveillance Program could not be attributed to GBS with confidence because specific serotypes of *C. jejuni* are associated with the development of GBS; a serotyping test was not performed for these isolates. The detected percentage of *C. jejuni* could only serve to motivate future studies that will examine the true proportion of GBS attributed to *Campylobacter* infection, and to readdress the potentially undiscovered or underreported cases of *C. jejuni*-induced GBS in South Africa.

The detection of *Campylobacter* by sex showed males to be slightly more associated with campylobacteriosis as compared to females, represented by 54% for case patients from the *Campylobacter* Surveillance Program, and by 54% for case patients from the Rotavirus Surveillance Program respectively. Conversely, it was females from the AFP Surveillance Program from which *Campylobacter* was more frequently detected, accounting for 55%. Extended studies involving the screening of stools of AFP patients for *Campylobacter* would need to be conducted to ascertain whether or not females are the risk group for GBS than males (documented risk group for GBS) in South Africa. Statistically, the sex-specific differences noted in all surveillance programs included in the study were not significant ($p>0.05$). The precise risk factors associated with sex-specific differences for campylobacteriosis in South Africa can only be addressed by continued long-term epidemiological studies.

For age of patients, three age-groups were identified as the highest risk groups as concluded by data from the *Campylobacter* Surveillance Program; at risk groups were children (35%; <5 years), young adults (12%; 25-34 years), and the elderly (10%; >65 years). At risk groups identified from the Rotavirus Surveillance Program were children aged <1-12 months (62%) and 13-24 months (23%), and those aged <1-2 years (37%) and 3-4 years (32%) from the AFP Surveillance Program. The high incidence of *Campylobacter* among these age risk groups requires interventions that

will control and prevent the persistent burden of disease in these age groups. Although sex-related differences of incidence held true with previous reports of men being more frequently implicated than females; age- and sex-related patterns of campylobacteriosis in this study revealed female infections to surpass those of males at certain age groups and the findings were statistically significant (p-value 0.02). Female at risk age-groups were those aged 25-34; 35-44; and >65 in years from the *Campylobacter* Surveillance Program, an observation which requires further investigation to determine risk factors associated with these groups in South Africa for control and prevention strategies to be put in place.

A pseudo-seasonal pattern for *Campylobacter* infections was identified for isolates from the *Campylobacter* Surveillance Program; a pattern that was characterized by a surge in the warmer months, followed by a rapid decline in the winter months. Summer, autumn, and spring may be seasons associated with incidence of *Campylobacter*; however, further studies would need to be carried out to deem these preliminary findings valid, because seasonal patterns for *Campylobacter* in developing countries are not well defined. Isolates from the Rotavirus and AFP Surveillance Programs lacked a marked seasonal pattern, with identified peaks even during winter.

All isolates from the *Campylobacter* Surveillance Program were sporadic cases, with no reported outbreaks during the course of the study, however, one questionable significant peak during the fourth quarter of 2014 and first quarter of 2015 was observed, accounting for 54% and 36% respectively, particularly in the Gauteng province. Significant conclusions to such observations can only be answered once a baseline study, with a suitable denominator is established, from which meaningful conclusions can be made, whether or not these numbers reflected an outbreak.

Cases of invasive *Campylobacter* infections were rare, remaining as low as 0.6% and associated more with children than adults. Other emerging *Campylobacter* species should in future be monitored, as some of the identified non-*C. jejuni*/*C. coli* isolates were associated with the invasive cases of *Campylobacter*.

