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**THE EPIDEMIOLOGY OF
CAMPYLOBACTER JEJUNI IN
COMMERCIAL BROILER FLOCKS
IN NEW ZEALAND**

A thesis submitted in partial fulfilment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

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New Zealand

NAOMI BOXALL

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Abstract

When written in Chinese, the word 'crisis' is composed of two characters - one represents danger, and the other represents opportunity.

SAUL DAVID ALINSKY

New Zealand maintains the highest incidence rate of human campylobacteriosis of the industrialized countries (334.2 cases per 100,000 in 2002), it accounts for more than 56% of all disease notifications in the country. New Zealand is unique globally, with a 'notification-based surveillance system for notifiable diseases that is complemented by laboratory reporting. In other countries (Australia, US, UK), the notification system is entirely laboratory based. Thus, the high incidence of Campylobacteriosis in humans may be related to the methods of reporting rather than the reality of the disease situation. However, the reason for such high incidence has not yet been fully elucidated, and several studies conducted in New Zealand and overseas have implicated the consumption of poultry meat as the main cause of human infections.

The reduction or elimination of *Campylobacter jejuni* in the food chain, particularly from poultry meat products, is a major strategy in efforts to control campylobacteriosis. One approach to this is to prevent *C. jejuni* colonization of broiler chickens. This approach has been used to control *Salmonella* contamination of poultry, but the measures put in place for control of *Salmonella* have not controlled *C. jejuni*. It is generally unknown how frequently *C. jejuni* colonizes commercial broiler chickens in New Zealand, or what could be done to prevent these infections from occurring. The present study was undertaken in order to describe some of the basic epidemiology of *C. jejuni* in commercial broiler flocks in New Zealand.

The thesis is intended to further describe the epidemiology of colonisation of commercial broiler chickens by *C. jejuni* in NZ, and present possible risk factors that could be controlled in future to decrease the number of positive flocks of birds that are processed.

The thesis set out to elucidate first the extent of *C. jejuni* colonisation of birds, flocks and farms while the birds were on the farm, having had minimal risk of exposure to Campylobacter spp., by sampling 15 birds in 80 flocks belonging to two companies prior to the first partial depopulation, an event during which the flock are exposed to potentially contaminated fomites and biosecurity levels are dropped, doors opened and personnel movements are extensive. The resulting prevalence estimates are 25.6% of farms, and

12.5% of sheds, are likely to be used to rear broiler chickens colonised with *C. jejuni*. When a positive flock is discovered, 76.9% of the birds are likely to be colonised with *C. jejuni*. These figures are results across the whole study population of farms and sheds, as there were no significant differences between prevalence estimates between companies.

Following this prevalence estimation, a longitudinal study was conducted involving 12 sheds, to determine whether the environment or the birds were colonised with *C. jejuni* first. Although 12 sheds were observed every other day from day 14 to the end of the rearing period, it was determined that the birds were positive either first, or at the same time as the environment. Having said that, the sensitivity of the testing method for the environment was dubious, as there were instances where a shed that had positive samples collected on one occasion appeared negative the next, before returning a positive result on the third consecutive sampling occasion.

A cross-sectional study of 810 flocks was undertaken to determine the most relevant risk factors for colonisation of the broiler chickens with *C. jejuni*. Because of the vertically integrated structure of the poultry industry, these 810 flocks corresponded to data collected from 77 farmers about their farms and the 219 sheds on those farms. The caeca from ten birds from each flock processed were pooled and examined for the presence of *C. jejuni*. These results were used to create a case definition, such that the flocks could be analysed with the questionnaire data, and different risk factors were seen in each season. More flocks reared for Company One were colonised by *C. jejuni* than for Company Two. Protective factors included having hard (i.e. gravel, asphalt or concrete) pathways to the growout houses, being near to another broiler farm, using the reticulated town water supply for the birds drinking water, using tunnel or crossflow shaped growout houses, using a Chore-Time™ feed delivery system within the growout house and chlorinating the water supply to the birds (only in winter). The odds of raising flocks colonised with *C. jejuni* increased if rodents were seen on the farm, if the growout houses were constructed with a concrete nib wall, if gas heaters were used during brooding, if cattle were farmed on the property, or if workers were employed on the farm. Sanitising the annex at least as frequently as once per run decreased the odds during summer, and tended to have a similar effect in other seasons.

Chlorinating the water supply appeared to have a protective effect in only one season, though the trend appeared towards protection in the other seasons. The risk factor was validated by sampling the drinking water that broilers chickens had access to for the FAC to see whether the levels that were present in the drinking water could have an effect on *C. jejuni*. 11 sheds that were known to chlorinate the water were sampled to determine

whether they met the drinking water standards for humans in NZ, or met the requirements presented by one of the companies involved. Only three sheds met the human drinking water standards for FAC, and two of these (one from each company) met Company Two's requirements.

This thesis is for both regulatory and industry stakeholders to assist with developing risk management approaches to diminishing the number of *C. jejuni* positive flocks. Where management practices are altered, it is hoped that the efficacy of such practices be measured by examining the changes in the rates of *C. jejuni* colonization within the industry.

Acknowledgements

I would maintain that thanks are the highest form of thought, and that gratitude is happiness doubled by wonder.

GILBERT CHESTERTON

Firstly, I'd like to acknowledge the farmers who tend the growout houses utilised in this study, for their willing acceptance of the intrusion of study personnel and for also helping tremendously during the questionnaire process. Several staff in the processing plants who collected the viscera and caecal samples for long periods of time, without slowing production aided and abetted this project.

This work could not have been undertaken without the support of Tegel (NZ) Foods Ltd, and Inghams Enterprises (Pty) Ltd, notably the broiler management teams and the laboratory staff. The Poultry Industry Association of New Zealand (PIANZ) are acknowledged and commended for providing the funds necessary to research this area. This study would not have been possible without TechNZ funding of the author.

It is widely acknowledged within academia that the undertaking of a Ph.D. is to indicate slight madness. Either that, or a reluctance to step outside the cossetting warmth of the academic environment. This is not the reason I decided to attempt this project: no, the supervisors for my Masters of Veterinary Studies prompted me. So saying, I'd like to extend a wary thank you to Stan Fenwick (aka. The Mad Scotsman) and Dirk Pfeiffer. Both of these gentlemen were instrumental in my taking on this Ph.D., and both left the country soon after they had persuaded me! Appendix B (only available in the 'paperback version'!) is included especially for Stan.

Peter Davies subsequently stepped into the breach to supervise me, and with the help of Nigel Perkins, they forced me to the other side. There were times when I didn't do nearly enough work: and they had no hesitation in letting me know this. I think I'm appreciative! Cord Heuer has managed to maintain his esteemable cool, even when I was next to tears during the examination and amendment-making process. The numbers are all his fault...I tried to do without them. Roger Morris made sure that I finished it all, despite my (high) heels dragging, so this really wouldn't be here were it not for him.

So many people have influenced, nurtured and supported me during this exhausting and often all-encompassing task; to list them all would push me over my page limit (ha ha) but I

hope the following people, in particular, realise how important they are to me. Vivienne Boxall (my mother), the most elegant and strong-willed person I know: I hope I grow up to be like her one day! Thanks go also to the ultimate iron-fist-in-kid-glove combination Yvonne Hilder (my grandmother). And if it weren't for Ron Boxall (my father), I never would have pursued a career in science. Thanks Dad, I think. 'Job satisfaction' and 'employability' all sounded quite reasonable when I was 14!

Liorah Atkinson has called me "ffffd" for years now; so thanks for the continued support doll. I'm looking forward to being across the road with the pink rinse and the 24 cats, fingers twitching the curtains in a semaphoric language only she will understand. David Woods has been marvellously good company, he is a friend and editor par excellence, with whom I hope I remain friends for many years. My flatmates and the extended 'family' thereof (Joseph Glancy, Chris Farnsworth, Emma Thompson, Emily Maire, Paul Martin - and Ana Puke too!) must be sick and tired of me celebrating 'thesis-related' milestones ("you have finished the book - CHAMPAGNE!") yet are (too?) often prepared with a sympathetic bottle of bubbles.

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Now if I could just get a little golden statuette....? <queue music>

Table of Contents

ABSTRACT	III
ACKNOWLEDGEMENTS	VII
TABLE OF CONTENTS.....	IX
TABLE OF FIGURES.....	XIII
TABLE OF TABLES	XV
INDEX	XVII
INTRODUCTION.....	21
CHAPTER 1 LITERATURE REVIEW.....	25
1.0. A HISTORICAL INTRODUCTION TO THE GENUS <i>CAMPYLOBACTER</i>	28
1.1. TAXONOMY AND CHARACTERISTICS OF THE GENUS <i>CAMPYLOBACTER</i>	30
1.1.1. <i>Classification</i>	30
1.1.2. <i>Campylobacter spp. in the Environment</i>	33
1.2. SUBTYPING OF <i>CAMPYLOBACTER JEJUNI</i>	34
1.2.1. <i>Phenotyping</i>	34
1.2.2. <i>Genotyping</i>	36
1.2.3. <i>Analysis of Genotyping Results</i>	40
1.3. <i>CAMPYLOBACTER IN HUMANS</i>	43
1.3.1. <i>Clinical Manifestations</i>	43
1.3.2. <i>Host Defences</i>	44
1.3.3. <i>Mortality</i>	44
1.3.4. <i>Morbidity and Cost</i>	45
1.3.5. <i>Extraintestinal Infection</i>	45
1.3.6. <i>Sequelae</i>	46
1.3.7. <i>Treatment</i>	47
1.3.8. <i>Pathogenesis and Virulence Factors</i>	47
1.3.9. <i>Epidemiology</i>	49
1.4. SOURCES OF <i>CAMPYLOBACTER</i> IN HUMANS	57
1.4.1. <i>Production Animals</i>	57
1.4.2. <i>Companion Animals</i>	60
1.4.3. <i>Water and Seafood</i>	61
1.5. <i>CAMPYLOBACTER AND BROILER CHICKENS</i>	62
1.5.1. <i>Pathology of Campylobacter jejuni in the Chicken</i>	63
1.5.2. <i>Epidemiology of Campylobacter jejuni on Broiler Farms</i>	64
1.5.3. <i>Investigations</i>	72

1.5.4. Control and Prevention	72
-------------------------------------	----

CHAPTER 2 THE PREVALENCE OF CAMPYLOBACTER JEJUNI IN COMMERCIAL BROILER CHICKEN FARMS IN NEW ZEALAND..... 77

2.0. INTRODUCTION	80
2.1. MATERIALS & METHODS.....	81
2.1.1. Poultry population.....	81
2.1.2. Cloacal sampling.....	83
2.1.3. Bacteriological examination	83
2.1.4. Strain typing by pulsed-field gel electrophoresis	84
2.2. RESULTS	86
2.3. DISCUSSION	92
2.4. REFERENCES	95

CHAPTER 3 WHICH IS COLONIZED BY CAMPYLOBACTER JEJUNI FIRST: THE CHICKEN OR THE ENVIRONMENT?..... 99

3.0. INTRODUCTION	102
3.1. MATERIALS AND METHODS	103
3.1.1. Longitudinal Study.....	103
3.1.2. Isolation of Campylobacter jejuni	104
3.1.3. Pulsed-Field Gel Electrophoresis Strain Typing	104
3.2. RESULTS	107
3.2.1. Longitudinal Study.....	107
3.3. DISCUSSION	110
3.4. REFERENCES	112

CHAPTER 4 PREHARVEST FACTORS ASSOCIATED WITH CAMPYLOBACTER JEJUNI COLONISATION OF BROILER FLOCKS IN NEW ZEALAND 115

4.0. INTRODUCTION	119
4.1. MATERIALS AND METHODS	121
4.1.1. Sensitivity testing.....	121
4.1.2. Flock information.....	121
4.1.3. Bacteriological examination	122
4.1.4. Statistical Analyses.....	123
4.2. RESULTS	124
4.2.1. Sensitivity testing.....	124
4.2.2. Descriptive results.....	124
4.3. DISCUSSION	133
4.4. REFERENCES	139

CHAPTER 5 CHLORINE IN COMMERCIAL BROILER DRINKING WATER 145

5.0. INTRODUCTION	148
5.1. MATERIALS AND METHODS	149
5.1.1. Growout houses.....	149
5.1.2. Chlorine Concentration.....	149
5.2. RESULTS	151
5.2.1. Temporal Differences in FAC Concentration.....	151
5.2.2. Spatial Differences in FAC Concentration.....	152
5.3. DISCUSSION	153
5.4. REFERENCES	155

CHAPTER 6	GENERAL DISCUSSION	159
REFERENCES FOR LITERATURE REVIEW AND GENERAL DISCUSSION	171
APPENDIX A		203

Table of Figures

FIGURE 1. ANNUAL INCIDENCE RATES OF CAMPYLOBACTERIOSIS IN INDUSTRIALISED COUNTRIES. DATA PREDOMINANTLY OBTAINED FROM [WWW.GIDEONONLINE.NET](http://www.gideononline.net) AND [HTTP://EARTHTRENDS.WRI.ORG/TEXT/POPULATION-HEALTH/COUNTRY-PROFILE.HTM](http://earthtrends.wri.org/text/population-health/country-profile.htm)..... 52

FIGURE 2. SAMPLING PROCEDURE FOR PREVALENCE STUDY. YELLOW CELLS INDICATE THE AGE OF THE FLOCKS WHEN SAMPLED. THE PINK CELLS HIGHLIGHT THE ‘SAME TIME PERIOD’ SUCH THAT THE LAST BIRDS IN THE FIRST FLOCK SAMPLED HAVE NOT BEEN KILLED BEFORE THE FIRST BIRDS OF THE LAST FLOCK WERE HATCHED..... 81

FIGURE 3. HISTOGRAM TO SHOW NUMBER OF GROWOUT HOUSES BY NUMBER OF POSITIVE SAMPLES 90

FIGURE 4. PFGE PROFILES AND DENDROGRAMS OF THE MAJOR CLONES OF *C. JEJUNI* ISOLATED FROM BROILER CHICKEN FLOCKS USING *KpnI* AND *SmaI* (ABOVE AND BELOW, RESPECTIVELY) 91

FIGURE 5. THE RESULT OF ENVIRONMENTAL AND CHICKEN TESTING ON ALTERNATE DAYS AFTER 14 DAYS OF AGE IN 12 GROWOUT HOUSES. SAMPLING BEGAN AT DAY 14, BUT CAMPYLOBACTER JEJUNI WAS NOT ISOLATED UNTIL DAY 24, AS SHOWN..... 109

FIGURE 6. AVERAGE FREE AVAILABLE CHLORINE (FAC) CONCENTRATION AT 5 SITES IN ONE GROWOUT HOUSE AT DIFFERENT TIMES OF THE DAY (ERROR BARS INDICATE 95% CI) 151

FIGURE 7. BOX PLOT OF FREE AVAILABLE CHLORINE (FAC) MEASUREMENTS WITHIN EACH GROWOUT HOUSE. THE HORIZONTAL LINE IN EACH BAR INDICATES MEAN FAC MEASUREMENT..... 152

Table of Tables

TABLE 1. THE 16 SPECIES OF CAMPYLOBACTER	31
TABLE 2. RAW DATA FROM PREVALENCE STUDY, OBSERVING CAMPYLOBACTER JEJUNI WITHIN FARMS, GROWOUT HOUSES AND BIRDS.....	86
TABLE 3. PREVALENCE ESTIMATES AND 95% CONFIDENCE INTERVALS FOR FARM, GROWOUT HOUSE AND BIRD PREVALENCE ESTIMATES.....	87
TABLE 4. PULSED-FIELD GEL ELECTROPHORESIS PATTERNS OF C. JEJUNI WHEN CLEAVED WITH KPNI	88
TABLE 5. PULSED-FIELD GEL ELECTROPHORESIS PATTERNS OF C. JEJUNI WHEN CLEAVED WITH SMAI	89
TABLE 6. NUMBER OF BROILER CHICKEN SAMPLES FROM WHICH C. JEJUNI WAS RECOVERED (n=8). THE SHADED AREAS INDICATE SAMPLING PERIODS THAT OCCURRED AFTER THE FIRST PARTIAL DEPOPULATION OF THE GROWOUT HOUSE.	107
TABLE 7. SUMMARY OF GROWOUT HOUSE SAMPLING	126
TABLE 8. DESCRIPTIVE DATA FOR EACH VARIABLE FOR ALL SEASONS. N=NUMBER OF GROWOUT HOUSES EXAMINED, No=NUMBER OF GROWOUT HOUSES IN EACH CATEGORY, C+=PERCENTAGE OF GROWOUT HOUSES POSITIVE FOR C. JEJUNI, SE=STANDARD ERROR, OR=CRUDE ODDS RATIO, 95% CI = 95% CONFIDENCE INTERVAL OF OR.....	127
TABLE 9. RESULTS OF MULTIVARIATE LOGISTIC REGRESSION ANALYSIS OF SIGNIFICANT RISK FACTORS ASSOCIATED WITH COLONISATION BY CAMPYLOBACTER JEJUNI	131

Index

AFLP	Amplified fragment length polymorphism
AIDS	Acquired Immunodeficiency Syndrome
Breeder	From age 0 to 24 weeks, birds are considered pullets. From 24 weeks on they are full-fledged breeder hens.
Broiler	Young (normally six weeks old) male or female birds weighing (1.36kg - 1.59kgs)
CDT	Cytolethal distending toxin
CE	Competitive Exclusion
CFU	Colony forming units (i.e. viable cells)
Chick	Newly hatched broiler chickens
DNA	Deoxyribonucleic Acid
DNases	Enzymes that degrade DNA in a non-specific manner
Fla	Flagellin (gene or protein)
GBS	Guillain-Barre syndrome
GC	Guanine & Cytosine (i.e. two of the four components of DNA)
GP	General Practitioner
H ₂ S/TSI	Hydrogen sulphide production on triple sugar iron agar
HeLa	Cells of the first continuously cultured human carcinoma strain (from cancerous cervical tissue of <i>Henrietta Lacks</i>)

HLA B7	Human histocompatibility (HLA) surface antigen encoded by the b locus on chromosome 7
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Kbps	Kilo (1000) base pairs
Layer	These specialized birds have been bred to be finely honed egg producing animals and are very different from the breeder lines. They produce the table eggs sold in stores.
mCCDA	Modified <i>Campylobacter</i> blood free selective agar
MEE	Multi-locus enzyme electrophoresis
MLST	Multi-locus sequence typing
MPN	Most Probable Number
NaCl	Sodium chloride
NCTC	National Collection of Type Cultures
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	p(otential of) H(ydrogen); the logarithm of the reciprocal of hydrogen-ion concentration in gram atoms per litre
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
Rpm	Revolutions per minute
TSA	Trypticase Soy Agar
UK	United Kingdom
UPGMA	Unweighted pair-group method

USA	United States of America
UV	Ultraviolet (light)
Vero	African Green Monkey (<i>Cercopithecus aethiops</i>) kidney cells

Introduction

"Where shall I begin, please your Majesty?' he asked. 'Begin at the beginning,' the King said, gravely, 'and go on till you come to the end: then stop.'"

- LEWIS CARROLL

In the last five decades, *Campylobacter* spp. have emerged as the most common cause of bacterial foodborne gastroenteritis in several industrialized countries, with the number of cases of campylobacteriosis often surpassing the number of cases of salmonellosis, this is particularly so in New Zealand. *Campylobacter coli* can cause the disease but the predominant cause is *C. jejuni*. New Zealand is unique globally, with a 'notification-based surveillance system for notifiable diseases that is complemented by laboratory reporting. In other countries (Australia, US, UK), the notification system is entirely laboratory based. Thus, the high incidence of Campylobacteriosis in humans may be related to the methods of reporting rather than the reality of the disease situation.

There are several transmission routes for *Campylobacter* spp. to infect humans. However, the foodborne transmission route is the route by which the majority of cases are infected, then a reduction of the overall *Campylobacter* spp. burden in the food chain will result in a reduction in the number of cases of disease. This reduction is thus a major strategy in efforts to control the incidence rate of campylobacteriosis. The main thrust of these efforts focuses on poultry products, although, as yet no case-control studies in human populations have suggested what fraction of campylobacteriosis is caused by consumption of poultry meat.