Antimicrobial-resistant *Campylobacter* species are a growing concern, an observation dictating the pressing need for control strategies and strict regulations of antibiotic use. *Campylobacter* isolates in this study were extensively resistant against ciprofloxacin and tetracycline, but showed more susceptibility to the macrolides (erythromycin and azithromycin), particularly erythromycin, with a total of 90% susceptible isolates. The monitoring of *Campylobacter* antimicrobial susceptibility profiles is essential, in order to continually assess the clinical usefulness of recommended drugs; such as macrolides which are gradually gaining resistance, and to put regulations in place to control and prevent the spread of resistant isolates. Cases of MDR *Campylobacter* were identified which was accounted for by 3.1%, however, more MDR *Campylobacter* species may be in circulation, current observations may be underreporting *Campylobacter* MDR cases due to difficulties encountered when growing cultures for antimicrobial testing.

Although the collection of animal isolates to source attribute *Campylobacter* infections was not successful, with only 18 isolates received between January 2014-December 2015, species of *Campylobacter* identified were the same as those from human isolates, with a predominance of *C. jejuni* and less of *C. coli*. The generated antimicrobial susceptibility profiles were similar to those of human isolates; extensive ciprofloxacin and tetracycline resistance was observed, with isolates showing complete susceptibility against the macrolides. MDR *Campylobacter* isolates were not identified from the animal sample set. A systematic approach of collecting animal isolates may form preliminary data that will aid in source attributing human *Campylobacter* infections in South Africa, and to reveal if there is a link between antimicrobial resistance patterns seen in isolates from animal husbandries and human clinical isolates.

MLST based on WGS data analysis was employed to study the genetic population structure of *C. jejuni*, which was the most prevalent species of *Campylobacter*. MLST proved to be an effective typing tool, discriminating strains based on their relative difference in their MLST loci on the basis of seven conserved housekeeping genes. High diversity and a weakly clonal lineage defined the molecular biology of *C. jejuni* isolates detected from the *Campylobacter* Surveillance Program study; the diversity was further substantiated by the discovery of isolates with new alleles and STs yet to

be assigned in the PubMLST database. Of all the identified STs, ST-227 and ST-572 were the most predominant STs, and ST-206 complex and ST-21 complex were the most predominant clonal complexes grouping the *C. jejuni* strains in our current study. MLST aided in the identification of clones from geographically different regions; ST-227 and ST-206 complex represented the most common strains of *C. jejuni* in the Gauteng Province; ST-7997 and ST-21 complex represented the most common strains of *C. jejuni* in the Western Cape Province. Isolates of animal hosts and human hosts were compared using MLST, which revealed a few shared STs and clonal complexes between animal isolates (chickens and bovine) and human isolates, represented by ST-227, ST-257, ST-7997, ST-21, and ST-52. However, transmission of *Campylobacter* from animal to human could not be deduced in our current study because the animal study was not well established. STs of animal *C. jejuni* strains that did not overlap with human *C. jejuni* strains were also identified, represented by ST-381, ST-613, and ST-1932.

Although animal isolates were relatively of a small sample size, the diversity that exists between these isolates could be established by the number of identified sequence types and clonal complexes. These findings can form a foundation from which large scale feasible studies comparing veterinary and human *C. jejuni* isolates in South Africa can further be carried out to source track infections and alleviate the problem of limited data explaining the molecular epidemiology of *Campylobacter* in both the clinical and veterinary sectors in South Africa. Furthermore such findings may help guide the development of vaccines against the most virulent and prevalent species of *Campylobacter*.

In South Africa, there are still research gaps regarding *Campylobacter* and studies such as this emphasise the importance of this organism in the public health domain. Although data in this study is still preliminary, with the possibility of epidemiological artefacts, it may offer guidance for future studies with similar study objectives.

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Appendices

Appendix A: Housekeeping genes for *Campylobacter* MLST

The MLST scheme for *Campylobacter* is based on the sequence identification of multiple loci located in seven housekeeping genes. The following seven loci were chosen for *C. jejuni* MLST scheme (protein products are shown in parentheses): *aspA* (aspartase A), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyl- transferase), *pgm* (phosphoglucomutase), *tkt* (transketolase), and *uncA* (ATP synthase alpha subunit) (Dingle *et al*., 2001; Schouls *et al*., 2003).