One approach to the reduction of *C. jejuni* in the food chain is to prevent *C. jejuni* colonization of broiler chickens. The hypothesis tested in this suite of studies is that there are possible risks of colonisation of *C. jejuni* to which broiler chickens are exposed during the rearing period and these can be elucidated and controlled such that fewer flocks will be colonised with *C. jejuni*. However, it is again generally unknown how frequently *C. jejuni* colonizes commercial broiler chickens in New Zealand, or what could be done to prevent these infections from occurring. The present study was undertaken in order to describe the epidemiology of *C. jejuni* in commercial broiler flocks in New Zealand.

Objectives:

1. To determine what proportion of birds and flocks are colonised with *C. jejuni*, and what proportion of farms are used to house them during the rearing period
2. To examine the possible environmental sources of *C. jejuni* to the birds and whether the birds or the environment are colonised first
3. To quantify some potential risks of exposure, and determine when they have the greatest effect and
4. To validate one of those risks.

The research presented in this thesis is novel within the country. Similar research has been conducted overseas, but this is not always applicable to the New Zealand growing situation. In particular, New Zealand has a much higher human Campylobacteriosis rate than the countries that have conducted research into poultry carriage of *Campylobacter* spp. consequently it seemed likely we could expect our results to differ wildly. This research is necessary to determine whether commercial broilers are, in fact, likely to have an effect on the rate of human campylobacteriosis in New Zealand, but is just the first step of many required to determine the total effect.

While the industry wishes to present the public with a healthy source of nutrition that is not contaminated with *Campylobacter* spp, it is important to know how much of the infection is due to the chickens merely 'catching' *Campylobacter* from their growing environment within the poultry shed. Once these facts have been determined, steps can possibly be taken to reduce the number of chickens entering the processing plant colonised or contaminated with *Campylobacter* spp. If the number of colonised birds on the farm is lower than we anticipate, then it is likely further studies will be required into the processing of the poultry meat, wholesale poultry meat management, and retail management of the same - to find out exactly which stages increase the risk to the consumer. It is highly likely that these gaps will be the focus of the many researchers dedicated to decreasing human campylobacteriosis rates in New Zealand in the future - this is just the beginning.

Chapter 1: The review of the literature covers the history and taxonomy of *Campylobacter*, the subtyping methods used to distinguish between species and strains of *Campylobacter* spp., the effect that *Campylobacter* spp. have on humans, animal sources of *Campylobacter* spp., specifically the current relationship between *Campylobacter* spp. and broiler chickens.

Chapter 2 is a research paper that describes a prevalence study that determined the proportion of birds, flocks and farms that had been, or contained birds that had been colonised by *Campylobacter jejuni*.

Chapter 3 is a research paper that describes a longitudinal study conducted in 2001, where samples were tested for *Campylobacter jejuni* from the growout house environment, food, water, and the broiler chickens every other day over the last three weeks of the growout period.

Chapter 4 is a research paper whereby the *Campylobacter jejuni* results from caecal samples from the broiler chickens collected at the poultry processing plant were collated with questionnaire data in order to identify potential risk factors for the broiler chickens.

Chapter 5 is the final research paper is a risk factor validation study that measured the true amount of Free Available Chlorine (FAC) delivered to the broiler chickens in the drinking water.

Throughout the thesis, the term 'campylobacteriosis' is fundamentally interchangeable with '*Campylobacter enteritis*'; and describes the condition resulting from an infection of a human by *Campylobacter* spp. or *C. jejuni* (where specified).

Each chapter is the manuscript of a paper published in or submitted to a peer-reviewed journal. Each chapter is written in a style to suit the readership of a particular journal and the format of the manuscript is that required by the journal, however, for the sake of consistency, all references are presented in the same style.

The list of references used in the Literature Review and General Discussion can be found at the end of the thesis; the references for each chapter are at the end of each chapter. Pages, figures and tables have been numbered sequentially throughout the thesis.

LITERATURE REVIEW

Literature Review

The trouble with the world is that the stupid are cocksure and the intelligent are full of doubt.

BERTRAND RUSSELL

1.0.	A HISTORICAL INTRODUCTION TO THE GENUS <i>CAMPYLOBACTER</i>	28
1.1.	TAXONOMY AND CHARACTERISTICS OF THE GENUS <i>CAMPYLOBACTER</i>	30
1.1.1.	<i>Classification</i>	30
1.1.2.	<i>Campylobacter spp. in the Environment</i>	33
1.2.	SUBTYPING OF <i>CAMPYLOBACTER JEJUNI</i>	34
1.2.1.	<i>Phenotyping</i>	34
1.2.2.	<i>Genotyping</i>	36
1.2.3.	<i>Analysis of Genotyping Results</i>	40
1.3.	CAMPYLOBACTER IN HUMANS	43
1.3.1.	<i>Clinical Manifestations</i>	43
1.3.2.	<i>Host Defences</i>	44
1.3.3.	<i>Mortality</i>	44
1.3.4.	<i>Morbidity and Cost</i>	45
1.3.5.	<i>Extraintestinal Infection</i>	45
1.3.6.	<i>Sequelae</i>	46
1.3.7.	<i>Treatment</i>	47
1.3.8.	<i>Pathogenesis and Virulence Factors</i>	47
1.3.9.	<i>Epidemiology</i>	49
1.4.	SOURCES OF <i>CAMPYLOBACTER</i> IN HUMANS	57
1.4.1.	<i>Production Animals</i>	57
1.4.2.	<i>Companion Animals</i>	60
1.4.3.	<i>Water and Seafood</i>	61
1.5.	CAMPYLOBACTER AND BROILER CHICKENS	62
1.5.1.	<i>Pathology of Campylobacter jejuni in the Chicken</i>	63
1.5.2.	<i>Epidemiology of Campylobacter jejuni on Broiler Farms</i>	64
1.5.3.	<i>Investigations</i>	72
1.5.4.	<i>Control and Prevention</i>	72

1.0. A Historical Introduction to the Genus *Campylobacter*

A bacterium from the genus *Vibrio* was implicated in various abortions in pregnant ewes during 1913. The bacterium was isolated and caused abortions in experimentally infected pregnant cows (250). The first definitive link between *Vibrio* and enteritis was made 18 years later, in 1931, when a *Vibrio* was the cause of winter dysentery in cows (108).

A further 26 years on, in 1957, Dr. E King described the first cases of human *Vibriosis*. She called the *Vibrio* isolated “related” *Vibrio* as it differed in thermotolerance to *Vibrio fetus* (124). Related *Vibrio* caused 12 cases of human enteritis between 1944 and 1967, all cases were diagnosed using blood cultures (44, 151, 261, 263). In 1971, an Australian team of researchers (40), and, concurrently, a Belgian team published separate reports detailing the findings of the related *Vibrio* in stool cultures from two cases of *Vibriosis* (46). The related *Vibrio* was isolated from 30 people when historical stool samples from 1000 patients were tested. It was stated, “it is probable that a large number of cases of diarrhoea are caused by the related *Vibrio*”, a statement which, 30 years later, can be described as an understatement.

The first critical study of the classification of *Vibrio*-like, curved, microaerophilic bacteria was made in 1973 (250). *Vibrio jejuni* was transferred to the genus *Campylobacter*, a genus described initially in 1963 by the same researchers. *Campylobacter* (Greek for ‘curved rod’) has an outer wavy membrane, complex cytoplasmic membranes and individual flagellar basal granules (250). The bacteria are oxidase positive, non-spore forming, Gram-negative, slender (0.5-8µm in length and 0.2-0.8µm in width) and exhibit a curved, S-shaped or spiral morphology. A single polar flagellum is present at one or both ends of the cells. Motility is characterised by darting or corkscrew-like movements. Some species produce coccidial forms in old cultures or those exposed to air (31, 178, 204).

Campylobacters belong to a distinct group of specialized Gram negative bacteria designated rRNA superfamily VI [1]. Apart from the genus *Campylobacter*, the group also contains *Arcobacter* and *Helicobacter*. *Arcobacter* are closely related to campylobacters and some cause intestinal infection in humans. *Helicobacter pylori* is well known as a cause of gastritis and peptic ulcer disease, but there are other *Helicobacter* species that cause infection of the human gut. A feature common to all these bacteria is that they are adapted to colonize the surface of the mucous membranes of the alimentary and reproductive tracts. This adaptation is reflected in their morphology. The combination of spiral shape and long

polar flagella leads to rapid motility that enables the organisms to "corkscrew" their way through mucus with a facility denied to conventional bacteria

1.1. Taxonomy and Characteristics of the genus *Campylobacter*

In 1963, when *Campylobacter* was first described, the genus comprised of two species. At present, the genus contains 16 species, and six subspecies. The 16s rRNA gene, used to determine phylogenetic relationships among all living organisms, played a major role in a previous extensive rearrangement of *Campylobacter* taxonomy. By use of DNA-rRNA hybridisations, relative phylogenetic positions have been determined, following cross-referencing with other phenotypic and genotypic data.

1.1.1. Classification

During the 30 years following the differentiation of the *Campylobacter* genus from *Vibrio*, groups of *Campylobacter*-like organisms were detected, described and identified as new species, or as different biotypes of existing species. When phylogenetic studies in the late 1980's revealed genotypic heterogeneity among the species thus identified, three major clusters, rRNA homology groups, were formed (247). *Campylobacter* was thus divided into three genera with revised genus descriptions, and new names were proposed for the remaining two genera: *Arcobacter* and *Helicobacter*.

The genera *Campylobacter* and *Arcobacter* form a family of gram-negative, nonsaccharolytic bacteria with microaerobic growth requirements and a low G+C content: the *Campylobacteraceae*. Members of this family are encountered mainly as commensals or parasites in humans or domestic animals. The family also contains misclassified species (*Bacteroides ureolyticus*) and strains originally described as 'free-living *Campylobacters*' (*Sulfurospirillum* sp.) (201).

At present, the genus consists of the 16 species shown in Table 1.

Species	subspecies	Biovar	Predominant Host Tissues	Consequences of Infection	Year	Reference
consensus			Human Oral Cavity	human periodontal disorders, diarrhoea(?)	1984	(93)
showae			Human Oral Cavity	dental plaque (?)	1993	(60)
curvus			Human Oral Cavity	human periodontal disorders	1991	(247)
rectus			Human Oral Cavity	human periodontal disorders	1991	(247)
gracilis			Human Oral Cavity, abscesses, lesions	human deep tissue infection	1985	(107)
sputorum		sputorum	Human Oral Cavity, feces, abscesses, lesions		1975	(133)
			Cows, Sheep, Pigs Genital tract, aborted material, feces			
		faecalis	Cows, Sheep Feces	human diarrhoea	1965	(67)
		paraureolyticus	Cattle, humans Feces	human diarrhoea	1998	(166)
hominis			Human Oral Cavity		1989	(147)
mucosalis			Pig Intestine and oral cavity	porcine intestinal disorders	1975	(133)
fetus	fetus		Aborted fetus Placenta, stomach contents	bovine and ovine abortion	1913	(146)
	venerealis		Cows Vaginal mucus, semen, prepuce	bovine and ovine infectious infertility	<1966	
hyointestinalis	hyointestinalis		Pig Intestine	porcine proliferative enteritis	1983	(73)
	lawsonii		Pig Stomach	unknown	1995	(167)
lanienae			Human (abattoir workers)		2000	(140)
jejuni	jejuni		Poultry, Cows, Sheep, Human, Environment Feces	human diarrhoea	1957	(123)
	doylei		Human gastric tissue, diarrhea, blood	unknown	1988	(170)
coli			Pig feces	human diarrhoea	<1977	
lari			Birds, animals, water, shellfish Feces	human diarrhoea	1984	(30)
upsaliensis			Cats, Dogs feces	human diarrhoea, abortion (?), abscess	1987	(165)
helveticus			Cats, Dogs feces	unknown	1992	(221)

Table 1. The 16 species of *Campylobacter*

Campylobacter contains DNA with a Guanine and Cytosine (GC) content between 29 and 36% (250). Most species are microaerophilic, although some show a range of oxygen tolerance, some are almost anaerobic, and others grow best in the presence of 5-10% oxygen (51). The aerobic species of *Campylobacter* were reclassified as genus *Arvobacter* in 1991 (247).

Most *Campylobacter* cells are slender, spiral, curved rods, 0.2 – 0.8 μm wide and 0.5 – 5.0 μm long. Cells of some species are predominantly straight or curved rods, and aged cells may appear to be coccoid, a degenerative (not dormant) stage. Cells of most species are motile, with characteristic corkscrew motility powered by a single polar unsheathed flagellum at one or both ends. However, *C. showae* has polar bundles of two to five flagella and *C. gracilis* is aflagellate (51).

It is acknowledged that the minimum growth temperature for *C. jejuni* and *C. coli* is around 30°C. However, this does not mean that metabolic activity ceases at lower temperatures, implying that there is a potential for the organisms to adapt to environmental stresses even when they are unable to grow. The metabolic activity (ATP production, catalase activity and respiration by oxygen uptake) in *C. jejuni* has been demonstrated at temperatures as low as 4°C (164). Chemotaxis toward formate and aerotaxis toward microaerophilic conditions has also been demonstrated at temperatures down to 4°C, illustrating the potential for the organism to migrate to conditions that might extend survival in the environment. The physiology of *C. jejuni* is complex and there are a number of chemicals in the environment that the organism can use as terminal electron acceptors (120). This comparative versatility may enable the organism to metabolise in diverse anaerobic environments outside the host.

C. jejuni is thought to die rapidly in the presence of oxygen and under dry conditions. However, studies show that *C. jejuni* can survive much better in vivo than in vitro. For example *C. jejuni* has been isolated from dry beach sand (269), which contradicts laboratory studies on survival in drying liquid droplets (94). One proposed explanation for this unexplained resilience is that *Campylobacter* may form viable but non-culturable (VNC) cells. In an original concept of this state, cells change from a spiral morphology to a coccoid form and become undetectable by normal culture techniques, but retain the potential to be resuscitated to an infectious form. While the evidence for VNC formation by *C. jejuni* remains equivocal, the ability for *Vibrio* to become VNC has been well characterised (207), and several other pathogens have also been described as undergoing a VNC transformation. A more recent theory suggests that only a few strains of *Campylobacter* transform to VNC cells. Those that retain their spiral morphology undergo a gradual loss of ability to maintain homeostasis (235), and so presumably there is a period where cells are

VNC followed by death. This latter theory is independently supported by studies where vibrioid cells were observed in chicken shed water supplies but could not be cultured (174), and have also been observed in a continuous culture microcosm where spiral cells persisted in biofilms (95). In studies on the survival of clinical and poultry isolates at 4°C, four from six poultry isolates became coccoid after ten days incubation, while only two from seven clinical isolates became coccoid. If a functional VNC state is found to be real, then this has significant implications for our knowledge about the survival of *C. jejuni* in the environment.

1.1.2. *Campylobacter* spp. in the Environment

When an investigation into a small rural area of New Zealand (Ashburton) in the South Island, using PFGE and Penner serotyping to examine the strains of *Campylobacter jejuni* isolated, it was shown that New Zealanders living in rural South Canterbury live in an environmental sea of *Campylobacter*. The organisms are ubiquitous in animal and bird reservoirs, which in turn contaminate surface water and the terrestrial environment through their infected faeces. Any person living and working in this environment is likely to be heavily exposed to this micro-organism. This study has also shown, at least among people with campylobacteriosis, that risk behaviour is common, including consumption of unpasteurised milk and drinking water (rural and urban). Given that many of the supplies are likely to be located on rural properties the most probable source of any *Campylobacter* contamination would be from livestock. An exception might be roof water where contamination from birds may also be significant, however only one human case indicated that their water supply was sourced from rainwater. A large river water study was conducted (The Freshwater Microbiological Programme, Ministry for the Environment) to investigate the presence of both pathogens and indicators in water. This study identified both the presence and numbers of *Campylobacter* in a high percentage of rivers. *Campylobacter* was detected in 60% of water samples from the 25 recreational fresh water sites tested throughout NZ (241). This study highlights the continued importance of genotyping isolates of *Campylobacter* using discriminatory methods such as those described in 1.3.9.4 and 1.4, such that we can begin to trace the strains, where they came from, and where they currently exist.

1.2. Subtyping of *Campylobacter jejuni*

Campylobacter demonstrate considerable ecological diversity. Most taxa appear to be pathogenic and may be associated with a wide variety of diseases in animals and humans. Accurate identification of these organisms is required to provide important clinical and epidemiological information in particular for:

- a) tracing sources and routes of transmission,
- b) identifying and monitoring temporal and geographical patterns exhibited by strains of phenotypic (or pathogenic) importance, and
- c) developing and testing strategies for control processes applied within the food chain.

A variety of approaches have been used to distinguish between species. Subtyping of *Campylobacter* spp. is an important aspect of epidemiological studies. Criteria for subtyping include the following: cost, ease of use, and discriminatory power. Phenotypic methods such as serotyping and phage typing are cheap and relatively easy to perform. Genetically based methods (e.g. *fla* typing, PFGE) have enhanced sensitivity and discriminatory power, consequently they show the most promise for research purposes.

1.2.1. Phenotyping

Most diagnostic laboratories use biochemical tests for speciation of *Campylobacter* spp. (248), for which various schemata have been described. Most schemata are tables of tests used to differentiate taxa within groups: catalase-positive, catalase-negative, and thermophilic species.

Biochemical tests useful in distinguishing between *Campylobacter* spp. include the following: α -haemolysis, catalase, hippurate hydrolysis, urease, nitrate reduction, selenite reduction, H₂S/TSI (trace), indoxyl acetate hydrolysis. Substrate (growth) related tests include the following: 25°C, 42°C, minimal medium, MacConkey, glycine, NaCl, and cefoperazone. Resistance profiles are conducted using two antibiotics: cephalothin and nalidixic acid.

There are now comprehensive differential schemata available in textbooks compiled from data pooled from several resources. However, two problems remain:

a) Lack of standardisation

Frequent and notable discrepancies have been discovered between results obtained from laboratories performing the same tests on the same isolate. This compromises the validity of schemata designed by collating data from several sources.

b) Lack of objectivity

Most schemata approach identification by observing whether an unknown isolate performs similarly to known taxa; more importance is placed on a single test result that is considered an essential strain characteristic than on other tests. This may lead an investigator to misclassify a species (e.g. hippurate-negative *Campylobacter jejuni* subsp. *jejuni* exhibits similar characteristics to *C. coli* on the basis of the hippurate test result).

The first strategy to objectively approach the identification of *Campylobacter* spp. was the biotyping scheme formulated in 1984 by Bolton *et al.* (30), which allowed researchers to distinguish between six taxa, with species identification occurring through the results of the first three tests (growth at 25°C, cephalosporin resistance and hippurate hydrolysis). Other clinically relevant taxa have since been described, so the truncated number of taxa used in this schema is today regarded as a limitation.

In 2000, a computerised schema of nearly 70 biochemical tests differentiated virtually all *Campylobacter* strains examined (168), but the large number of tests, and the computing environment required to analyse the results, limit the suitability of the schema for most laboratories.

While there are considerable difficulties in identifying *Campylobacter* spp. and their relatives by means of classical phenotypic tests, a recent study suggests that there is considerable correspondence between genomic relationships and groupings already determined, such that most strains are accurately identified to the species or sub-species level (168).

1.2.1.1. Serotyping

In 1980, Penner and Hennessy described a passive haemagglutination procedure for serotyping *Campylobacter jejuni* subsp. *jejuni* (then *fetus* subsp. *jejuni*) on the basis of soluble heat-stable antigens (179). The Penner method uses passive haemagglutination, whereby the supernatant from a boiled cell suspension is used to sensitize erythrocytes that are then mixed with antisera to demonstrate agglutination. This system recognizes 65 serotypes in total, and comprises 47 antisera for *C. jejuni* and 18 antisera for *C. coli* (180).

Lior *et al*, in 1982, published a serotyping method that detected variation in heat-labile antigens (139). This schema (a slide-agglutination test) recognizes 130 serotypes of *C. jejuni*, *C. coli* and *C. lari*. A separate schema was later proposed to accommodate *C. upsaliensis*. The proportion of isolates that give a result from serotyping can be low, but, in general, non-typeability in human and veterinary strains is less than 20% (161).

The main disadvantage of these methods is the lack of commercially available, high-quality antisera. Production of antisera to the large number of strains for either of the typing systems would be impractical for most diagnostic laboratories, therefore isolates from various food and animal sources are not always serotyped. This minimizes the opportunities to compare isolates using this method.