Table: Sequenced fragment sizes (bp) for *C. jejuni*.

Locus	Fragment size (bp)
aspA	477
glnA	477
gltA	402
glyA	507
tkt	498
pgm	459
uncA	489

(Dingle *et al*., 2001).

Appendix B: TE buffer for crude genomic DNA extraction

TE buffer (10mM Tris, 1mM EDTA pH 8.0)

10 mL 1M [*]Tris (pH 8.0)
2 mL 0.5M [ϕ]EDTA (pH 8.0)

Two milliliters (mL) of 0.5 molar (M) EDTA at pH 8.0 was added to 10 mL of 1 M Tris at pH 8.0 and the solution was diluted to final volume of 1000 mL with 988 mL sterile water and autoclave.

*Tris (Merck, Darmstadt, Germany)
[ϕ]EDTA (Sigma Aldrich, St. Louis, USA)

Appendix C: Preparation and validation of QIAamp Fast DNA Stool Mini Kit

Preparation of reagents in QIAamp Fast DNA Stool Mini Kit for extraction

- The precipitants in Buffer AL and InhibitEX Buffer were dissolved by incubating in a water bath set at 55 °C and each of the buffers were thoroughly mixed by vortexing.

- Buffer AW1 supplied as a concentrate was prepared to a working solution by adding of 25 mL absolute ethanol (100 %) to the concentrate and thoroughly mixed by vortexing.

- Buffer AW2 supplied as a concentrate was prepared to a working solution by adding of 30 mL absolute ethanol (100 %) to the concentrate and thoroughly mixed by vortexing.

- Buffer ATE was mixed by vortexing before use.

- Proteinase K was mixed by inverting the tube up and down.

To validate the extraction kit, a stool specimen from a healthy individual was spiked with 0.5 McFarland bacterial lysate in normal saline [Diagnostic Media Products (DMP), NHLS, RSA] prepared from *C. jejuni* (strain C12.2 - WHO EQA 2012) and *C. coli* (strain C9.1 - WHO EQA 2009) control strains respectively (100 µL bacterial lysate into ~200 mg stool specimen), following which DNA was extracted as outlined by the extraction protocol. Extracted DNA from spiked stool specimens yielded the expected PCR results for each control strain.

Appendix D: *Campylobacter* real-time PCR

Table: *Campylobacter* target gene sequences and primer/probe sequences.

Species	Target gene	PCR *primer/probe	ϕPrimer/probe €sequence (3'-5')	Product size
C. jejuni	*mapA*	*mapA*-F1	CTGGTGGTTTTGAAGCAAAGATT	95 bp
		mapA-R1	CAATACCAGTGTCTAAAGTGCGTTTAT	
		mapA-probe1	**6FAM** - TTGAATTCCAACATCGCTAATGTATAAAAGCCCTTT - **BHQ1**	
C. coli	*ceuE*	*ceuE*-F1	AAGCTCTTATTGTTCTAACCAATTCTAACA	103 bp
		ceuE-R1	TCATCCACAGCATTGATTCCTAA	
		ceuE-probe1	**Cy5** - TTGGACCTCAATCTCGCTTTGGAATCATT -**BBQ**	
Non-*C. jejuni/C. coli*	*16SrRNA*	Camp -F2	CACGTGCTACAATGGCATAT	108 bp
		Camp-R2	GGCTTCATGCTCTCGAGTT	
		Camp-probe P2	**Cy3** - CAGAGAACAATCCGAACTGGGACA - **BBQ**	

*All primers were synthesized by Inqaba, Pretoria, RSA.

ϕAll probes were synthesized by Roche, Johannesburg, RSA.

€ Target gene sequences were obtained from Best *et al*., (2003); Lund *et al*., 2004; and Botteldoorn *et al*., 2008.