1.2.1.2. Phage typing

In 1985, a phage typing system for distinguishing between strains of *Campylobacter jejuni* and *C. coli* was described (78). The 14 virulent bacteriophages used were recovered from poultry faeces in the United States. An additional system was developed in 1992, which extended the original bacteriophage collection to include five phages isolated from chicken litter in Canada. Seven hundred and fifty four *Campylobacter* isolates, 672 *C. jejuni* isolates and 82 *C. coli* isolates received from human and non-human sources from 17 different countries, were phage-typed. Overall, 80.6% of the total isolates were typable (122). A further schema was developed in 1990 that combined six of the American phages with ten isolated from various sources (including pig and poultry manure, and sewage effluent) in the United Kingdom (196).

In combination, phage typing, serotyping, and biotyping have provided excellent results, however, carrying out all three types of tests may be too costly for most laboratories (173).

1.2.2. Genotyping

Genotypic methods measure chromosomal differences in relatively stable genomic structures, whereas phenotypic methods measure characteristics that may not be expressed in a stable manner.

In 2000, a review was carried out to evaluate Penner serotyping, ribotyping (automated ribotyping), *flaA* typing, Pulsed-field Gel Electrophoresis (PFGE), and Random Amplified Polymorphic DNA (RAPD) as typing methods. These methods were assessed for their use on *Campylobacter jejuni* isolates from humans, animals and environmental sources during outbreaks and related to sporadic cases. Penner serotyping was determined to be useful for

rough aggregation of typing a large quantity of isolates, and to compare against serotypes seen in other countries, from other time periods etc. Riboprinting and *flaA* typing were 'fairly' discriminative, and useful for screening high numbers of isolates: with automation and standardisation, riboprinting could be considered a definitive typing system. However, PFGE and RAPD were determined the most discriminative methods, and, being based on the entire genome of the bacteria, they were most useful in measuring genetic similarity in outbreak situations (162).

1.2.2.1. *Fla* Typing

The motility of *Campylobacter* spp. have been described above. The flagellar filaments are formed by a protein subunit, encoded for on the genome by a gene designated *fla*. *Campylobacters* have two flagellin genes, *flaA* and *flaB*. In *Campylobacter jejuni*, the two genes are in tandem, separated by approximately 0.2 kilobases (kb). The genes are conserved, with 92% identity between *flaA* and *flaB* genes in individual isolates; however, the *fla* genes differ between isolates, thereby providing the basis of a typing scheme.

Primers for the highly conserved sequences at the N- and C-terminal regions of encoded proteins are synthesised, and polymerase chain reaction (PCR) is used to amplify a 1.7-kilobase sequence. The product is then digested, to produce restriction fragment length polymorphisms (RFLP's) after electrophoresis. The method is quick and simple, though different laboratories design their own schema, which can hinder comparison of results. Furthermore, alternative genes have been used in conjunction with *flaA* to increase discriminatory power of this PCR based typing scheme (185). The level of discrimination for *fla* typing is higher than serotyping, but lower than pulsed-field gel electrophoresis (PFGE) (68).

It is presumed that there is an association with the heat-labile serotyping scheme (Lior) and the flagellin subunits, thus several attempts have been made to relate *fla* patterns to Lior serotypes. Studies have indicated that specific *fla* types can be found in several serotypes (37). However, individual Penner serotypes, ribotypes, or PFGE clones may display more than one *fla* type.

1.2.2.2. Ribotyping

Ribotyping, based on the detection of restriction fragment length polymorphisms (RFLP) containing ribosomal RNA genes, was introduced in 1992 (66). The discriminatory power of the technique is dependent on the choice of enzyme and the gene targeted. In 1996, a method was developed in which the 16S gene of strains belonging to all 47 heat-stable

serotypes of *Campylobacter jejuni* were examined, showing 100% typeability (69). Although the typeability for this technique is high, it is time consuming and technically complex, therefore rendering it unsuitable for routine subtyping.

1.2.2.3. Random Amplification of Polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA (RAPD) typing methods produce short sections of DNA amplified by an arbitrary primer (e.g. ten-mer). The resulting amplicons are different sizes depending on the locations of the primer sites in the genomes of different strains, thus the banding patterns are suitable for comparison of isolates within bacterial species. The level of discrimination for *Campylobacter jejuni* has been demonstrated as equal to that of PFGE techniques (160).

While the technique has high discrimination and typeability, reproducibility is difficult between laboratories. Factors associated with the lack of reproducibility include the type of thermocycler used in the PCR method, and the source of Taq DNA polymerase. However, as the availability of commercially prepared reagents increases, the likelihood of future standardisation also increases.

1.2.2.4. Amplified Fragment Length Polymorphism (AFLP)

AFLP is a high-resolution genotyping method, first developed for use in plants. It has recently been applied to the genotyping of *Campylobacter* spp. (54). The method involves digesting the genome with two restriction endonucleases, ligating oligonucleotide adapters to the fragments, and then amplifying a subset of the fragments based on the ligated adapters. Two specific methods have been developed for subtyping *Campylobacter* spp. (54, 137), which use different restriction endonucleases. Comparative studies of molecular subtyping techniques suggest that AFLP is equally as discriminatory as PFGE (137). Once again, complex banding patterns are the primary result of this method; hence interpretation of the results is not standard. Similarly to PFGE, the equipment used for this method is expensive, and may prohibit the use of the technique by some laboratories.

1.2.2.5. Multi-locus Sequence Typing (MLST)

In 1991, a multi-locus sequence typing (MLST) technique that uses comparative DNA sequencing of conserved housekeeping genes to characterize organisms was developed for *Campylobacter* spp. (49). These are the same genes used in multi-locus enzyme electrophoresis (MEE); hence the typing systems are analogous. In a recent study, clonal complexes recognised by MLST correlated with the strain associations previously described using MEE and contained some isolates indistinguishable by PFGE (195). The technique

is available to laboratories around the world, and there is the opportunity to create a global virtual library of isolates that can be expanded for use as a reference (<http://www.mlst.net>).

1.2.2.6. Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed Field Gel Electrophoresis (PFGE) is a technique used to separate especially long strands of DNA by length in order to tell differences among samples. It operates by alternating electric fields to run DNA through a flat gel matrix of agarose. Specialized equipment is required, consisting of a gel rig with clamped electrodes in a hexagonal design, a chiller and pump, and programmable power supply.

Genotyping of *Campylobacter* spp. isolates collected from an outbreak of gastroenteritis was first applied in 1984, analysing the restriction endonuclease digestion patterns produced by high frequency cutting enzymes (33). However, the complex patterns produced are difficult to interpret and are unsuitable for general typing schemes. Consequently, the alternate method using infrequent-cutting enzymes was applied to *Campylobacter jejuni* in 1991 (270). PFGE offers the advantage of high discrimination (similar only to that of amplified fragment length polymorphism (AFLP)) as well as reproducibility and high typeability. The drawbacks of this method are:

- c) Preparation of the DNA-containing Agarose blocks is time-consuming and tedious, though some rapid methods are becoming more available (189);
- d) Occasionally, DNases produced by some strains of *Campylobacter* degrade the DNA prior to electrophoresis, and must be deactivated by formaldehyde pre-treatment of the cells (75);
- e) The apparatus for this technique are expensive and specialised;
- f) The infrequent-cutting enzymes used to produce PFGE profiles do not digest the DNA of some strains;
- g) Interpretation of results can be complex.

PulseNet, the National Molecular Subtyping Network for Foodborne Disease Surveillance, is the Center for Disease Control and Prevention's (CDC) network of public health laboratories. These laboratories perform PFGE on foodborne bacteria. The network permits rapid comparison of these fingerprint patterns through an electronic database. PulseNet provides critical data for the early recognition and timely investigation of outbreaks, thus reducing the burden of foodborne disease. The National Microbial Typing Database in New Zealand is also equipped to perform a similar function for New Zealand, and indeed, may be extended to the Asia-Pacific region.

Due to the sheer volume of work having been performed using PFGE on *Campylobacter* spp. it is highly advisable that we maintain this method for temporal comparisons. However, as the cost involved in performing MLST decreases, and more laboratories are able to conduct this technique, it should start being used first in addition to PFGE, then instead of. The National Microbial Typing Database in New Zealand has been created to be sufficiently flexible to cope with new procedures as and when they become commonplace.

1.2.3. Analysis of Genotyping Results

The first parameter to be examined is the relationship and similarities between the lanes. These must be calculated before clustering methods can be of use. Similarity can be expressed mathematically as an Index that ranges from 0 to 1 (1 suggests indistinguishable isolates). There are several methods to calculate this, such as the Dice Coefficient, the Jaccard Coefficient, and the Euclidean Distance Squared. They differ in the weight of the number of positive and negative matches. Whether a distance or a similarity is calculated is of no importance as they can be transformed into each other.

The three methods use the same numbers, merely in different ways. A mathematical equation that can be written as a vector is constructed. It represents the bands found in each lane. When comparing PFGE profiles, the vector S contains B elements ($S=[s_1, s_2, s_3 \dots s_B]$), where B is the number of band types in the lane's band set.

S_i is 1 if the band type is found in the lane.

S_i is 0 if the band type is not found.

If S and T are two vectors representing two samples with lanes that are from epidemiologically related isolates then the similarity index can be calculated as described.

1.2.3.1.1. Dice Coefficient

This coefficient was first called a coincidence index, measuring the chance of one species (band) occurring in the same sample (lane) as another, given the second species (band) is present. (13) The formula can be explained as "the sum of bands that appear in both lanes divided by the sum of all bands in both lanes, multiplied by 200".

$$\text{Similarity} = 200 \times (\sum (s_i, t_i)) / (\sum (s_i + t_i))$$

$$\text{Distance} = 100 - \text{similarity}$$

1.2.3.1.2. Clustering Methods

The following definitions are used in the clustering methods described.

- P and Q are indices indicating two clusters to be joined to a single cluster.
- K is the index of the cluster formed by joining P and Q.
- I is the index of any remaining clusters other than P, Q or K.
- NP is the number of samples in the Pth cluster.
- NQ is the number of samples in the Qth cluster.
- N is the number of clusters in the Kth cluster formed by joining the Pth and Qth cluster ($n=np+nq$).
- Dpq is the distance between cluster P and Q.

The algorithm is the same for each of several clustering methods; only the formula determining the minimum distance differs. The space conserving cluster method: UPGAMA gives the most plausible clusters and is least affected by outliers.

1.2.3.1.3. Unweighted Pair Group Method (UPGAMA)

$$dk_i = (np/n) \times dp_i + (nq/n) \times dq_i$$

This is also called Weighted Average Linkage. The foregoing method can be generalised by weighting the distances with the number of objects of the groups involved. The equation translates to the cluster being formed based on the distance to p and q proportional to the number of individual objects within p and q. The fusion of two groups depends on the least mean of all possible distances between the single objects. (118) As long as the rate of nucleotide substitution is constant, UPGAMA shows good performance.

1.2.3.2. Issues with Dendrograms

The weakness of hierarchical methods such as these is that early decisions are permanent. The mathematical formulae are calculated within a computer algorithm contained within a program (GelCompar, Diversity Database etc). The output, rather than a series of numbers or tables, is a dendrogram. The information given in this format is a lot more intuitive than that in tables. A diagram represents the hierarchical evolutionary relationship between isolates of strains. Initially intended to determine the grand pattern of evolution and aid with taxonomy, these techniques have been refined to the genetic level, where they can be used to differentiate between strains and hypothesise the evolution of different isolates.

The tree output indicates quantitatively the relationships between different strains. It can be used in an outbreak situation or in surveillance systems. The problem with the latter is that

PFGE typing may be too sensitive for extensive surveillance as it readily detects genomic rearrangements and requires the aforementioned detailed phylogenetic analysis to track subclonal evolution. Also, the more divergent the genomic DNA patterns between strains, the less accurate the relatedness appears on interlaboratory evaluation.

However, in the case of an outbreak of a particular disease, PFGE has been proved a useful tool. It must be accompanied by an epidemiological study, as random sampling based on no prior knowledge is not only expensive, but may lead to incorrect conclusions and wrongful 'control' procedures.

1.2.3.3. Genetically Related

Genetically related isolates are termed clones. Clones are described as "isolates that are indistinguishable from each other by a variety of genetic tests (e.g., PFGE and ribotyping) or that are so similar that they are presumed to be derived from a common parent (237). Given the potential for cryptic genetic changes detectable only by DNA sequencing or other specific analyses, evidence for clonality is best considered relative rather than absolute.

Interpretative criteria for determining relatedness between isolates have been proposed for outbreaks of pathogens (237). However it is more difficult to apply these criteria over the time period of longer-term studies. Studies that collect samples over a period of more than one year require careful interpretation of results.

One of the future aims of Surveillance Networks is to establish interpretative criteria by ongoing collection of data from varied geographies in the United States. This will allow a more robust interpretation of numerical estimates of relatedness based on indistinguishable patterns and a small number of differing bands. It is recognised that there are differences in genome stability between pathogenic species. For example, *Escherichia coli* O157:H7, is considered a highly clonal organism and has a stable genome and therefore single band differences may signal unrelatedness (81). This is in contrast to *C. jejuni*, which is now regarded as genetically diverse with a high frequency of DNA recombination events within and between organisms (132). Therefore one to three PFGE band differences may be interpreted as signaling a degree of relatedness. Caution must be taken against over interpretation of results and epidemiological information must be used to confirm linkages (81).

1.3. Campylobacter in Humans

1.3.1. Clinical Manifestations

In 1979, 514 patients with diarrhoea were studied to review the clinical and epidemiological features of campylobacteriosis (25); little has changed since that initial description. The prodromal phase of campylobacteriosis (fever, headache, myalgia, and malaise) can present about 12 to 48 hours before the onset of diarrhoea (213). A mean incubation period from point-source outbreaks has been estimated at 3.2 days (range 18 hours to 8 days) (214), longer than most intestinal bacterial infections. Onset is often abrupt, with cramping pains quickly followed by diarrhoea. This abdominal cramping is apparent with *Campylobacter* infections, but not those of other enteritic infections. Rigors have been recorded, and fever may be sufficiently high to cause convulsions in children. The prodromal symptoms may obscure a correct diagnosis, particularly in patients for whom the abdominal symptoms have not appeared; these patients often have more severe illness than those whose illness begins with diarrhoea. When it does appear, diarrhoea is commonly profuse, watery, bile-stained, and sometimes prostrating at the beginning of the illness. After one or two days, blood may appear in stools, suggesting infection of the colon and rectum. The infective dose is considered to be very low; infection has been induced in humans with as little as 500 colony forming units (CFU) (24). Patients secrete *Campylobacter* in their faeces for several weeks post-recovery, unless antibiotics have been used for treatment (115), however, there is no evidence of transmission having occurred from a recovered patient.

In more than 70% of patients, there is a four-fold increase in specific IgG titres and blood and polymorphonuclear leukocytes are seen in stool samples (25). Infection in developing countries tends to result in watery diarrhoea, whereas the predominant symptom of cases from developed countries is acute inflammatory enteritis.

Infection begins at the jejunum and upper ileum, however it soon spreads to the rest of the ileum and the colon. Crypt abscess formation and acute inflammatory changes in the mucosa occur, with lesions indistinguishable from those found in *Salmonella* and *Shigella* infections. Mesenteric adenitis is a common symptom, and bacteria have been isolated from inflamed lymph nodes (213). Recovery from enterocolitis, when the infection spreads to the colon, is very slow, and can result in a mistaken presumptive diagnosis of Crohn's disease or chronic ulcerative colitis (238).

1.3.2. Host Defences

Both *Campylobacter jejuni* and *C. coli* are susceptible to the bactericidal action of normal human serum, although there is a *C. jejuni* strain that is resistant (42, 217). Specific IgG, IgM, and IgA antibodies appear in serum after about five days of illness, peak within two to four weeks and then decline over several months. IgA antibodies appear locally in intestinal secretions in response to infection. Those specific for flagellar and surface proteins confer protection from reinfection with homologous strains and possibly a range of other strains (24).

In developing countries, where repeated infection is common in early childhood, infection rates decline with age and fewer infections are associated with diarrhoea. At the same time, there is a rise in IgA antibody levels in serum (27). There also appears to be cross immunity between different strains in these endemic areas (A. Daniel, personal communication). Campylobacteriosis is almost absent in older children and adults in developing countries, but whether this is a result of continued exposure is not clear.

1.3.3. Mortality

The mortality rate in the USA resulting from gastroenteritis caused by subsequent complications of *Campylobacter jejuni* is unknown because the infection is not reported as a cause of death on death certificates (262). Nineteen cases of *Campylobacter* abortion have been described: nine due to *C. foetus* subsp *foetus*, 9 to *C. jejuni* and one to *C. coli* (212). Mortality tends to be low, seen more in elderly or immunocompromised patients or those suffering from another serious disease.

The most recent estimates of mortality were obtained by calculating death rates from the US FoodNet Surveillance system (150), but this assumes that the deaths attributable to the foodborne infections were limited to the acute phase of infection; the confounding effect of co-morbidity was overlooked. A cohort study of data matched from the Danish civil registration system, the national registry of enteric pathogens, the national registry of patients and the cancer registry provided a more robust calculation of morbidity for enteric diseases. Of these diseases, the acute relative mortality for campylobacteriosis, once adjusted for co-morbidity, was five (i.e. the mortality rate for patients with a *Campylobacter* infection was five times higher than for the background population). The authors of that study also noted significant excess long term mortality up to one year after infection with *Campylobacter* (88).

1.3.4. Morbidity and Cost

In New Zealand, it is estimated that there are 75,345 cases of campylobacteriosis per year, including 9000 GP visits, 250 hospital admissions, 18 cases of long-term illness and two deaths. These infections cost the country approximately 378,000 days of productivity (85). Using these figures, and taking the cost of foodborne infectious disease (per case) as \$462, campylobacteriosis costs New Zealand almost \$35million each year, with long term sequelae accounting for most of that cost (202).

1.3.5. Extraintestinal Infection

1.3.5.1. Bacteraemia

Bacteraemia is an occasional feature of campylobacteriosis (215) and septicaemia is prominent in immunocompromised individuals or those at extreme ranges of age (217). It is likely that bacteraemia occurs more often, especially in those individuals who develop fever or rigors, however it goes undetected due to the following reasons:

- h) blood cultures are rarely performed so early in the illness;
- i) *C. jejuni* is resistant to the bactericidal properties of normal serum;
- j) Not all methods to detect bacteraemia are sensitive for *Campylobacter* spp. (253).

Most cases of focal infection with *C. jejuni* arise as a result of bacteraemia combined with immunodeficiency or a predisposing condition (214).

1.3.5.2. Abortion and Perinatal Infection

Some species of *Campylobacter* have a predilection for uterine tissues of ruminant animals, but that is not demonstrated in humans, although abortions have occurred as a result of *Campylobacter* infection. In 1986, a review of the 19 abortions caused by *Campylobacter* spp. described in the then current literature were reviewed (212). Nine of those were probably caused by *Campylobacter jejuni*. Since then, more occurrences have been described (48), though it does appear that pregnant women can develop the infection without untoward effect on the fetus (273).

1.3.6. Sequelae

1.3.6.1. Reactive Arthritis

Following enteritis caused by *Salmonella*, *Campylobacter* or other intestinal bacteria, reactive arthritis can develop, generally within 14 days of the onset of diarrhoea. The ankles, knees, wrists and small joints of the hands and feet are most commonly affected and the duration of arthritis can range from several weeks to several months, occasionally a year (91). The presence of tissue antigen HLA B27 predisposes patients to reactive arthritis, which occludes the calculation of reactive arthritis frequency in patients with campylobacteriosis (6).

1.3.6.2. Guillain-Barre syndrome

Guillain-Barre syndrome (GBS), first described in 1916, is an autoimmune-mediated disorder of the peripheral nervous system. It is estimated that 15% of patients recover completely, 3-8% die, and the remaining surviving patients suffer varying degrees of physiological, neurological or physical deficits (217). It is an acute inflammatory demyelinating polyneuritis marked by paralysis, pain, and wasting muscles, and has replaced poliomyelitis as the most frequent cause of acute neuromuscular paralysis in developed countries. Affected persons develop weakness of the limbs, respiratory muscles and areflexia. The disease is generally self-limiting, with the least muscular capability occurring within two to three weeks, followed by recovery over weeks to months.

A recent study in Sweden demonstrated that incidence rates of GBS in people that had a laboratory confirmed *Campylobacter jejuni* infection differed between age groups, with no cases in the under 20 years age group (n=8711), 14 cases per 100,000 in the 21-59 years age group, and 248 cases per 100,000 in the over 59 years age group. The risk of developing GBS in the two months following *C. jejuni* infection was approximately 100 times higher than the risk for the general population (145). While the incidence of *C. jejuni* gastroenteritis is high, GBS is rare, suggesting that only a few serotypes of *C. jejuni* may be responsible for GBS; although nothing is known about the GBS triggering mechanisms of *C. jejuni* (217), and discussion about whether the severity of *C. jejuni* infection is associated with risk of GBS continues.

1.3.7. Treatment

Treatment of a human with campylobacteriosis usually includes the replacement of fluid and electrolytes lost through diarrhoea. Antimicrobial therapy is unlikely to be of much use, because the patient is likely to be in recovery when they present with symptoms to a general practitioner (GP) and bacteriological results are obtained. The effectiveness of ciprofloxacin and other fluoroquinolones has been severely compromised following the emergence of fluoroquinolone-resistant strains in the early 1990's (187). Most quinolone-resistant *Campylobacter* are still susceptible to erythromycin, though dual resistance has been recently found emerging in communities of men who have sex with men (72).

1.3.8. Pathogenesis and Virulence Factors

Despite the importance of *Campylobacter* as an enteric pathogen, there is a paucity of knowledge about the mechanisms by which the bacteria cause disease. The recent completion of the genome sequencing of *Campylobacter jejuni* NCTC 11168 may pinpoint genetic determinants of virulence.

No remarkable difference has been seen in the expression of pathogenic genes responsible for the expression of adherence, invasion, colonization and cytotoxin, found in *C. jejuni* isolated from a variety of sources (45).

1.3.8.1. Motility

Motility is conferred by the polar flagellaum, and the 'corkscrew' motion allows *Campylobacter* to penetrate through the mucus layer in the intestine (159). The flagellaum comprise of two *fla* protein subunits A and B, described previously (see above). Mutants that do not express subunit A but do express subunit B produce truncated, stubby flagella, and are completely non-motile. Mutants that cannot express the B subunit show slightly decreased motility, but are still capable of movement (255). When the gene *pflA* was mutated, it resulted in a non-motile bacterium, with a paralyzed flagella (271). Aflagellate mutants have been used to demonstrate the importance of flagella to *Campylobacter jejuni* colonization and pathogenesis (157, 255).

1.3.8.2. Chemotaxis

The ability to detect, and then move up and down chemical gradients has been shown as vital to *Campylobacter* colonization. Non-chemotactic mutants were incapable of colonising the intestines, using a mouse model (236). *Campylobacter* can metabolize mucin, thus is well

adapted to survive in the mucus film of the caecal and cloacal crypts (204). The ‘corkscrew’ motility of the organism allows it to move through the thick layers of mucus towards these environments (217), attracted by two chemotactants: the glycoprotein component of mucus, and L-fucose (a terminal sugar of mucin). A motile, non-invasive strain was discovered when the *cheY* gene was disrupted (272); it is hypothesised that this gene affects the rate of flagellar motor switching. Other genes possibly related to chemotaxis are ten chemoreceptor genes containing methyl-accepting chemotaxis protein domains.

1.3.8.3. Adhesion

Campylobacter jejuni crosses through the mucus layer of the gut, and adheres to the epithelial cells that lie beneath. Some bacterial cells then invade the host cells, causing mucosal damage and inflammation; it is unknown whether this is the cause of diarrhoea. Experiments suggest invasiveness is stronger in ‘fresh’ clinical isolates (passages decrease invasiveness), and that some strains are more invasive than others (267). Flagellar importance in adherence and invasion has been demonstrated, as reduced-motility mutants show reduced adherence and no invasion at all (271). Adhesins identified include the PEB1 and cadF proteins. PEB1 mutants showed decreased adherence and invasion of HeLa cells, and their ability to colonize mice was compromised (177). CadF mutants cannot bind fibronectin, a cell surface glycoprotein, and were thus unable to colonize newly hatched chicks (275).

Recent studies have indicated the presence of a microtubule-dependent (actin-filament independent) gut-invasion mechanism, through which at least some *C. jejuni* strains may cause disease (128).

1.3.8.4. Invasion

The study of inflammation markers has resulted in the proposal that it is the invasion of the epithelial cells that triggers inflammation (64). Host cell invasion has been studied *in vitro* using several different cell lines; invasion is dependent on newly synthesised *Campylobacter* proteins and host cell signal transduction. The *claB* protein mutant strain adhered to INT407 cells but was not capable of invading these cells, and the expression of seven other proteins was also affected (127). Phosphorylation is one of the host cell processes that has been discovered to be vital to invasion by the bacteria. The same study indicated that calveolae, the plasma membrane invaginations present in a variety of mammalian cells, are also important, as they are implicated in endocytosis (268).

Mutations in specific virulence factor genes *cadF*, *dnaJ*, *pldA* and *ciaB* impair the ability of *Campylobacter jejuni* to colonize the caecum of chicks, such that the birds can tolerate massive inocula (274), suggesting that these genes influence the virulence of the strain.

1.3.8.5. Toxins

While invasion may give rise to inflammation alone, it does appear that the level of invasion may be insufficient to be the sole cause, prompting searches for the toxic activity of *Campylobacter jejuni*. Although cytotoxins and enterotoxins have been reported, genes encoding toxins other than the cytolethal distending toxin (CDT) have yet to be isolated (254). It is likely that strain and assay differences are responsible for the differences of toxic activity that are described. Cytolethal distending toxin causes cells to become slowly distended and eventually leads to cell death. While most *C. jejuni* and *C. coli* strains carry the *cdt* gene, there are differences in the amount of CDT produced. It is not yet clear why *C. jejuni* produces high titres of the toxin, while *C. coli* produces less (184). A recent study indicated that 100 of 101 isolates of *C. jejuni* contained the *cdt* gene and also showed cytotoxic effects in a Vero cell line (16). However, since some *cdt*-negative strains continue to exhibit cytotoxic effects, it is likely there are alternate toxigenic genes yet to be described.

1.3.9. Epidemiology

The majority of cases of campylobacteriosis are sporadic cases (36, 213). There are several transmission routes for *Campylobacter* spp to infect humans. In New Zealand, *Campylobacter jejuni* was commonly found in faeces from dairy cows, beef cattle, sheep and ducks, chicken carcasses, sheep offal and surface waters. Preliminary analysis of Penner types was suggestive of transmission to humans from dairy and beef cattle and possibly from sheep (87). Having said that, in 2003, there were 14,786 cases of campylobacteriosis, 202 caused by *C. jejuni*, and 56.4% of which were either known or suspected to have been caused by foodborne transmission. This is in alignment with the common preconception that campylobacteriosis is primarily a foodborne disease.

The epidemiology of campylobacteriosis reveals two distinct patterns. One involves an outbreak in which a large number of people develop clinical symptoms; the other pattern is that of a sporadic or single isolated case (262).

1.3.9.1. Foodborne Enteritis Surveillance

Surveillance of foodborne illness is severely complicated by under-reporting. Many cases are not reported because the ill person does not seek medical care, the health-care provider does not obtain a specimen for diagnosis, the laboratory does not perform the necessary diagnostic test, or the illness or laboratory findings are not communicated to public health officials (150). In 1999 the degree of under-reporting (i.e. the factor between the number of reported cases and the number of cases that actually occur in the community) was calculated at 7.6 for campylobacteriosis in England (260). As we cannot clearly define the proportion of cases of gastroenteritis that are foodborne in origin, surveillance reports must be considered to be those of 'gastroenteritis' cases, rather than foodborne gastroenteritis. The underreporting in New Zealand has not yet been estimated, but is thought to be similar to that experienced by UK, despite the different notification systems, because we only report on laboratory confirmed cases.

Rapid detection of *Campylobacter* spp. is vital in food hygiene to aid the prevention of foodborne diseases. Procedures for the isolation and identification of *Campylobacter* spp. are labour intensive and time consuming, and several steps are necessary: pre-enrichment, enrichment, plating on selective agars, and biochemical identification (150). Many food and clinical laboratories are ill-equipped to isolate and identify *Campylobacter jejuni* due to technical challenges and operational costs (262). *Campylobacter jejuni* exists in low concentrations in foods, so isolation must be achieved in the face of normal resident flora. This, and the time factor, makes full epidemiological studies on *C. jejuni* complex.

For foodborne disease cases linked to poultry, *Salmonella* and *Campylobacter* create the highest risks in comparison to other agents (262).

1.3.9.2. Incidence

There has been a steady annual increase in the number of reported cases of campylobacteriosis in New Zealand since the disease was made notifiable in 1980. There were 14,731 cases of campylobacteriosis in New Zealand in 2003, i.e. 394 cases per 100,000. Campylobacteriosis is the most common cause of gastroenteritis in New Zealand, causing over six times the number of cases as salmonellosis. In 2002 there were 515 hospitalisations due to campylobacteriosis, from the 7735 cases for whom this data was recorded (6.7%) (219).

Using the degree of under-reporting described above, the real incidence of campylobacteriosis in New Zealand may be as high as 2995 cases per 100,000 people per

year compared to the current reported rate of 394 cases per 100,000. The reported rate of campylobacteriosis in New Zealand far exceeds the rates observed by other industrialised countries, as summarised in Figure 1. The cause of the much higher incidence rate in New Zealand is unknown.

It is not clear whether the high incidence rate seen in New Zealand is a reporting artefact of our notification system, or the fact that we report across the entire nation, rather than use sentinel sites as the US FoodNet surveillance system does. For instance, California, often considered to have similar geographical features and climate as New Zealand, has an incidence of 18 cases per 100,000, which is higher than the US national incidence reported: 13.3 cases/100,000. Only selected counties are included in the FoodNet surveillance conducted in California, so it is highly likely that the California (state) incidence is an underestimate of the real incidence (92).

In New Zealand, an outbreak is defined (generally) as two or more cases linked to a common source, a community wide or person-to-person outbreak or any other situation where outbreak investigation or control measures are used or considered. However, in several instances where one person has transmitted Campylobacteriosis to another member of the family (perhaps a mother preparing food for a child), it is not always considered an outbreak (the definitions are interpreted differently by different Public Health Units). Other definitions of an outbreak include statements such as: "The incidence of a disease in a population above that which would normally be expected at any given time or location." The actual number of cases could vary from one (e.g. measles) to over a hundred (e.g. influenza) depending on the specific condition." so the current outbreak definition for New Zealand (i.e. $n \geq 2$ cases) need further clarification depending on the circumstances.

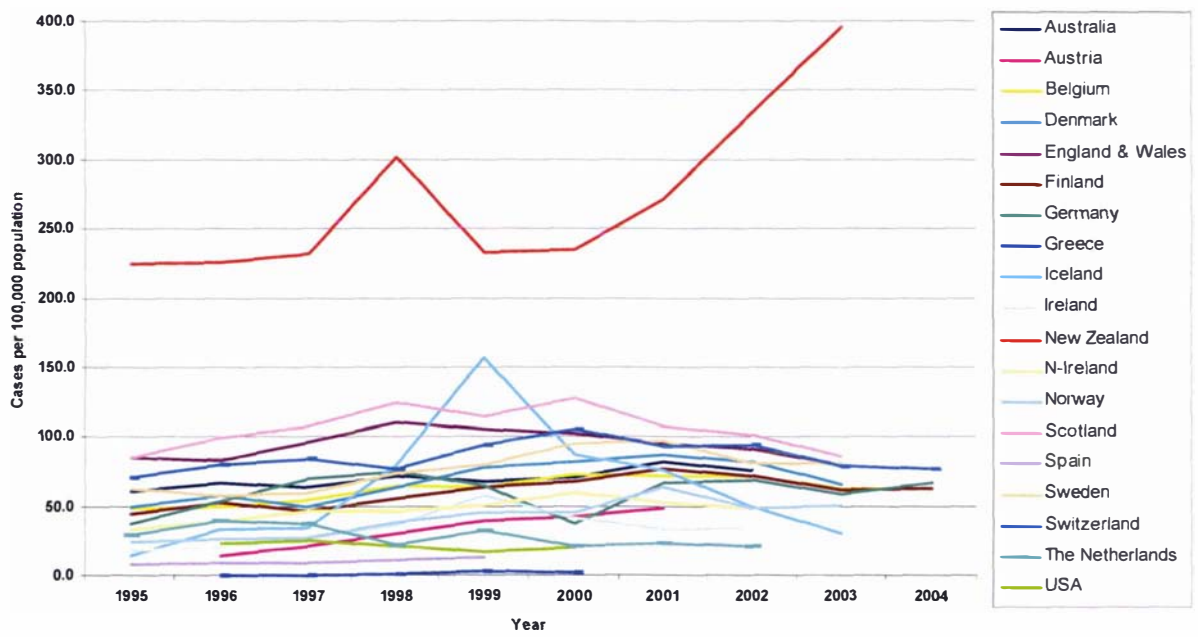


Figure 1. Annual incidence rates of campylobacteriosis in industrialised countries.

Data predominantly obtained from www.gideononline.net and <http://earthtrends.wri.org/text/population-health/country-profile.htm>

1.3.9.3. Characteristics

There are distinct age- and sex-specific patterns exhibited by cases in New Zealand. In 2002, the incidence in the 0-4 years age group was 599.1 per 100,000 children, significantly higher than the national average rate. A peak was also seen in the 20-29 year age group. These peaks correspond to times of weaning and when persons set up housekeeping on their own and prepare foods (36, 204). In all age groups, more males than females were affected, a similar to patterns seen in other industrialised countries (219).

Notification rates continue to suggest seasonality, with most cases reported in the summer months of December, January and February. This matches data from other industrialised countries, where there are peak incidences during summer months (163). Seasonality, however, is not so evident in tropical and sub-tropical countries, but as these countries tend to be less developed, there may be confounding factors involving the different epidemiology of campylobacteriosis in more endemic situations (see below).

The disease affects people of all ages, and the prevalence of infection is insufficient to induce herd immunity in developed countries. In developing countries, infection is so frequent that babies become immune within the first year or so of life, so the disease rarely affects older children and adults. Under these hyperendemic conditions, partial immunity to campylobacteriosis is acquired from breastfeeding such that many childhood infections are symptomless (213).

Patients with AIDS appear to be more susceptible to campylobacteriosis. A survey conducted in Los Angeles County, USA, indicated that the average incidence of campylobacteriosis in AIDS patients was 519 per 100,000, compared to the general population rate of 13.3 per 100,000. A similar difference is probable for patients with other immunocompromising conditions (217). Similar data is unavailable in New Zealand, where the incidence of AIDS is 0.45 cases per 100,000 per year (219).

1.3.9.4. Source of Infection

The major sources of campylobacteriosis are assumed to be the consumption of poultry, raw milk, untreated water and contact with pets (161). Undercooked poultry is assumed to be the single most important cause of sporadic cases, but other foods are also very likely sources, as *Campylobacter* spp. can be easily isolated from many different types of foods: lamb (226); veal (76); minced beef, sausage, sheep, cattle and pork offal (29); oysters (3); pork and beef (130); and also from recreational water (225).

There have been several studies conducted worldwide which attempted to identify risk factors for campylobacteriosis. One such study identified three risk factors for campylobacteriosis: eating fully cooked broiler chicken (OR=4.7), eating broiler chicken reported to be raw or undercooked (OR=9.0), contact with a cat or kitten (OR=9.0). There was no duplication between the serotypes of *C. jejuni* in the humans and those found in the cats, and the cats were therefore not considered a source of *C. jejuni* for humans (47). A case-control study in south-eastern Norway found poultry consumption to be associated with campylobacteriosis (116). It was further indicated that perhaps poultry and cattle could be major sources of human campylobacteriosis cases (161). In King County, Washington State, USA, a case-control study showed that consumption of broiler chicken and Cornish game hen was responsible for more than doubling the risk of *C. jejuni* enteritis (84). Another case-control study in Colorado, USA, in 1982 indicated a significant correlation between handling or preparation of raw chickens and the occurrence of confirmed *C. jejuni* enterocolitis (97). In 1997, the Centers for Disease Control and Prevention (CDC) in the United States stated that a primary source of human campylobacteriosis is poultry products (79).

Although cows may be colonised with higher numbers of *Campylobacter*, a bovine carcass is treated in a vastly different manner to that of a chicken carcass. First of all, the skin of a bovine carcass is removed, and then the gut system is removed and kept separate from the meat of the animal. In contrast, multiple chickens are plunged into the same bath, with their gut still in situ. The heated bathwater allows the feather follicles to dilate, allowing for easier defeathering of the bird. However, it is also the perfect opportunity for *Campylobacter* to escape the cloaca and colonise the meat and skin of other chicken carcasses. In fact, plugging the cloaca of chickens prior to their entry into the scalding tank has been shown to be effective in reducing the number of colonised chicken carcasses leaving the defeathering process within a commercial plant (23).

To counter these findings, a multicentre investigation of laboratory-confirmed cases of campylobacteriosis, in 1995, showed that both consumption of chicken at home and handling in the domestic kitchen of the whole chicken bought raw, with giblets, were significantly associated with reduced risk of becoming ill with campylobacteriosis (5). Similarly, a national case-control study conducted in the United Kingdom showed that neither domestic handling nor eating of chicken were risk factors for acquiring campylobacteriosis. However, this may have been due to the complications associated with tracing poultry sources when very large suppliers are involved (175).

Campylobacter serotypes from chickens, cattle and swine have been compared with the most common serotypes isolated from humans in Canada (156), and a similar study has been carried out in Holland (161). In England, serotypes from food and environmental samples isolates were compared with isolates from patients (71). In New Zealand, 50% of human isolates had DNA restriction digest patterns which were indistinguishable from poultry isolates (114). The coincidental appearance of fluoroquinolone resistance in human and poultry isolates also suggests a connection between the two groups (36, 57).

It has been demonstrated in the Netherlands, that elimination of *Campylobacter* spp. during sewage treatment is occasionally incomplete; the organism could be isolated from both sedimented sludge and surface water at the discharge points of selected sewage plants (125). It is possible that the use of untreated water from sewage plants as drinking water for animals or in irrigation could be a source of contamination (217).

Most risk factors (other than poultry consumption) for campylobacteriosis are minimized through simple behavioural changes, such as avoidance of unpasteurised dairy products and untreated drinking water. Careful hand washing after handling animal carcasses, live cattle or calves, and puppies is also an important preventative measure (56).

1.3.9.5. Outbreaks

In New Zealand in 2002, campylobacteriosis caused 51 outbreaks (of a total of 337 recorded disease outbreaks), though not all (33) of the resulting outbreak investigations identified a source of infection (32). Six of the outbreaks in 2002 were discovered to be caused by *Campylobacter jejuni* (many are not speciated so this does not reflect a total proportion). Thirty-seven of these outbreaks had a suspected source or vehicle of infection, and poultry meat was implicated in 28 of these (75.7%). Thirty-four outbreaks were foodborne, one of which was likely to have occurred as a result of an infected food handler preparing food that was consumed by 11 people. In total, there were 239 cases of campylobacteriosis involved in outbreaks during 2002, an average of 4.7 people involved in each outbreak. Forty-one people were hospitalised, but no one died.

Indeed the time taken to isolate and identify *Campylobacter* spp. from food, water or environmental samples occludes the ability to determine the source of infection in an outbreak situation. There are some good examples of outbreak investigations resulting in the identification of a source in the literature as described.

A 1995-6 outbreak of campylobacteriosis in Denmark was spread by contaminated water; contamination of the water supply was traced back to contamination of ground water due to a break in a sewage pipe (59). Another water-borne campylobacteriosis outbreak occurred in Fife, Scotland, in 1995, when the public water supply was contaminated by stream water into which treated sewage had been discharged (110). Despite the fact that contaminated water has caused outbreaks, raw seafood has not been implicated as a cause (3).

An outbreak among Austrian youth centre children in 2000 occurred due to unpasteurised milk from a nearby farm (134), and other children in a day nursery in the UK were infected with *Campylobacter jejuni* having consumed milk from bottles where magpies had pecked the lid (190). More recently in 2002 in Wisconsin, USA, cow leasing to circumvent regulations prohibiting the sale of unpasteurised milk caused the infection of five people with *C. jejuni* (2). Thirteen of 20 people who attended a meal in Utah at which raw milk was served, developed campylobacteriosis (183).

An outbreak investigation in southern England resulted in a single outbreak serotype of *C. jejuni* being isolated from chickens that had been supplied to a training college, trainee chefs, and patrons who ate at the attached restaurant being affected. Eventually the investigation successfully tracked the outbreak source to the wholesalers of origin, and traced back to the original farm of origin (175).

Another poultry related outbreak occurred in Germany, affecting five people at a barbeque. Testing of isolates gathered from a poultry flock in Austria and the slaughterhouse used to process that flock suggested that the outbreak strain of *C. jejuni* had colonized the slaughterhouse, and was cross-contaminating poultry carcasses there (9).