Campylobacter primer/probe mix preparation

- 70 µl of PCR/molecular grade water
- 30 µl of 100 µM mapA-F1 primer
- 30 µl of 100 µM mapA-R1 primer
- 6 µl of 100 µM mapA-probe1 probe
- 60 µl of 100 µM ceuE-F1 primer
- 60 µl of 100 µM ceuE-R1 primer
- 12 µl of 100 µM ceuE-probe1
- 60 µl of 100 µM Camp-F2 primer
- 60 µl of 100 µM Camp-R2 primer
- 12 µl of 100 µM Camp-probeP2

Primers and probes were received as lyophilized products, which were resuspended in TE buffer at pH 8.0 according to instructions outlined by the manufacturer to make 100 µM working solution. The *Campylobacter* primer/probe mix for real-time PCR was prepared by adding reagents outlined above into a 1.5 mL amber tube and vortexed to mix and spun down. The mix was stored at -20°C.

Volume of genomic DNA added into a 50 µL real-time PCR reaction.

- Crude genomic DNA from culturable bacterial isolates-1µL
- Crude genomic DNA extracted from non-culturable bacterial isolates in transport media-4µL
- Genomic DNA and nucleic acid from clinical specimens-7 µL

Control strains for *Campylobacter* real-time PCR

i. *C. jejuni* positive control - (**strain C12.2 - WHO EQA 2012**)

ii. *C. coli* positive control - (**strain C9.1 - WHO EQA 2009**)

iii. Non-template control (NTC) – (autoclaved water)

Interpretation of Real-time PCR results

Positive results were indicated by an amplification plot showing a typical real-time PCR sigmoidal curve with samples showing calculated threshold cycle (C_t) values on the SDS 7500 software. The '*C. coli*_ceuE' detector was shown in a grey colour, the '*C. jejuni*_mapA' detector was shown in a blue colour, the 'Campy_16SrRNA' detector was shown in a red colour, and the 'IPC' detector was shown in an orange colour (Figure below). The C_t values were interpreted differently for bacterial cultures and clinical specimens. For bacterial cultures a C_t value of 15-30 (positive results ranged between C_t values of 15-25) was accepted as a positive result, and for clinical isolates a C_t value of 15-38 (positive results ranged between C_t values of 25-35) was accepted as positive.

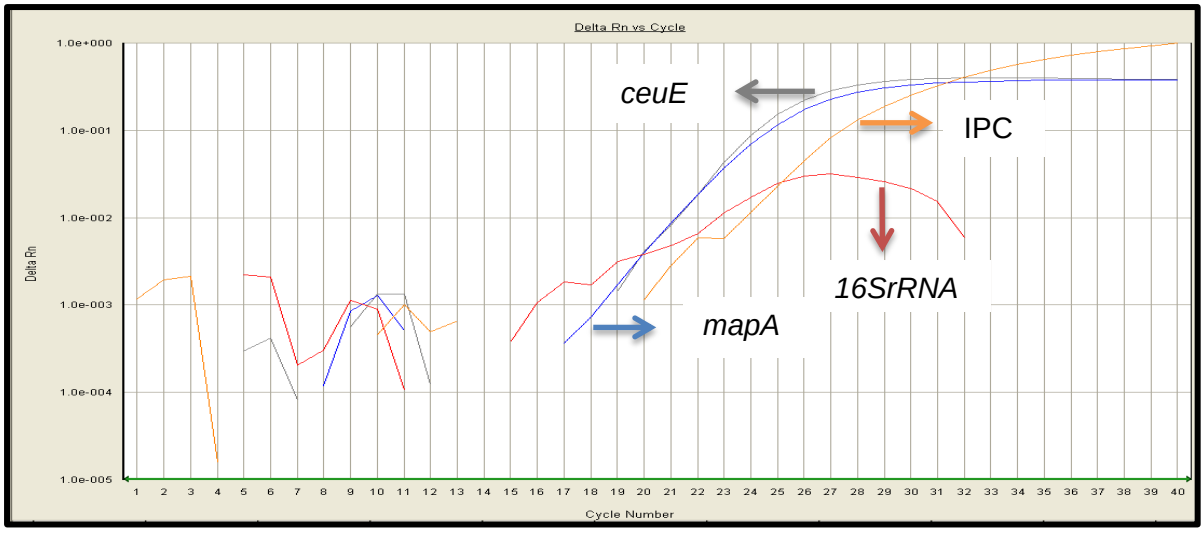


Figure: Real-time multiplex PCR results showing sigmoidal curves and C_t values of amplified target genes.