An outbreak amongst summer camp dwellers in Wisconsin, USA, in 1997 indicated that cross contamination had occurred in the kitchens and the tuna-salad was the vehicle by which 79 people were infected with *C. jejuni* (191). This was the second reporting of such an unusual vehicle causing an outbreak of foodborne disease, the first: an outbreak of Hepatitis A in seven of 11 persons attending a picnic (101). Since then, tuna salad sandwiches have been implicated in an outbreak of rotavirus gastroenteritis among college students in 2000, though it should be noted that chicken salad sandwiches were equally likely to have been the vehicle of transmission (1).

1.4. Sources of *Campylobacter* in humans

1.4.1. Production Animals

Case control studies have revealed a significant association between human campylobacteriosis and prior handling or consumption of raw or undercooked poultry products. The extent to which this poultry factor impacts upon human illness is undetermined. Due to the perceived implication that contaminated poultry meats are the primary food associated with human illness, a great deal of research into *Campylobacter jejuni* has been poultry-orientated. However, *C. jejuni* has been isolated from several other food types, including raw milk, beef, lamb and seafood, so these sources must also be considered. The slaughtering process of each of the red meat producing animals could lead to cross contamination of muscle tissue with intestinal contents. To this effect, high percentages of carcasses (sheep, cattle, pigs) have been colonized with *Campylobacter* spp. post-slaughter (230) but it is also thought that the forced air-chilling of the carcasses overnight kills the majority of organisms on the surface, as there are seldom recoveries from retail meats (142). Cooked meat products are rarely contaminated with *Campylobacter* spp., and the sporadic cases resulting from consumption of cooked meats are considered to be caused by cross-contamination from raw meat products.

1.4.1.1. Poultry

The thermophilic *Campylobacter* spp. are considered commensal organisms for poultry, residing in the ideal environment of the intestines at 41°C without causing clinical illness. Poultry have large numbers of *Campylobacter* spp. on their skin, feathers and in their intestines when they enter the slaughterhouse. Measurements of 10⁵ CFU/g of caecal content have been documented from birds slaughtered and sampled at the farm, while those birds that had been transported to the processing plant contained 10⁶ CFU/g of caecal content. Levels of *Campylobacter* spp. on birds at the farm were 10³ CFU/carcass, but this increased to 10⁷ CFU per carcass after transport to the processing plant (229).

The final slaughterhouse stages of washing and chilling the carcasses with chlorinated water reduce the contamination, but do not eliminate it. Air chilling has been shown to rid the carcass of more organisms than immersion chilling (197).

1.4.1.2. Cattle

Campylobacter jejuni can cause mastitis in cows (131). *Campylobacter* abortions in cattle are mostly caused by *C. fetus* subsp. *venerealis*, and in cattle herds examined in one study, *C. jejuni* serogroups were demonstrated in the intestine of cattle, though only a single case of abortion was due to *C. jejuni* (218).

Several species of *Campylobacter* are capable of causing mild or moderate bovine enteritis, with experimental infection. *C. jejuni* and *C. fetus* subsp. *fetus* caused a febrile enteritis in ruminating and milk-fed calves, and intestinal lesions similar to those found in man were also observed (8).

1.4.1.2.1. Dairy Cattle

Unpasteurised milk is a well documented cause of outbreaks of campylobacteriosis (2, 34, 96, 134, 183, 190, 211), the largest to date occurring in 2500 school children in the United Kingdom in 1980 (111). Although *Campylobacter* can be regularly isolated from cow faeces, they are seldom present in bulk milk tank samples (52) and if they are present, there are low numbers of the organisms. The mean level of contamination has been estimated using the Most Probable Number (MPN) method at 16 ± 30 organisms per 100ml milk (98). The pasteurisation procedure is sufficient to eliminate this risk. Pasteurised milk has only caused problems where birds have pecked at foil tops of milk bottles (190, 220).

Bovine *Campylobacter* mastitis was suspected and experimentally induced, before naturally occurring infection was detected. Only the inoculated quarter of the udder became infected. Reports of the natural disease are few, but it is probably under-diagnosed. *Campylobacter* mastitis is often subclinical, particularly during the early stages when campylobacters are excreted in large numbers; once the milk has become granular and loaded with cells *Campylobacter* counts have fallen. Infection is probably uncommon, otherwise more outbreaks of campylobacteriosis would be observed among the population of people that drink unpasteurised milk every day (213).

Raw milk is generally thought to be contaminated from faecal matter at the point of collection, though direct contamination as a result of udder infection by *Campylobacter* spp. has been described (169). Shedding occurs predominantly in young animals, and becomes seasonal in adult cows (222). *Campylobacter* spp. cannot survive the ripening and production stages of cheese making as the cells are susceptible to desiccation (11). Yoghurt is not a

suspected vehicle for campylobacteriosis because of both the low pH level, and the presence of lactic acid, to which the organism is sensitive (43).

1.4.1.2.2. Beef Cattle

Meat producing cattle, like dairy cattle, also excrete *Campylobacter jejuni* in their faeces. Higher excretion rates are reported in the summer months than in winter (28). Carriage rates have been reported as high as 89% of a 306-head herd of beef cattle during one year shedding an average of 610 organisms (MPN) per gram of fresh faecal material (223). This is higher than the number of organisms shed by poultry of 230 CFU/ gram of caecal content (22).

Though beef faecal material may contain more *Campylobacter* cells, it is less likely to come into contact with the meat of the beef animal during processing - due to the differences in processing as described previously. This means that the risk of being infected with *Campylobacter* spp. from the consumption of beef is far less than that of consuming chicken. Human exposure to the faecal material of beef cattle is likely to be far lower than exposure rate to chicken faeces, purely through the different processing practices.

1.4.1.3. Pigs

Campylobacter spp. were first described in pigs in 1944 and were believed to be the causative agent for swine dysentery. Today, *Brachyspira hyodysenteriae* is the principal agent of swine dysentery, although there is evidence that *Campylobacter coli* and anaerobic bowel flora might play a part in the pathogenesis of the disease. While *C. jejuni* can be recovered from the intestinal contents of pigs, *C. coli* is found more often (156), though it seems to be only a minor source of campylobacteriosis for humans in New Zealand (114). Although this is the case, there has been at least one spontaneous abortion in a human following infection by *C. coli* in New Zealand (65) and more elsewhere (212). Both bacteria have been recovered from pork livers collected after evisceration, though *C. coli* was recovered from more livers than *C. jejuni* (153). Studies have indicated that individual pigs could harbour up to eight types of *Campylobacter* during the fattening period (257).

For many years *C. mucosalis* and more recently *C. hyointestinalis* have been linked with porcine proliferative enteropathy (PPE). The association is strong, in that either bacterium may be found in large numbers in lesions, yet neither is commonly isolated from the intestines of healthy pigs. It is possible to transmit the disease with filtrates of diseased mucosa, but not by inoculation with pure cultures of the *Campylobacter* spp. (213).

1.4.1.4. Sheep

The disease classically associated with *Campylobacter* spp. infection in sheep is epizootic abortion, known for many years as vibronic abortion. It has also been known to cause abortion in a goatherd (86). Twenty two percent of sheep abortions studied were deemed caused by *C. jejuni* (249), although *C. fetus* subsp. *fetus* is the most common cause of abortion in sheep.

In a study examining the prevalence of thermotolerant *Campylobacter* spp. in lamb carcasses, 92% of the 360 small intestine samples were positive for thermotolerant *Campylobacter* spp., at an average concentration of \log_{10} 4 CFU/gram (MPN) of intestinal content (224).

The role of campylobacters as enteropathogens of sheep is ill defined. Mild scouring in flocks is commonly associated with outbreaks of *Campylobacter* abortion. There are occasional reports of severe outbreaks of scouring among weaning lambs, apparently due to *Campylobacter* spp. (213). Experimental infection of lambs with *C. jejuni* or *C. hyointestinalis* has produced at most, mild intestinal lesions and mild mucoid diarrhoea (240).

1.4.2. Companion Animals

Campylobacter jejuni was isolated from the small intestine of weaned 4-6 week old rabbits, that had shown watery diarrhoea without fever (249). Domestic ferrets are also susceptible to infection with *C. jejuni*. They develop disease closely resembling that seen in man and have been proposed as a suitable model for human infection (70).

Campylobacter spp. carriage rates are generally low in rats, mice, guinea-pigs and rabbits, but high rates have been found in hamsters. Oral challenge with *C. jejuni* or *C. coli* resulted in infection without illness, but reduced carbohydrate absorption was also seen in young mice (213).

Diarrhoea was caused by *Campylobacter jejuni* in 13% of scouring dogs presented to veterinarians in Hungary (249). Dogs have been seen to excrete *C. helveticus*, a catalase-negative species of *Campylobacter*. Experimental infection of healthy puppies with *C. jejuni* has resulted in colonization without illness or only mild enteric disease (141).

Campylobacter upsaliensis, *C. jejuni* and *C. coli* have been recovered from faecal samples obtained from domestic and stray dogs in Australia at a rate of 34%, 7% and 2% respectively (14). Carriage rates of *Campylobacter* spp. in healthy dogs and those with

diarrhoea presented to Norwegian veterinarians were not significantly different, approximately 25% (198).

Campylobacter spp. are common in cats, with carriage rates up to 18% of healthy cats and 16% of cats with diarrhoea reported from Norway (198). Coinfection with *Helicobacter* spp. and *Campylobacter* spp. was observed in 33% of the 64 commercially reared clinically healthy cats from which microaerobic bacteria were isolated (208). Different countries report different carriage rates however, with researchers in Denmark observing a 5% carriage rate in 42 healthy kittens (82).

1.4.3. Water and Seafood

1.4.3.1. Water

Campylobacter spp. contamination of surface water is likely to originate from faecal contamination by wild birds, domestic animals or sewage effluent (59, 239, 251). It is recognised as a source of campylobacteriosis outbreaks and is suspected to play a role in contamination of farm animals (110, 126).

Campylobacter spp. have also been isolated from streams, seawater and other recreational waters (129, 225, 239). While not intended for human consumption, accidental ingestion can account for exposure to the organism, and may cause some sporadic cases in the community.

Drinking water supplies are generally chlorinated, thus are regarded as being free of *Campylobacter* spp., provided safety procedures are maintained to prevent contamination with untreated water (26). An increased risk of infection has been associated with drinking water from non-urban supplies (100).

1.4.3.2. Shellfish

Shellfish have been contaminated with *Campylobacter* spp.; there are reports of Pacific Oysters, raw clams and raw mussels causing human illness (3, 58). Studies conducted to determine the predominant type found in seafood implicate gulls as a reservoir, as *C. lari* is the most commonly found species.

1.5. *Campylobacter* and Broiler Chickens

An association between poultry and sporadic cases of human campylobacteriosis has been demonstrated in several studies (47, 84, 97). The consumption of fresh (as opposed to frozen) chicken has been associated epidemiologically with outbreaks of gastroenteritis due to *Campylobacter jejuni*, both in the United Kingdom and the United States (84, 90). They are also common inhabitants of the intestinal tracts of other food animals (see above).

C. jejuni, *C. coli* and *C. lari* are well adapted to avian species, a feature reflected by the frequency of carriage in wild birds, the facility with which they colonize poultry, and their optimum growth temperature of around 42°C. However, the pattern of *C. coli* infection in broilers is generally more transient than that seen with *C. jejuni* and infection is usually associated with raising swine on the same farm property (79).

Infection is readily established from small inocula (193). Live chickens are frequently colonized with *C. jejuni* and the intensive nature of poultry production can facilitate cross-contamination with the result that a high proportion of chicken carcasses may be contaminated with *Campylobacter* cells (99).

Broilers do not show signs of disease at the time of initial colonization so it is presumed that *C. jejuni* enters a non-essential commensal relationship in the intestinal tract of the bird (62). *C. jejuni* tends to be apathogenic in commercial chickens, turkeys and ducks (204). When broiler chickens are first colonized with *C. jejuni*, mild diarrhoea is probably the only manifestation of colonization, but it is more likely there would be no sign at all (238). However, three-week-old Japanese quails (*Coturnix Coturnix japonica*) displayed diarrhoea for two weeks after oral inoculation with *C. jejuni* from a child with diarrhoea (144) demonstrating the possibility of clinical symptoms among the family *Phasianidae*.

Colonization with *C. jejuni* typically occurs between days 14 and 49 of the seven week growout period and if it occurs, the proportion of colonized birds is presumed to be high at the time of slaughter (234). Infected chicks can excrete *C. jejuni* for up to 63 days housed under conditions that inhibit coprophagy (204). Shedding of *C. jejuni* persists for long periods in flocks housed on litter, at least until 12 weeks of age in broiler strains or up to 42 weeks in breeders (113, 204). Coprophagy may partly explain the rapid transmission, and it also has been shown that the virulence of an invasive *C. jejuni* isolate can be enhanced by passage through chicks (199). Within days of exposure to *C. jejuni*, the pathogen spreads

rapidly to virtually all the chickens in the flock (99, 206), however it is possible to raise entire flocks free from *Campylobacter* spp. (136).

1.5.1. Pathology of *Campylobacter jejuni* in the Chicken

Campylobacter spp., for the most part, appear not to have a clinical effect on poultry. However, throughout the United States and Europe during the 1950's, an epidemic of 'vibrionic hepatitis' was later attributed to *Campylobacter jejuni* (17). Mortality of 10-15% was seen in birds greater than eight weeks of age, and the disease affected egg-layers more than broilers (probably because broiler chickens do not live as long as eight weeks). It is not clear why this disease has decreased in importance in the following decades, though the introduction of cages to layer flocks may have decreased the mortality due to vibrionic hepatitis. The disease still affects ostriches and has been attributed to infection by *C. jejuni* serotype 8 (181).

In 1981, it was demonstrated that 32% of three-day-old chicks died when orally challenged with 10^7 CFU of *C. jejuni*. When challenged with only 90 bacteria; 90% of the birds developed watery diarrhoea (193). This finding was repeated in 1984, and *C. jejuni* was also found in the spleens, livers and hearts within six days of oral challenge by up to 10^6 CFU (200). Another study in 1984 demonstrated that infection in chicks 12 hours after hatching resulted in onset of diarrhoea, but infection at 3 days of age with 10^9 CFU failed to produce any detectable clinical change (258).

Pathogenic isolates derived from human patients with enterocolitis induced diarrhoea of short duration in newly hatched chicks (200). Host factors relating to *C. jejuni* colonization influenced the susceptibility of commercial strains of chickens. A significant difference in the number of 1-day-old chicks colonized was noted among three broiler strains (200, 232). Colonization appeared to be dependent on the strain of the bacteria and the size of inoculum but independent of the age of the chicks (62).

There are two criteria that correspond to *C. jejuni* disease: one is a distended intestinal tract, often with foamy intestinal contents that suggests exposure to a toxigenic *C. jejuni*; the second is a red or mottled yellow liver surface which indicates the presence of an invasive *C. jejuni* (39). However, not all chicks with the above symptoms yield *C. jejuni* from the intestinal tract. Hepatic haemorrhagic lesions are associated with *Campylobacter* infections in poultry and some birds exhibit swollen intestines but normal livers (148).

Gross lesions of *Campylobacter* infection in neonatal chicks comprise distension of the jejunum with accumulation of mucus and fluid or disseminated areas of haemorrhage (204). Infection of chicks at the time of hatch resulted in focal hepatic necrosis (39). Microscopic lesions attributed to *C. jejuni* infection include oedema of the mucosa of the ileum and caecum, with *C. jejuni* present in the brush border of enterocytes (204). In more severe cases mononuclear infiltration of the submucosa and villous atrophy, resulted in intraluminal accumulation of mucus, erythrocytes and mononuclear and polymorphonuclear cells (258).

Campylobacter jejuni can be present in the intestinal tract of chickens in populations at a range of 10^5 to 10^9 CFU/gram. It colonizes primarily the lower gastrointestinal tract; principally the caeca, large intestine, and cloaca where densely packed cells localise in mucus within crypts (19, 55). The organism is not in apposition with the outer membrane of the microvilli and *Campylobacter* cells subsist in the mucosal film. Despite the apparently commensal nature of *Campylobacter* spp. infection in chickens, there is a strong systemic specific immune response to *Campylobacter* spp. (101).

It has been suggested that strains of *C. jejuni* introduced into a poultry flock may not have equal opportunities for growth and spread. Certain types may easily infect and be frequently isolated, whereas others may become overgrown and not be isolated at all (244). Laboratory experiments with broilers challenged with two different *C. jejuni* serotypes showed a complete dominance of one type over the other within a week (103). Whether spread is independent of serotype is unknown but could be determined by feeding experiments using multiple isolates of a variety of serotypes (39).

The exact duration of colonization and shedding is unknown, but it is accepted that the bacteria are commonly present in the blood and liver and may be excreted for up to three months (19). In broiler chickens, this may mean that they will be shedding *Campylobacter* cells when they go to slaughter.

1.5.2. Epidemiology of *Campylobacter jejuni* on Broiler Farms

National surveillance studies looking for *Campylobacter* spp. in chickens are fraught with difficulty: fragmented poultry industries, geographic dispersal of farms, and the poor organisation of smaller, organic farms make random sampling and sample collection at the farm level challenging. The collection method (cloacal swabbing) requires catching and

handling the birds, a point that may occlude the ease of data collection. Several studies have tested retail chicken for the presence of *Campylobacter* spp. but as the rate of cross-contamination within a poultry processing plant is extremely high, these results are not representative of the farm situation.

1.5.2.1. Prevalence and Distribution

Several prevalence studies have been carried out overseas, and a range of flock prevalence's have been determined from sampling at the processing plant, from: 18% in Norway (117) to 24% in Finland (7), 27% in Sweden (20), 42.5% in Denmark (256) and 76% in England (99). In all of these studies, *Campylobacter jejuni* was the most prevalent species identified. The comparison of these studies is difficult, as different sample collection and isolation methods were used for each. If the differences seen are real, and not an artefact of the different sampling and/or identification methods, then parameters such as number of animals per farm, climatic conditions and distance between farms may influence the infection rate. The prevalence of poultry carcass contamination is related to the prevalence of flock infection, and both vary widely (77, 259).

Seasonal variation in flock prevalence has been described in some countries: Canada (242), Norway (117), The Netherlands (104), Denmark (15, 256) and has had opposing views in others (England (99, 252)). All studies identified a peak prevalence in summer except in Norway, where there is a peak in Autumn (117). This autumnal peak has also been observed in black-headed gulls (*Larus ridibundus*) in Sweden (35) and a higher proportion of samples collected in autumn from juvenile migrating passerines and shorebirds has also been observed (251).

1.5.2.2. Vertical Transmission

Despite maternal infection it is considered unlikely that vertical transmission occurs under commercial conditions. However speculation continues as to whether vertical transmission does indeed occur, and whether sampling regimes are insufficiently rigorous to detect colonization in the younger birds.

Chicks appear not to be infected with *Campylobacter* spp. at hatch, and the means by which young broiler birds are infected remain unclear (234). Different RAPD types have been identified from parent flocks and isolates and their progeny (182). The finding of *Campylobacter*-negative broiler flocks originating from *Campylobacter*-positive parent flocks (breeders) is an indication that vertical transmission does not occur (246). Experimental

transmission studies have shown that newly hatched chicks can easily become colonized (258). Breeders remain alive for longer periods than broiler chickens, and the percentage of *Campylobacter jejuni* colonized breeders has been shown to decrease from 72-100% of the flock at seven weeks of age to 20-46% of the flock at forty-two weeks of age (193).

To obfuscate the issue, a study conducted in 1996 shows a different picture, suggesting that the lack of strain diversity exhibited by *C. jejuni* in the broiler chickens, and the intermittent high positivity of growout houses were evidence for common source *C. jejuni* introduced by vertical transmission rather than contamination (176). There is obviously a requirement for rigorous examination into this particular field of *Campylobacter* transmission within poultry, but as yet, there are few field results to support the thesis of vertical transmission.

Examination of layers for commercial table-egg production indicated that hens demonstrated to be faecal shedders of *C. jejuni* did not produce infected eggs (205). Furthermore, a study conducted in 1984 demonstrated that layers caged individually and provided with *C. jejuni*-free drinking water and feed were less likely to excrete *C. jejuni* than those that roamed freely within a growout house. However, throughout the study, some birds did not excrete a detectable number of *C. jejuni* despite being communally raised among birds later identified as *C. jejuni* excreters. Rates of faecal excretion of *C. jejuni* were not correlated to egg production. *C. jejuni* was present on the shell of about 1% of the eggs from *C. jejuni*-excreting hens, but not in any of the contents of the eggs, and the organism did not penetrate into the eggs examined (50).

Laboratory studies have indicated that shell penetration by *C. jejuni* occurs at an extremely low rate and that the shell membranes serve as an effective barrier to the infection of the albumen (50). The organism can be introduced into eggs by immersion in *C. jejuni* or by contamination of the shell followed by application of pressure and temperature differential treatments. However, these experimental procedures certainly do not reflect commercial conditions (50, 158). *Campylobacter* spp. have been recovered from the inner shell and membranes of eggs that have been refrigerated, or those that have been stored in a solution of *Campylobacter* cells (50, 102, 158).

While the idea of vertical transmission is permanently discussed among interested scholars, there has never been definitive evidence to support the theory that it occurs. *Campylobacter* is seldom isolated from hatchery samples - despite researchers finding DNA sequences that appear to indicate the presence of *Campylobacter* (143). When parent flocks are colonised with *Campylobacter*, they are often a different strain to those colonising broiler flocks (80). Still, the aspect of vertical transmission continues to be contentious - supported by the

frequency distribution of *Campylobacter* types for chickens supplied by two hatcheries over a 5-year period showing marked dissimilarity (135). These findings suggested an association between the isolation rate and type of *Campylobacter* isolates in broiler chickens and the hatchery supplying chicks. The lack of diversity of types and the intermittent high positivity of sheds is evidence for a common source of *C. jejuni* introduced by vertical transmission rather than contamination at the hatchery or during transportation. However, the typing method used in the latter study was serotyping - not a particularly discriminatory method but possibly the best between 1989 and 1994. It is this author's opinion that if indeed vertical transmission does occur, it occurs at such a low rate, that the risks of colonisation are higher for birds via horizontal transmission, so work should be focussed on this in the future. Once horizontal transmission from the environment ceases to be a method of introduction of the bacteria to the birds, then energies can be put into preventing vertical transmission.

1.5.2.3. Horizontal Transmission

The scientific community appears to agree that while vertical transmission may be considered unlikely, the most likely source of *Campylobacter* spp. to the broiler chickens is through horizontal transmission. However, it is not known exactly where this transmission may occur, or when during the growout period it is most likely to occur.

1.5.2.3.1. Delivery and "Placement"

There have been few studies focussed on the delivery and placement schedule of chicks into the growout houses on farm. Those that have been conducted have primarily concentrated on the hatchery as a means for contamination of chicks (89, 119, 176, 182). However, where longitudinal studies have been conducted on broiler farms and have included sampling of fomites associated with chick delivery (including: delivery crates, delivery truck tyres and workers boots), *Campylobacter* spp were not isolated from the fomites (79).

1.5.2.3.2. Growout Houses (Sheds)

It has been proposed that the age of a growout house may have an effect on the transmission of *Campylobacter jejuni* to susceptible chicks. Results from several studies indicate that neither the state of the growout house (new or old), nor the *Campylobacter* status of the flock prior to the susceptible chicks influenced the likelihood of infection (188). New growout houses compared with old ones indicated that neither the time of

Campylobacter spp. introduction nor the rate of colonization in flocks was influenced by whether the house was new or had previously been used (79). Consecutive production cycles generally showed different serotype patterns and *Campylobacter*-negative flocks were observed following positive ones, negating the likelihood of transmission from one cycle to the next via a persistent contamination of the broiler house (103, 172). On occasions when there were numbers of different serotypes on the farm adjacent growout houses were different in the types present, suggesting that transfer between the growout houses was not the predominant mode of transmission (20, 174).

In New Zealand, the all-in-all-out policy is adhered to. At the end of the run, all of the articles inside the shed are removed from the shed, and thoroughly cleaned. This includes drinker lines, feeder lines, heaters, boots etc. The litter is removed from the shed *in toto* such that the concrete floor can be cleaned prior to the placement of the following flock. This particular management practise is slightly different from that practised in the United States of America, where litter remains in the shed to be reused as a floor for the following flock. Fresh litter is added before each placement, though the old litter is only removed a few times per year (E. Line, personal communication). In some English sheds, the flooring is not treated wood shavings, but is wire mesh. Experimentally, there were fewer isolations of *Campylobacter* from caged broiler chickens than from those reared on a litter floor (266). In Quebec, there is a fundamental difference in the way that the broiler chickens are raised. Instead of having sheds next to each other on the farm, as is the case in New Zealand, sheds are placed one atop the other. Birds are placed in one end, and then retrieved from the other end of the shed at the end of the run. Farmers walk through the sheds less frequently than in New Zealand farms (once per week, instead of twice per day) and the birds are left to themselves for most of the growout period (E. Nadeau, personal communication).

1.5.2.3.3. Food and Water

Experimentally, water colonized with *Campylobacter jejuni* can infect chickens rapidly, with up to 64% of the birds colonized within three days of exposure and 89% within one week (206). Field observations from several different sources indicate that food may play a minor role in the introduction of *Campylobacter* spp. to the broiler chickens (10, 20, 105, 174, 192, 233).

The role of disinfection of drinking water remains an enigma. Some research has identified that disinfecting drinking water has protected the broiler chickens from colonisation (117,

174) while others have not (20, 105, 136). Drinking water supplies are normally chlorinated, and contamination of the drinking water tends to follow rather than precede flock infection (117, 174). It has been suggested that *Campylobacter* spp. can survive in the biofilms on the inside of the water reticulation system, which would justify the chlorination and regular dismantling and thorough scrubbing of the supply system (243). Most studies agree that once *Campylobacter* spp. have entered the growout house, the feed and water distribution systems have the potential to enable within-growout-house transmission, especially given the coprophagic nature of the broiler chickens. Rapid horizontal spread of *C. jejuni* within three days of infection of chicks with water containing 10^5 CFU/ml has been demonstrated (206). If birds are provided with water from an open, non-chlorinated source, then this could be a possible source of infection (204).

1.5.2.3.4. Environment

Perhaps the most important factor in preventing transmission of *Campylobacter* spp. to the broiler chickens is the environment of the growout house and consequently the biosecurity measures taken by farmers to keep the environment and the birds separate. Even variations in environmental temperature have been described as having an effect on the initial colonization of *C. jejuni* in broilers (265).

Flies, fomites, wild birds and vermin can serve as vehicles to introduce infection into flocks. However, if these were the only source of infection, it is unlikely that a single serotype of *Campylobacter* would have persisted in the broilers on the farm (174). Also, there have been no occurrences of flies, air, food or water being colonized by *Campylobacter* spp. prior to the colonization of the broiler chickens (21, 79) though in one study, the bacterium isolated from the broilers had been previously isolated from workers' boots and a wild bird on the same farm (79). In studies where wildlife have been examined for *Campylobacter* spp. colonization at the same time as the birds within the growout house; some suggest that rodents are a likely source (10, 233), while others claim the opposite (79, 109). In a study conducted in 1997, various species of wildlife around the growout houses were found to be positive for *Campylobacter* spp., but there was no correlation between this and the onset of flock colonization (79).

An Irish study conducted in 1995 examined the strain types of *C. jejuni* isolated from domestic animals on the same farm as colonized broiler chickens, and observed that most serotypes isolated from the laying hens, pigs, sheep and cattle were different from those isolated from the broilers at the same time (172). An alternative study conducted three

years later in the Netherlands noted that a particular RAPD type of *C. jejuni* was isolated from both the farmers' boots and a broiler flock during the same broiler cycle. The authors of this study proposed that transmission of *C. jejuni* from the cattle to the broilers (and vice versa) on this farm may have occurred via the farmers' footwear (246).

It is clear that while the same strain of *C. jejuni* can be seen in the environment around the growout house as in the broiler chickens, this does not indicate the direction of transmission. It indicates that transmission is possible, or that there was a common source that infected both the environment and the broiler chickens, possibly at the same time. There is a great deal of confusion as to where *Campylobacter* spp. comes from when they colonize the birds. This remains despite more than 20 years of valuable research into the potential sources of transmission.

1.5.2.3.5. Litter

Survival of *Campylobacter jejuni* in damp litter, prolonged periods of shedding by colonized birds, and coprophagy contribute to persistence of infection in flocks (203). Experimental work has shown that autoclaved litter artificially contaminated with *Campylobacter* spp. can infect chickens under laboratory conditions (152). These infected chickens shed *Campylobacter* spp. until at least nine weeks after being transferred to a growout house with a wire floor that prevented coprophagy, demonstrating the potential role of litter in the perpetuation of transmission (152). Most field observations indicate that the birds were colonized prior to isolation from litter; thus it is thought that litter becomes cross-contaminated and is not a source of the organism (79).

1.5.2.3.6. Insects

There is a possibility that *Campylobacter jejuni* may be introduced to a susceptible flock by colonized insects entering the shed. The most popular insect seen in a poultry growout house is the darkling beetle (*Alphitobius diaperinus*). There is evidence to suggest that the presence of the beetle may allow successive flocks of chickens to become colonized with *Salmonella typhimurium* (12), however, the role of the beetle in the colonisation of the chickens with *Campylobacter* spp is not as clear. *Campylobacter* serotypes from darkling beetles inside Dutch broiler houses were identical to the ones isolated from the broilers during the same run, though this study concluded that horizontal transmission from one broiler flock to the next one via a persistent contamination within the broiler house did not seem likely (172). Having said that, the presence of the beetles in the growout house annex increased the risk of colonisation in French broiler flocks, as determined through a risk factor study

(188). Similar work has been undertaken in New Zealand where a large number of *Campylobacter* subtypes were isolated from both beetles and broilers in four sheds, indicating that *Campylobacter* colonization of poultry is likely to arise from a number of different reservoirs. However, a set of genetically distinct isolates were common to the broiler flocks and to the beetles suggesting that *Alphitobius diaperinus* may serve as a source of *Campylobacter* contamination of poultry (18).

1.5.2.3.7. Catching and Processing

A recent New Zealand study examined the fomites used by the commercial companies for collecting the broiler chickens from the farms and demonstrated that the truck beds, the truck wheels, the drivers' boots, the catchers' boots, the pallets, the crates, and the forklift wheels were contaminated with *Campylobacter jejuni* cells prior to leaving the processing plant en route to a growout house (186). This was also found in Britain at the same time (216).

It has been suggested in Denmark that partial depopulation should be minimalised to decrease the number of birds colonized with *C. jejuni* that are processed for consumption (83), and other studies have alluded to the need for increased hygiene measures during the catching process (41, 74).

If *Campylobacter* spp. are present within the intestinal tract of broiler chickens at slaughter, broiler carcasses can become extensively contaminated during the slaughter process. Up to 50% of broiler flocks may be infected at slaughter and it is suspected that most of the birds in an infected flock carry the organism. Faecal samples prior to slaughter contain sizeable concentrations of *C. jejuni* cells (10^7 CFU/g faeces) (174). The popular habit of feed-withdrawal prior to slaughter is likely to increase the frequency of *Campylobacter* spp. isolation from the crop, which may provide another source of *Campylobacter* spp. contamination of carcasses at processing (38). This leads to the contamination of equipment, working surfaces, process water, and air. The large volumes of water used during the processing of the birds contributes to the distribution and survival of the organism, and complicate in-plant control (22).

The processing of the birds is as follows. The neck is cut, and the birds are almost immediately plunged into a bath of scalding water, held at 50-60°C. This heat opens the skin follicles, which facilitates defeathering. During this and the evisceration stage, leakage of intestinal contents contributes to carcass contamination. An experiment to determine how many CFU's were present on a carcass during the processing stage indicated that birds carrying 10^7 CFU cloacally prior to entering the scald tank carried 10^3 after defeathering.

The same experiment plugged the cloaca of experimentally inoculated birds prior to entering the scald tank, which prevented the escape of contaminated intestinal content, and significantly reduced the number of detectable cells on the surface of the breast tissue (23).

It has been demonstrated that the incidence of *Campylobacter* spp. on broiler carcasses was significantly lower on those submitted to air- rather than immersion chilling (197).

1.5.3. Investigations

Most often, investigations into broiler farms or broiler wholesalers are driven by public health, alerted when there is an outbreak of human campylobacteriosis. One such investigation provided evidence for an association between the farm supplying chickens, and a large number of cases of campylobacteriosis in the community over a four-month period. These were notably persons working at, or eating at, a catering college and its associated restaurants served by the farm (175). The source was located to the main farm water supply, which had burst during the first week of placement of poultry. The water supply, distribution system and header tanks were found to be colonized with *Campylobacter* spp. when examined (175).

Water was again shown to be the predominant source of *Campylobacter jejuni* on a broiler chicken farm in southern England where an outbreak of campylobacteriosis in humans occurred in 1984 (174). A total of 60% of all 'source' and 'supply' water samples consistently showed evidence of *C. jejuni*, and the organisms were found throughout the water system, from the soil-water interface at the bottom of the 30m borehole, to the biofilm of the pipework within the chicken growout houses (174).

The single common factor among three farms with positive flocks was the involvement of the owner/manager with *Campylobacter*-positive cattle. Cattle were found to be positive concurrent with the flock infection and may well be one of the reservoirs that maintain the organism on the farm (79). Drinking water and worker's boots were also found to be positive concurrent with the flock infection – but not until AFTER the colonization had occurred in the birds (79).

1.5.4. Control and Prevention

Intervention measures have long been investigated as a means of preventing broiler chickens from being colonized by *Campylobacter* spp.. The ultimate goal of such measures is to provide broiler flocks free from *Campylobacter* spp., or with fewer individual colonized

birds. It was hoped that both of these outcomes would reduce poultry-associated human campylobacteriosis. Carcass cross-contamination occurs within the poultry processing environment on a large scale, and methods to reduce this are largely described as ineffective and expensive (149). Effective methods that can be used in processing plants include the use of air-chillers (197) and the freezing of carcasses (231). It is worthwhile noting that nearly all processing plants use chlorinated water at all stages. More likely intervention strategies include biosecurity measures on-farm and biological control mechanisms still under development.

1.5.4.1. Biosecurity Measures

Between them, several epidemiological studies have indicated that the following methods succeeded in reducing or preventing the colonization of broiler chickens by *Campylobacter* spp.: provision of clean, chlorinated drinking water, cleaning and disinfection of the broiler growout house between flocks, a 'stand-down' period between flocks where the growout house stands empty, a hygiene barrier in the entrance room to the broiler house (annex), hygiene practises (dipping boots in a disinfectant footbath, changing protective clothing and footwear, and washing and drying of hands), the prompt removal of dead birds, vermin control, and having no partial depopulations (41, 63, 74, 83, 109, 117, 174, 206, 245, 246).

While the strict application of some or all of these measures have proven successful, such measures are difficult to enforce all the time on all farms, and the measures do not eliminate the risk of re-introduction of *Campylobacter jejuni* at any future time (74). It has also been shown that the application of even the most stringent biosecurity measures does not prevent the broiler chickens from being colonized by *Campylobacter* spp. (209).

1.5.4.2. Biological Measures

Biological intervention strategies to decrease colonization by *Campylobacter* spp. include: competitive exclusion, immunisation and medication. Competitive exclusion (CE) microflora can be introduced to the broilers prior to challenge with *Campylobacter*. In 1988, a research group demonstrated that CE had no effect on the subsequent colonization of broilers (228). Eight years later, the same research group used a slightly different method. Rather than preparing pure cultures of *Campylobacter* spp. that had previously been isolated from humans or other broiler chickens, they prepared mucosal competitive exclusion (MCE) cultures using two new methods. One method was to scrape the mucin layer from

the caecal epithelia and suspend the layer in a pre-reduced anaerobic medium, the second was to deposit a 1mm segment of distal caeca which had been cleared of caecal contents into the same pre-reduced anaerobic medium. These liquids were then used to challenge the chickens. The group discovered that the undefined MCE contains a diverse composition of microflora, providing a wide-spanning protection against the diversity of strains within the *Campylobacter* genus that cannot necessarily be expected from a more narrowly defined set of bacteria - as previously used (227).

Early immunisation studies have indicated the potential for oral vaccines to prevent colonization of broiler chickens by *Campylobacter* spp.. This effort has been continued to discover that the flagellin antigen may be valuable for immunological control of intestinal infection with *Campylobacter jejuni* in chickens. Researchers maintain that further work is required to purify these as vaccine candidates by using methods that preserve conformational epitopes (264). Further work has indicated that anti- *Campylobacter* spp. maternal antibody can contribute to the lack of *Campylobacter* spp. infection in young broiler chickens in natural environments, providing further evidence supporting the feasibility of development of immunization-based approaches for control of *Campylobacter* spp. infection in poultry (194).

The lack of clinical symptoms of *Campylobacter* spp. colonization in the broiler chickens means it is uncommon to administer antibiotics for the purposes of control. However, it has been shown that the administration of an antibiotic treatment following another disease decreased the risk of a broiler flock being contaminated with *Campylobacter* spp. (188). That said, the rapid emergence of quinolone-resistant *Campylobacter* spp. coincided with the increased use of fluoroquinolones in human and veterinary medicine. The extensive use of enrofloxacin in poultry and the almost exclusive transmission route of campylobacter from chicken to man, in the Netherlands, suggested that the resistance observed was mainly due to the use of enrofloxacin in the poultry industry (57).

Researchers have looked at the effect of withdrawing antimicrobial growth promoters, to observe whether it would cause an increase in pathogen load. There was a decrease in *Salmonella* prevalence in broilers, chicken, swine, and pork and no change in the prevalence of *Campylobacter* in broilers. This shows that *Salmonella* and *Campylobacter* rates have not increased in food animal carriers since antimicrobial growth promoters were withdrawn in 1998. This finding, combined with evidence that the withdrawal has taken place without remarkably noticeable effects on the productivity in broilers and swine, is of particular importance in light of the emerging problem of antimicrobial drug-resistant human

pathogenic organisms, which are associated with the use of antimicrobial growth promoters (61).

**THE PREVALENCE OF *CAMPYLOBACTER JEJUNI*
IN COMMERCIAL BROILER CHICKEN FARMS IN
NEW ZEALAND**

The Prevalence of *Campylobacter jejuni* in Commercial Broiler Chicken Farms in New Zealand

The secret to creativity is knowing how to hide your sources.

ALBERT EINSTEIN

CHAPTER 1 THE PREVALENCE OF <i>CAMPYLOBACTER JEJUNI</i> IN COMMERCIAL BROILER CHICKEN FARMS IN NEW ZEALAND.....	77
1.0. INTRODUCTION.....	80
1.1. MATERIALS & METHODS.....	81
1.1.1. Poultry population.....	81
1.1.2. Cloacal sampling.....	83
1.1.3. Bacteriological examination.....	83
1.1.4. Strain typing by pulsed-field gel electrophoresis.....	84
1.2. RESULTS.....	86
1.3. DISCUSSION.....	92
1.4. REFERENCES.....	95

Cloacal samples were collected from 1200 broiler chickens from 80 distinct flocks during an eight-week period. The flocks represented a third of the production flocks in the north of the North Island, for two of the largest commercial broiler growing companies in New Zealand. Samples were examined for the presence of *Campylobacter jejuni*, which was isolated from 71 samples. These samples were collected from eight individual flocks, indicating a flock prevalence of 10%. The flocks were situated on seven farms, indicating a farm prevalence of 14.5%. The samples collected indicated a bird prevalence ranging from 6.6% to 100% infectivity. The results indicated that one company had a higher rate of flock infection than the other. This is the first time that cloacal sampling of broiler chickens for *Campylobacter jejuni* has been carried out on farms in New Zealand, and the results were used in the formation of sampling plans used in the subsequent studies. The isolates collected were genotyped using Pulsed-field Gel Electrophoresis (PFGE) to form the beginning of a poultry *C. jejuni* database for New Zealand.

2.0. Introduction

Campylobacter jejuni is the primary cause of bacterial gastrointestinal disease in New Zealand. The incidence rate of campylobacteriosis is 332.4 cases per 100,000 people per year (1). This rate has remained high despite efforts to reduce the risk of disease through public education programmes about food preparation and storage. Ingestion of poultry meat has been shown to be a risk factor for sporadic campylobacteriosis (12) and there is widespread public concern over the perceived risk of contracting foodborne disease associated with poultry meat products.

Poultry processing plants have been cited as a source of high levels of bacterial cross-contamination of carcasses (2, 3, 5, 6, 17, 21), and means of improving hygiene standards to prevent cross-contamination are very complicated, costly and not necessarily effective (22, 25). An alternative approach to control considers factors associated with *C. jejuni* infection in broilers at the farm level.