Table : Interpretation of *Campylobacter* real-time PCR results.

C. jejuni target gene (*mapA*)	*C. coli* target gene (*ceuE*)	*Campylobacter* species target gene (*16SrRNA*)	IPC	Result
+	–	+	+ or –	*C. jejuni* positive
–	+	+	+ or –	*C. coli* positive
–	–	+	+ or –	Positive for other non-*C. jejuni/C. coli Campylobacter* spp.
–	–	–	+	Negative for *Campylobacter* spp.

Appendix E: Extraction and quantification of DNA for WGS

Preparation of reagents in QIAamp DNA Mini Kit for extraction

- The precipitants in Buffer AL were thoroughly mixed by vortexing.
- Buffer AW1 supplied as a concentrate was prepared to a working solution by adding of 25 mL absolute ethanol (100%) to the concentrate and thoroughly mixed by vortexing.
- Buffer AW2 supplied as a concentrate was prepared to a working solution by adding of 30 mL absolute ethanol (100%) to the concentrate and thoroughly mixed by vortexing.
- Buffer ATE was mixed by vortexing before use.
- Proteinase K was mixed by inverting the tube up and down.

Preparation of Qubit™ Working Solution

- The Qubit™ Working Solution was prepared by diluting the Qubit™ reagent 1:200 in Qubit™ buffer.
- A total of 200 µL of Working Solution was prepared for each standard and sample.

Table: Assay tubes for DNA quantification using the Qubit™ dsDNA BR Assay kit.

	Standard Assay tubes	**DNA Sample** Assay tubes
Volume of **Working Solution** (Qubit™ reagent + Qubit™ buffer)	190 µL	199 µL
Volume of **Qubit™ Standard**	10 µL	-
Volume of **DNA Sample**	-	1 µL
Total volume in each Assay Tube	200 µL	200 µL

Appendix F: Rotavirus Surveillance Program study sites

Table: Study sites from the Rotavirus Surveillance Program.

Study sites	Province	Hospital Name
1	Gauteng	Chris Hani Baragwanath Hospital
2	Mpumalanga	Mapulaneng Hospital
3	Mpumalanga	Matikwana Hospital
5	KwaZulu-Natal	Edendale Hospital
6	Western Cape	Red Cross Childrens Hospital
8	Northern Cape	Kimberley Hospital
9	Polokwane	Polokwane Hospital
10	Free-State	Pelonomi Hospital

Appendix G: Ethics clearance certificate

R14/49 Ms Madile S Thobela et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140750

NAME: (Principal Investigator)	Ms Madile S Thobela et al
DEPARTMENT:	School of Pathology National Institute for Communicable Diseases Centre for Enteric Diseases
PROJECT TITLE:	Characterization of Campylobacter Isolates from a South African Population
DATE CONSIDERED:	25/07/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Karen Keddy and Dr Anthony Smith
APPROVED BY:	Professor A Woodiwiss, Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	25/09/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

M Thobela — Principal Investigator Signature

17/10/2014 — Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix H: Plagiarism declaration with Turnitin report