Commercial producers of broiler chickens in New Zealand regularly determine whether a flock is colonized with *Salmonella* spp., but *Campylobacter* spp. have not been included in testing procedures due to the more complicated procedures involved in growing these bacteria. Several prevalence studies have been carried out overseas, and a range of flock prevalences have been determined: 18% in Norway (19), 24% in Finland (2), 27% in Sweden (4), 42.5% in Denmark (30) and 76% in England (16). The prevalence of *C. jejuni* in New Zealand broiler flocks is not known.

Pulsed-field gel electrophoresis (PFGE) has been used to determine the molecular strain type of isolates recovered during outbreaks of campylobacteriosis in humans in New York (8), Kansas (13) and Finland (14), and also for distinguishing sporadic cases of disease in Denmark (23). The technique has been used to compare strains of *C. jejuni* carried by wild birds, with strains colonizing commercial broiler flocks and humans in the same geographical area in Sweden (9). The genetic diversity of *C. jejuni* isolates from retail poultry has been examined using PFGE in the United States, in order to identify sources of human infection (11).

The aim of this study was to determine the proportion of farms, growout houses and flocks that contained at least one bird colonised with *C. jejuni*. Over the course of the study, 77 birds were colonised with *Campylobacter jejuni*, residing in 8 growout houses.

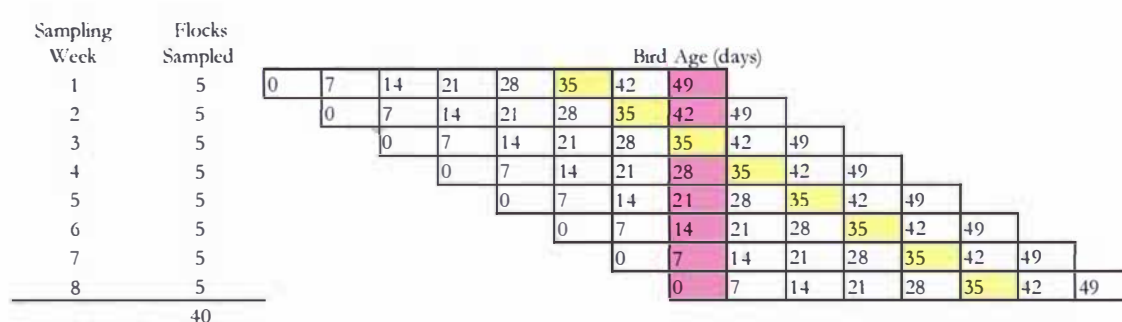
2.1. Materials & Methods

2.1.1. Poultry population

The target population for this study was all growout houses used to grow chickens for Company A and B, situated in the north of the north island of New Zealand. Flocks were included in the sampling procedure if they were within 50km of the processing plant and were 14 days old on the appropriate date of sampling. Where more than one flock met these criteria, flocks were selected using simple random selection.

During an 8-week period, 40 flocks were observed from each company. These flocks belonged to 51 farms, 23 to Company One, and 28 to Company Two. Birds were sampled 12-24 hours prior to the first partial depopulation between 31 and 36 days. It was possible to test a maximum of five growout houses per week, a constraint resulting from laboratory size and the available incubator space. This, as well as the need to keep all the chickens sampled within the same timeframe (i.e. the last chickens sampled hatched from eggs that had been laid by the time the first chickens were sampled – see Figure 2), allowed testing of 80 growout houses across the two companies. A two-stage sampling frame was not designed for this study, owing to the tight financial and laboratory constraints impinged upon the research. The decision to sample 40 flocks from each company arose from necessity and logistic reasons rather than calculation.

Figure 2. Sampling procedure for prevalence study. Yellow cells indicate the age of the flocks when sampled. The pink cells highlight the ‘same time period’ such that the last birds in the first flock sampled have not been killed before the first birds of the last flock were hatched.



The total population size was 77 farms, containing 219 growout houses (98 for Company One, 121 for Company Two).

WinEpiscope[®] was used to calculate the error of the final prevalence calculation given the growout house sample size and an expected growout house prevalence of 20%. The error calculated was an acceptable 13%. This meant that the growout house prevalence estimate finally calculated from the survey was within 13% of the correct value (95% CI).

Flocks were included in the sampling procedure if they contained birds of the appropriate age, prior to the first partial depopulation. Some growout houses on some farms had already undergone the first partial depopulation when others on the same farm were sampled. This sampling outline was chosen so that the major biosecurity breach of depopulation had not occurred - as it seemed an ideal opportunity in which *C. jejuni* could enter a growout house housing susceptible chickens.

Within the growout houses, a formalized random sampling plan was not employed. The investigator sampled three birds from each of five locations while walking through the growout house, representing the central area and each of four quadrants within the growout house floor plan.

WinEpiscope[®] was used to determine sample size, following the 'detection of disease' module. In a population of 10,000 (at least) birds, if the prevalence is 50%, then 15 samples were required to be sure that positive growout houses were not misclassified as negative. 15 birds was sufficient to correctly diagnose a growout house as positive or negative with a 95% CI if the prevalence was as low as 20%. Given a test sensitivity of 70% (27) and specificity of 100%, the chance of finding a positive bird if the population is truly free from disease is 0. The flock sensitivity was calculated as being 99.8%, using the following formula:

$$FSe = 1 - [\mu(1 - \alpha) + (1 - \mu)\beta]^n$$

Where:

μ = within flock prevalence (estimated at 50%)

α = test sensitivity (70%)

β = test specificity (100%), and

n = sample size (15 birds)

Clustering was examined using SAS Enterprise Guide[®] (SAS Institute GmbH, Neuenheimer Landstr. 28-30, D-69043 Heidelberg, Germany) at both the farm and the company level, such that the intra-class correlation could be calculated for both, to

determine whether the prevalences and 95% confidence intervals required adjustment due to the nested nature of the data.

2.1.2. Cloacal sampling

Birds were sampled by cloacal swab. This was taken by turning the bird upside down, supporting it with the left arm, while using the right hand to remove a sterile swab from a packet and insert into the cloaca. The bird was then gently placed upright onto the ground, and the swab broken off into a bijou of Bolton's broth, which was opened and closed with the left hand.

2.1.3. Bacteriological examination

Bolton's enrichment broth in 5 ml aliquots were maintained at 4°C for a maximum of 12 hours in an insulated container. Sterilized swabs were used to collect a cloacal sample from each bird and the swab was immediately broken off into the Bolton's broth in the bottle. The bottles were replaced in the insulated container and were transported to the company laboratory within 4 hours of the first sample collection. At the laboratory a positive control swab, taken from a sample of chicken faeces to which 100µl of a 48-hour *Campylobacter jejuni* reference strain NCTC 11351 in Bolton's broth had been added, was also inoculated into Bolton's broth.

All inoculated bottles were placed inside a 1.5L gas jar containing one 15g sachet of Campygen® (Oxoid Ltd. Basingstoke, Hampshire, England). Lids on the bottles were loosened to allow rapid equilibration of their contents with the gas jar atmosphere. Each gas jar was incubated at 42°C for 48 hours. After this time, the jars were opened and the bottles swirled to resuspend particulate matter. Swabs were used to inoculate plates of Modified *Campylobacter* Blood Free Selective Agar (mCCDA (Fort Richard®)) with suspension, and sterile loops were used to streak the growth over the remainder of the plate. Plates were incubated at 37°C for 48 hours inside a 3.5L gas jar containing three Campygen® sachets.

Plates were then examined for colonies showing typical morphology of *Campylobacter* (7). Suspected *Campylobacter* colonies were streaked onto one side of a divided Tryptic Soy Agar (TSA) plate and the other side was inoculated for confluent growth. A Gram-stain was also performed at this stage. Antibiotic disks (nalidixic acid and cephalothin, 30µg each (Fort Richard®), were placed on the area of confluent growth and the TSA plates were

incubated for a further 48 hours at 42°C in a 3.5L gas jar containing three Campygen® sachets.

Any TSA plates showing the typical *C. jejuni* pattern of antibiotic resistance (resistant to cephalothin, but susceptible to nalidixic acid) were used for further biochemical tests. These included the production of oxidase and catalase, and the ability to hydrolyze hippurate. If the collective results indicated the presence of *C. jejuni* then an Amies® transport swab of the growth was taken and the isolate was sent to Massey University for storage in glycerol broth at -70°C until strain typing could be carried out by PFGE.

2.1.4. Strain typing by pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was completed within four months of the original sample collection date using an adaptation of a generic method (20) as follows. The isolates were partially thawed, and a loopful of culture was streaked onto a TSA plate and incubated at 42°C in a microaerobic environment. Provided the growth was pure and no contaminants were present a swab was used to collect the entire growth from the plate and add it to 15ml of brain heart infusion broth.

The optical density of this broth at 610nm was determined and 100µl of cells were added to a microcentrifuge tube. The tubes were spun at 13000rpm for five minutes to form a cell pellet. The supernatant was removed and the pellet was resuspended in 150µl PETH IV buffer. The tubes were spun again, the supernatant was removed, and the pellet was resuspended in 50µl PETH IV buffer.

A 1% w/v solution of Pulsed-Field Certified Agarose (BioRad®) in PETH IV buffer was melted in a water bath. Eighty microlitres of molten cooled agarose were added to the re-suspended cells and the mixture was immediately transferred into a plug mould, which was placed on ice to assist solidification.

The plugs were removed from the mould, added to sterile microcentrifuge tubes containing one millilitre of lysis buffer and incubated in a water bath overnight at 56°C. Following incubation the plugs were transferred to plastic 50ml universal tubes, containing 10ml of TE buffer and the tubes were plunged into ice and rocked for 20 minutes. This washing process was repeated six times. The plugs were removed from the washing buffer, placed in sterile cryovials containing one millilitre of TE buffer and were refrigerated at 4°C until required.

The plugs were cut with a sterile scalpel, and one third was added to 100µl of restriction buffer before being kept on ice for 45 minutes. The restriction buffer was removed and 80µl of cutting buffer and 15 Units *KpnI* or *SmaI* restriction enzymes were added. After incubation for a further 45 minutes on ice, the plugs were incubated for 24 hours at 37°C or 25°C respectively.

A 1% w/v PFGE agarose gel containing 10% TBE buffer was poured into a mould. The 30-well 1.5mm Bio-Rad™ comb was added after the gel was poured to allow correct well formation. The gel was allowed to set at room temperature for one hour and was then placed in the electrophoresis chamber and covered with 2L 0.5% TBE buffer. The gel was pre-electrophoresed in the chamber for one hour at 6Vcm⁻¹ using the initial switch time, until the buffer had cooled to 14°C. Following pre-electrophoresis, gel forceps were used to push the plugs against the front wall of each well. Lambda and low range molecular weight ladders for PFGE (BioRad®) were added to every 6th lane (if more than ten isolates) and molten 1% w/v PFGE agarose was used to seal the wells.

The loaded gel was returned to the chamber and run using the following parameters: for *KpnI* gels a 4-40 second linear ramped switch time for 21 hours at 6Vcm⁻¹, for *SmaI* gels a 0.5-20 second linear ramped switch time for 23.5 hours at 6Vcm⁻¹.

The electrophoresed gel was removed and added to a 0.1% w/v solution of ethidium bromide (BDH®) in deionised water. The gel was stained for a minimum of ten minutes, and rinsed in more water. The Gel-Doc system (BioRad®) was used to illuminate the gel with UV light and to capture the resulting image in digital form. The digital image was analyzed using Diversity Database (BioRad Laboratories, Hercules, CA), and interpreted using guidelines previously suggested (29).

2.2. Results

Three measures of prevalence were determined: the farm prevalence (proportion of farms in each company that had at least one positive flock on the property), the growout house prevalence (proportion of growout houses in each company containing a flock colonized with *Campylobacter jejuni*), and the flock prevalence (proportion of birds within a flock that were colonized with *C. jejuni*).

Twenty-eight of 38 farms growing birds for Company One were tested (69%) and six contained flocks that were colonized with *C. jejuni*, indicating a farm prevalence of 21.4%. The farm prevalence for Company Two was 4.3% when one of the 23 farms tested housed a flock colonized with *C. jejuni*. The 95% confidence intervals, calculated using SAS Enterprise Guide® (SAS Institute GmbH, Neuenheimer Landstr. 28-30, D-69043 Heidelberg, Germany) were 5.2 – 37.6% for Company One and -4.7 – 13.3% for Company Two. As a crude comparison, the 95% confidence intervals around the farm prevalence calculations for each company overlap, consequently, the difference between the farm prevalence measurements for each company is not significant.

Table 2. Raw data from prevalence study, observing *Campylobacter jejuni* within farms, growout houses and birds

	Company	
	One	Two
Farms Positive	6	1
Farms Tested	27	21
Total Farm Population	39	38
Growout houses Positive	7	1
Growout houses Tested	40	40
Total Growout house Population	121	98
Birds Positive	71	6
Birds Tested	105	15
Total Bird Population	10,000+	10,000+

In Company One, seven of the 40 growout houses tested (17.5%) contained birds colonized with *C. jejuni*, whereas in Company Two, one growout house of the 40 tested (3%) contained birds colonized with *C. jejuni*. All prevalence estimates are shown with their confidence limits in Table 3.

Table 3. Prevalence estimates and 95% Confidence Intervals for farm, growout house and bird prevalence estimates

Company		Farm Prevalence	95% Confidence Intervals	True Farm Prevalence	95% Confidence Intervals
		One	21.4%	(5.2, 37.6)	43.4%
Two		4.3%	(-4.7, 13.4)	7.7%	(2.4, 13.0)

Company		Growout house Prevalence	95% Confidence Intervals	True Growout house Prevalence	95% Confidence Intervals	Adjusted Growout house Prevalence	95% Confidence Intervals
		One	18.4%	(12.3, 24.5)	21.2%	(14.2, 27.6)	21.2%
Two		2.3%	(-0.1, 4.7)	3.7%	(0.7, 6.6)	3.7%	(-1.2, 8.6)

Company		Bird Prevalence	95% Confidence Intervals*	True Bird Prevalence	95% Confidence Intervals
		One	40.0%	(27.4, 52.6)	57.1%
Two		67.6%	(62, 73.2)	96.6%	(82.6, 100)

Confidence intervals calculated using 95% CI's for a proportion, i.e. $\mu \pm \sqrt{p(1-p)/n}$

The flock prevalence estimate was used with the growout house sensitivity (89.6%) to estimate true growout house prevalence for each company. Survey Toolbox[®] (AusVet Animal Health Services, Wentworth Falls, NSW 2782, Australia) was used to perform this function, which results in a true prevalence for Company One of 21.2% (95% confidence intervals ranging from 14.2% – 27.6%). The true growout house prevalence for Company Two was 3.7% (95% confidence intervals range 0.70% - 6.6%). However, due to the high intra-class correlation of farms and growout houses (i.e. multiple measurements were collected from individual farms) of 0.315, a variance inflation factor was calculated (1.195) and used to adjust the confidence intervals of the true prevalence estimate. This widened the 95% confidence intervals such that they overlap between the two companies. It could be considered that because these two confidence intervals overlap, the differences in flock prevalence seen between the companies are non-significant.

Fifteen birds were sampled from each flock. The range of positive results from the 15 tests was 1-15. However, since only one growout house in Company Two was positive, and this growout house had a high proportion of positive birds, the 95% confidence intervals of the true prevalences do not overlap. This implies that growout houses in Company Two have a higher bird prevalence, though since the sample size was only one growout house, this is considered unlikely, especially in light of the fact that some growout houses in Company One exhibited a bird prevalence of 100% in the 15 bird sample from that growout house (see Figure 3). The cumulative frequency line in Figure 3 indicates the proportion of flocks that had a number of positive samples.

Of the eight positive growout houses identified in this study, *C. jejuni* was isolated from 77 of the 120 tested birds, thus the overall bird prevalence could be considered (64.2%). When the true prevalence is calculated, this becomes 91.7%, with 95% confidence intervals from 85.2 – 98.3%.

Two of the eight positive growout houses produced isolates with different PFGE band patterns (growout houses 1 and 4, from different farms). The same *SmaI* and *KpnI* patterns were found in five different growout houses, some of which were geographically distinct from one another. Five strain types of *C. jejuni* were identified with *KpnI* and five with *SmaI*. One growout house contained isolates exhibiting two strain types (Table 4 & 3).

Table 4. Pulsed-Field Gel Electrophoresis patterns of *C. jejuni* when cleaved with *KpnI*

Pattern	Number of Isolates	Percentage	N	Source Farm	Source Growout house	Company
KA	14	93.3%	15	1	3	Two
	2	13.3%	15	33	166	One
	15	100.0%	15	70	155	One
	10	66.7%	15	27	127	One
	8	53.3%	15	27	128	One
KB	1	6.7%	15	39	124	One
	13	86.7%	15	68	212	One
KC	6	40.0%	15	39	124	One
KD	7	46.7%	15	33	166	One
KE	1	6.7%	15	7	110	One
TOTAL	77					

Table 5. Pulsed-Field Gel Electrophoresis patterns of *C. jejuni* when cleaved with *SmaI*

Pattern	Number of Isolates	Percentage	N	Source		
				Farm	Growout house	Company
SA	14	93.3%	15	1	3	Two
	2	13.3%	15	33	166	One
	15	100.0%	15	70	155	One
	10	66.7%	15	27	127	One
	8	53.3%	15	27	128	One
SB	1	6.7%	15	7	110	One
SC	6	40.0%	15	39	124	One
SD	7	46.7%	15	33	166	One
SE	1	6.7%	15	39	124	One
	13	86.7%	15	68	212	One
TOTAL	77					

Figure 3. Histogram to show number of growout houses by number of positive samples

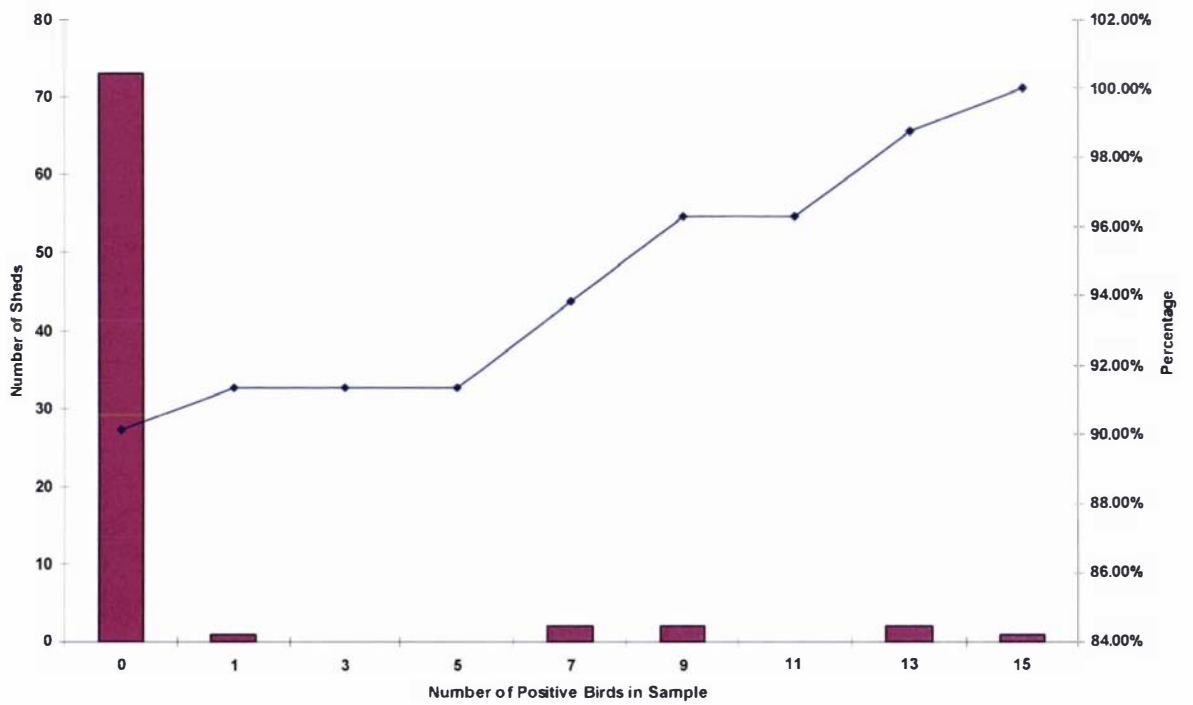
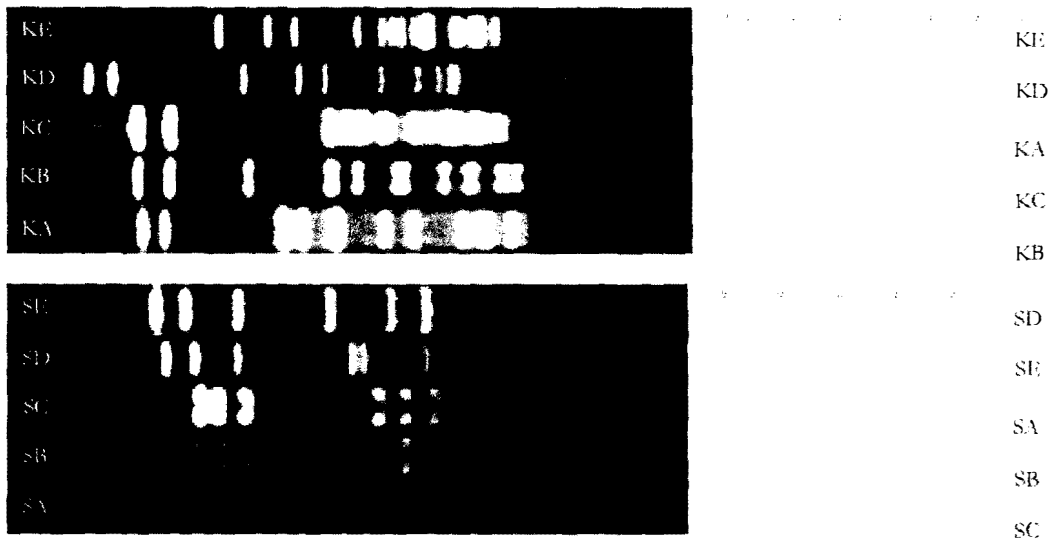


Figure 4. PFGE profiles and dendrograms of the major clones of *C. jejuni* isolated from broiler chicken flocks using *KpnI* and *SmaI* (above and below, respectively)



2.3. Discussion

Very few farms were being used to rear birds colonised with *Campylobacter jejuni*. A reason for this may be that the birds were sampled prior to the first major breach of biosecurity, the first 'cut' or 'partial depopulation'. This could have been estimated by re-sampling all of those flocks after the first partial depopulation, though because of the sampling constraints, this was not possible. When a flock was determined to contain birds colonised with *C. jejuni*, it was likely that the prevalence of colonised birds within that flock was 91.4%, a prevalence estimate much higher than those recorded elsewhere (4, 16, 19), though most other researchers have not calculated the true prevalence using the test sensitivity and the crude prevalence, and the tests conducted have also been different, at different stages of processing, therefore results are not truly comparable.