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

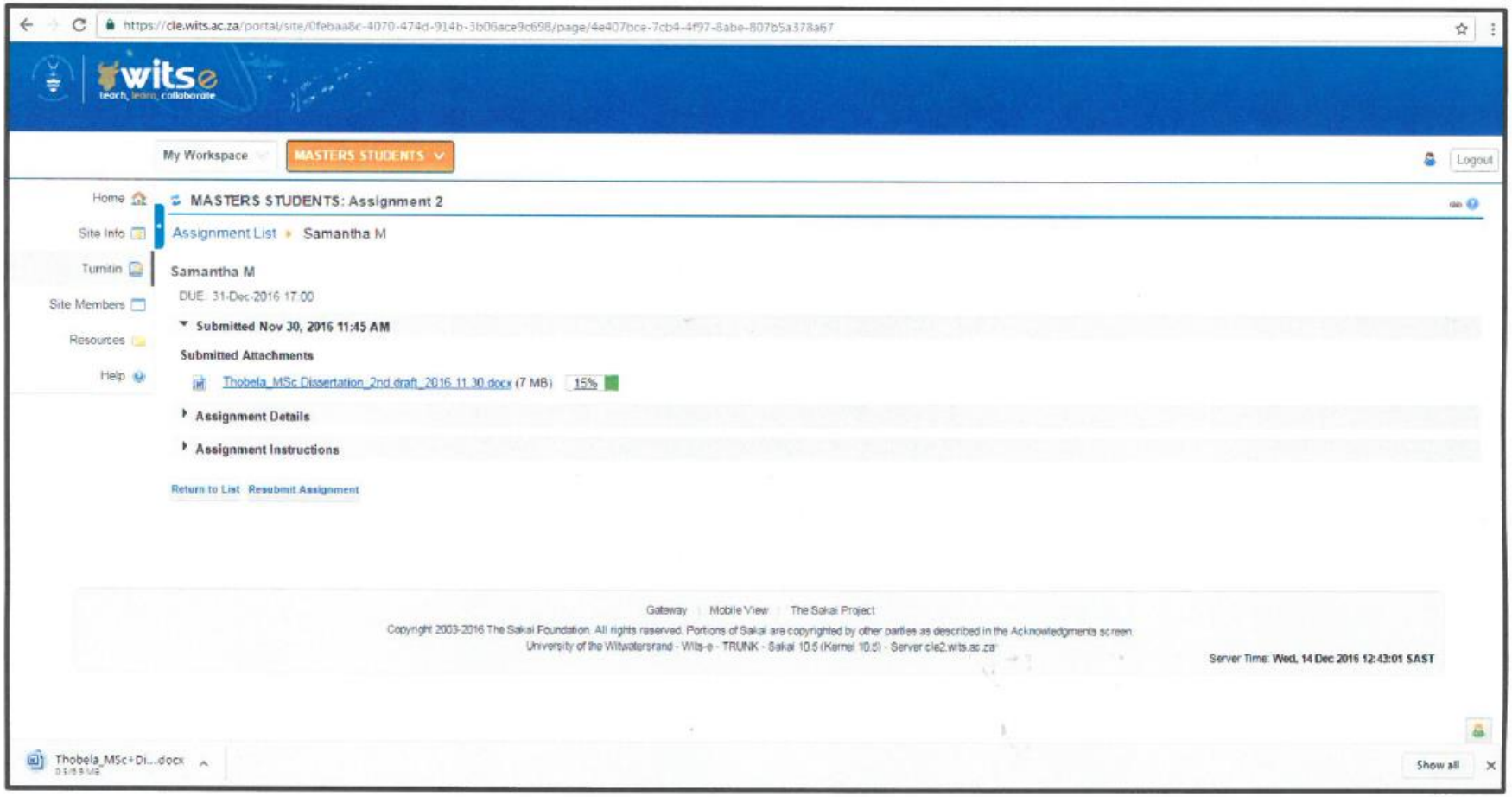
I Mandile Samantha Thobela (Student number: 374653) am a student registered for the degree of Master of Science in Medicine in the academic year 2014.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: M Thobela Date: 05 January 2017

1



DR. ANTHONY M SMITH
BSc (Hons). PhD

5/1/2017