The true within-flock prevalence does follow anecdotal reports that once *C. jejuni* has entered a flock of susceptible birds, it will colonise all birds to 100% within three days. Though this anecdote fails to be confirmed with prevalence data, the tests used to isolate or identify *C. jejuni* from the birds are not 100% sensitive, therefore it is likely that prevalence estimates are often underestimated.

Since so few growout houses are housing birds colonised with *C. jejuni*, it seems that implanting particular processing strategies may influence the scale of cross-contamination that occurs within the processing plant; perhaps by strategic processing (processing those birds from *Campylobacter*-positive flocks at the end of the day to minimise cross-flock-contamination). This procedure has certainly helped decrease the *Salmonella* spp. cross contamination (N. Kourkgy, personal communication), however, current culture-based detection methods for *Campylobacter* spp. take so long, and are so laborious, the flock may have become colonised by the time a negative test result is obtained. Perhaps the use of molecular techniques to observe for the presence of DNA from thermophilic *Campylobacter* spp. should be employed on an industry level, as this would allow the implementation of processing strategies at the very least.

Using the true within-flock prevalence (91.4%) and the test sensitivity data (70%), the effect of sample size on flock sensitivity can be evaluated for future studies. Indeed, to obtain a flock sensitivity of 100%, 22 birds from within that flock must be sampled individually.

Campylobacter jejuni was isolated from farms within one small area. This appears to be a product of placement schedules, truck movement or farm proximities though this was not observed or evaluated directly within this study.

The 80 growout houses tested in this study can be considered representative of the target population, in that they were reared with the same feed, the same water and light procedures etc. Although the researcher wore protective gear including face masks, hair nets and goggles, it is possible that this breach of biosecurity may have affected the results. Were the study to be done in future, it would be advisable to have the farmer collect the samples required on one of the two daily 'walk throughs' of the growout house, though this has the potential to add bias, as each farmer may conduct the sampling slightly differently.

Experimental studies were conducted to observe horizontal transmission via seeder chicks, and have indicated that 77% of exposed chicks were colonized after three days of contact with a seeder chick, increasing to 98% after seven days (26). This high proportion of positive birds was observed in only one of the positive flocks in this study. There are two possible explanations. Firstly, the organism may recently have been introduced and sampling occurred before the entire flock was colonized by *Campylobacter jejuni*. These flocks were sampled before any major breach of biosecurity had occurred as a result of the first partial depopulation, which supports the theory that the risk of infection increases as the age of the bird increases. Alternatively, the detection method used may have been less sensitive than estimated (27).

Figure 4 shows the PFGE strain types exhibited by isolates collected in this study. It is reported that a higher number of DNA fragments (bands) increases discriminatory power (29), so *SmaI* may not be the most suitable restriction endonuclease for analysing poultry isolates in New Zealand. However, it is routinely used for analysis of human isolates in New Zealand (15), thus to compare poultry and human isolates, *SmaI* is the enzyme of choice. The *SmaI* fragment sizes ranged from 35-364kbps. The enzyme *KpnI* produced a larger number of bands, with smaller bands occurring between 7-369kbps. In this study no difference was seen in the ability of either enzyme to distinguish between strains. Thirty-eight of the 68 (56%) isolates that underwent PFGE exhibited indistinguishable strains of *C. jejuni*, regardless of the endonuclease used, 58% of isolates collected from company A exhibited the same strain type.

The number of bands produced affects the Dice coefficient (10), the measurement of the relatedness of strain types. Thus, the *SmaI* dendrogram (Figure 4) indicates that types SC and SE were 94% similar, whereas the *KpnI* dendrogram indicates that the strains were 67%

similar to each other. This illustrates the difficulties experienced in analysing and interpreting relatedness in these bacteria based on PFGE patterns, especially in sporadic disease situations (as opposed to outbreaks of disease). The unweighted pair-group method (UPGMA) method was used to create these dendrograms. This method implies that evolution of new strains form bifurcating trees, and does not allow for a time factor. There is no evidence of a 'molecular clock' for *C. jejuni* at present, therefore it is impossible to determine how related the strains really are. Methods used in evolutionary biology have shown that neighbour-joining "splits" diagrams may be closer to the true evolutionary pattern between isolates (24), although when that method was applied to these strain types, the results were inconclusive.

Though this study was also attempted in summer, the methodology had not yet been perfected, consequently no *Campylobacter* spp were isolated from the chickens. Once the method had been finalised, in autumn, sampling again occurred in winter. Though this means that we have no comparison with the human peak of cases (which occur in summer/autumn) we can still compare strains of *Campylobacter* found in chickens and those found in humans, as there are still many human cases in winter. In a New Zealand study carried out in 1996/97, 50 of a possible 65 human isolates of *Campylobacter jejuni* exhibited an indistinguishable strain type to 53 of a possible 58 poultry isolates (15). However, human isolates of *C. jejuni* were not collected at the same time as poultry isolates during this study due to resource constraints.

This study was performed at companies whose farms were approximately 300km apart and the same strain of *C. jejuni* was isolated from five growout houses, one of which belonged to Company Two, the remaining four to Company One. Conversations with farm and company personnel did not allude to an identified vector of transmission of the strain between the two companies (the two flocks were sourced from geographically and genetically disparate hatcheries/breeder flocks), although wild animals and birds have long been speculated to cause the spread of *C. jejuni* in broiler flocks (18, 28).

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WHICH IS COLONIZED BY *CAMPYLOBACTER*
***JEJUNI* FIRST: THE CHICKEN OR THE**
ENVIRONMENT?

Which is colonized by *Campylobacter jejuni* first: the Chicken or the Environment?

Optimism is essential to achievement and it is also the foundation of courage and true progress

- LLOYD ALEXANDER

CHAPTER 3 WHICH IS COLONIZED BY *CAMPYLOBACTER JEJUNI* FIRST: THE CHICKEN OR THE ENVIRONMENT?..... 99

3.0.	INTRODUCTION.....	102
3.1.	MATERIALS AND METHODS	103
3.1.1.	<i>Longitudinal Study</i>	103
3.1.2.	<i>Isolation of Campylobacter jejuni</i>	104
3.1.3.	<i>Pulsed-Field Gel Electrophoresis Strain Typing</i>	104
3.2.	RESULTS	107
3.2.1.	<i>Longitudinal Study</i>	107
3.3.	DISCUSSION	110
3.4.	REFERENCES	112

Twelve sheds from two commercial broiler companies in New Zealand were observed from three weeks after the addition of one-day-old chicks through to the final depopulation. The chickens, food, water, litter, boot-dip, boots and the immediate environment of the shed were tested for the presence of *Campylobacter jejuni* in an attempt to elucidate the entry point of *C. jejuni* into the shed.

Seven of the 12 sheds housed birds colonized with *C. jejuni* by the final depopulation. Three sheds contained birds colonized with *C. jejuni* by the first partial depopulation, while the chickens in the remaining four sheds were colonized after the first partial depopulation. It is not clear whether partial depopulations increase the risk of *C. jejuni* colonization. In all cases, the birds were either colonized prior to or at the same time as the environment in which they resided.

Birds in one shed that were colonized with one clone of *C. jejuni* were colonized with a new clone after 10 days. Birds in another shed were colonized by a clone of *C. jejuni* different to that found in the environment of the shed.

3.0. Introduction

Campylobacteriosis is the most prevalent foodborne disease in New Zealand, with 332.4 cases per 100,000 people per year in 2002 (1). Most cases are caused by *C. jejuni*, although there are some illnesses due to *C. coli* (15). Case-control studies conducted in New Zealand and elsewhere indicate that contact with raw or undercooked poultry meat is one of the primary risk factors in human illness (6, 8, 11). Carcass cross-contamination is difficult to prevent at the poultry processing plant level (14), consequently alternative methods to decrease the likelihood of *C. jejuni* entering the processing plant are being considered (5, 22, 23).

Overseas models cannot always be applied to New Zealand, as the growing environment and management methods of commercial broiler flocks differs around the world. A prevalence study conducted on broiler farms used by two commercial poultry producers indicated that up to 32% of sheds housed birds colonized with *C. jejuni* (4). There is speculation as to the role of the 'partial depopulation' with regards to introduction of pathogens, a case-control study conducted in Denmark that observed risk factors for *Campylobacter* colonization of the broiler chickens indicated that this was important (7).

Experimental evidence has shown that within three days of the introduction of the bacteria, nearly 100% of the flock will be colonized (2). For this reason, and to prevent *C. jejuni* from entering the processing plant, it is necessary to test the hypothesis that a contaminated environment leads to the colonisation of the broiler chickens by *C. jejuni*.

3.1. Materials and Methods

3.1.1. Longitudinal Study

Between February and April 2001, chickens in 6 sheds from each of two companies, (A and B) were observed over a period of approximately 3 weeks. The birds and the environment were sampled every other day from 14 days of age until the final depopulation. Each growout house was from a distinct farm with no two farms sharing any boundaries. Growout houses A – F were from company A farms, growout houses G – L from company B farms.

Bolton's enrichment broth in 5 ml aliquots were maintained at 4°C for a maximum of 12 hours in an insulated container. These 12 sheds were selected because they were available. There are only 6 - 10 sheds at a time in each company that are placed in the same week - and are thus the same age. In this case, when there were 10 sheds placed at the same time, simple random sampling was carried out to select which sheds would be enrolled in the longitudinal examination. If the farmer did not wish to be part of the study, then an additional shed was selected using the same method. One criteria was that sheds were not on the same farm - which is often the case when placements are organised. Consequently, it was often the case that all farms that were in the same age cohort were sampled, but which shed from each of these farms depended on random chance.

Samples were collected from each farm and each sample was immediately added to 5mls of Bolton's enrichment broth. Samples included: eight cloacal samples from randomly chosen chickens from various parts of the growout house, five grams of sodden litter from beneath a drinker, five grams of feed from a feeder, five grams of feed from a hopper, fifty millilitres of water from a drinker inside the growout house, fifty millilitres of water from a tap between the header tank and the drinker lines, ten millilitres of liquid from the boot dip, a swab of a 5cm² area on the underside of the left gumboot used inside the growout house, and a swab of a 5cm² area from the first most likely footfall on the concrete path outside the annex.

Water samples were filtered through a 0.45micron Millipore™ (Billerica, Mass., USA) filter and the filters were added to 5ml of the enrichment broth (13)

3.1.2. Isolation of *Campylobacter jejuni*

The bottles were replaced in an insulated container and were transported to the company laboratory within four hours of the first sample collection. All inoculated bottles were placed inside a 1.5L gas jar containing one 15g sachet of Campygen[®] (Oxoid Ltd. Basingstoke, Hampshire, England). Lids on the bottles were loosened to allow rapid equilibration of their contents with the gas jar atmosphere. Each gas jar was incubated at 42°C for 48 hours. After this time, the jars were opened and the bottles swirled to resuspend particulate matter. Swabs were used to inoculate plates of Modified *Campylobacter* Blood Free Selective Agar (mCCDA (Fort Richard[®])) with suspension, and sterile loops were used to streak the growth over the remainder of the plate. Plates were incubated at 37°C for 48 hours inside a 3.5L gas jar containing three Campygen[®] sachets.

Plates were then examined for colonies showing typical morphology of *Campylobacter* (3). Suspected *Campylobacter* colonies were streaked onto one side of a divided Tryptic Soy Agar (TSA) plate and the other side was inoculated for confluent growth. A Gram-stain was also performed at this stage. Antibiotic disks (nalidixic acid and cephalothin, 30µg each (Fort Richard[®]), were placed on the area of confluent growth and the TSA plates were incubated for a further 48 hours at 42°C in a 3.5L gas jar containing three Campygen[®] sachets.

Any TSA plates showing the typical *C. jejuni* pattern of antibiotic resistance (resistant to cephalothin, but susceptible to nalidixic acid) were used for further biochemical tests. These included the production of oxidase and catalase, and the ability to hydrolyze hippurate. If the collective results indicated the presence of *C. jejuni* then an Amies[®] transport swab of the growth was taken and the isolate was sent to Massey University for storage in glycerol broth at -70°C until strain typing could be carried out by PFGE.

3.1.3. Pulsed-Field Gel Electrophoresis Strain Typing

Pulsed-field gel electrophoresis was completed within four months of the original sample collection date using an adaptation of a generic method (12) as follows. The isolates were partially thawed, and a loopful of culture was streaked onto a TSA plate and incubated at 42°C in a microaerobic environment. Provided the growth was pure and no contaminants were present a swab was used to collect the entire growth from the plate and add it to 15ml of brain heart infusion broth.

The optical density of this broth at 610nm was determined and 100µl of cells were added to a microcentrifuge tube. The tubes were spun at 13000rpm for five minutes to form a cell pellet. The supernatant was removed and the pellet was resuspended in 150µl PEXT IV buffer. The tubes were spun again, the supernatant was removed, and the pellet was resuspended in 50µl PEXT IV buffer.

A 1% w/v solution of Pulsed-Field Certified Agarose (BioRad[®]) in PEXT IV buffer was melted in a water bath. Eighty microlitres of molten cooled agarose were added to the resuspended cells and the mixture was immediately transferred into a plug mould, which was placed on ice to assist solidification.

The plugs were removed from the mould, added to sterile microcentrifuge tubes containing one millilitre of lysis buffer and incubated in a water bath overnight at 56°C. Following incubation the plugs were transferred to plastic 50ml universal tubes, containing 10ml of TE buffer and the tubes were plunged into ice and rocked for 20 minutes. This washing process was repeated six times. The plugs were removed from the washing buffer, placed in sterile cryovials containing one millilitre of TE buffer and were refrigerated at 4°C until required.

The plugs were cut with a sterile scalpel, and one third was added to 100µl of restriction buffer before being kept on ice for 45 minutes. The restriction buffer was removed and 80µl of cutting buffer and 15 Units *KpnI* or *SmaI* restriction enzymes were added. After incubation for a further 45 minutes on ice, the plugs were incubated for 24 hours at 37°C or 25°C respectively.

A 1% w/v PFGE agarose gel containing 10% TBE buffer was poured into a mould. The 30-well 1.5mm Bio-Rad™ comb was added after the gel was poured to allow correct well formation. The gel was allowed to set at room temperature for one hour and was then placed in the electrophoresis chamber and covered with 2L 0.5% TBE buffer. The gel was pre-electrophoresed in the chamber for one hour at 6Vcm⁻¹ using the initial switch time, until the buffer had cooled to 14°C.

The loaded gel was returned to the chamber and run using the following parameters: for *KpnI* gels a 4-40 second linear ramped switch time for 21 hours at 6Vcm⁻¹, for *SmaI* gels a 0.5-20 second linear ramped switch time for 23.5 hours at 6Vcm⁻¹.

The electrophoresed gel was removed and added to a 0.1% w/v solution of ethidium bromide (BDH[®]) in deionised water. The Gel-Doc system (BioRad[®]) was used to capture the resulting image in digital form. The digital image was analyzed using Diversity Database (BioRad Laboratories, Hercules, CA), and interpreted using guidelines previously suggested (20).

3.2. Results

3.2.1. Longitudinal Study

Laboratory constraints prevented more than eight birds being sampled from each growout house. Table 6 demonstrates the number of broiler chicken samples from which *Campylobacter jejuni* was isolated. Using WinEpiscope®, if eight of 10,000 birds tested negative for *C. jejuni*, then the maximum possible prevalence is 31%. Given the estimate of 70% prevalence, then the probability of diagnosing at least one animal as truly positive from eight samples, is 100%. This remains the case until the prevalence estimate is 10% at which point, the probability falls to 57%. In seven of 31 sampling occasions that yielded *C. jejuni* isolates (22.5%), the number of birds positive was less than four (n=8). Although this is not statistically indicative of lower prevalence, common sense suggests this is the case, in light of the fact that those seven occurrences were seen earlier in the life of the birds, when the flock prevalence is expected to be low.

Table 6. Number of broiler chicken samples from which *C. jejuni* was recovered (n=8). The shaded areas indicate sampling periods that occurred after the first partial depopulation of the growout house.

		Sampling time (i.e. the number of the visit to the shed)													
Shed	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
A	0	0	0	0	0	0	0	0	0	3	0	5			
B	0	0	0	0	0	0	0	0	0	4	6	6			
C	0	0	0	0	0	0	0	0	0	0	0	6	6	5	
D	3	2	0	1	3	4	3	0	4	5	8	6			
E	0	0	0	0	0	0	0	0	0	0	7	6	6	7	
F	0	0	0	0	3	1	3	0	4	4	6	8			
G	0	0	0	0	0	0	0								
H	0	0	0	0	0	0	0	0	5	5					
I	0	0	0	0	0	0	0								
J	0	0	0	0	0	0	0	0							
K	0	0	0	0	0	0	0	0	0	0					
L	0	0	0	0	0	0	0	0	0						

Figure 5 indicates the progression of *Campylobacter jejuni*-colonized sampling points in each of the 12 growout houses sampled. Seven (58%) growout houses housed birds that became colonized by *C. jejuni*. Two of those growout houses were colonized by *C. jejuni* prior to the first depopulation, and by the final depopulation, a further five growout houses contained birds colonized by *C. jejuni*.

The minimum age of isolation of *C. jejuni* from birds was 24 days. The average age of the birds at final depopulation in company A was 46 days old, whereas company B depopulated growout houses earlier (final depopulation average age of 32 days). Partial depopulations occurred in all but one growout house studied at an average age of 36 days and 32 days (company A and B respectively). In growout houses A, D, F and H, *C. jejuni* was isolated on one occasion, and then was not able to be found on the next sampling occasion.

In growout houses A, B, C, D, F and H there was a progression of colonized sampling points. In two of these cases (growout houses B and H), *C. jejuni* was isolated from the chickens at the same time as being found in the environmental samples. In the remaining growout houses (A, C, D, and F) the birds appeared to have been colonized by *C. jejuni* prior to the environment becoming contaminated, as *C. jejuni* was not isolated from the environmental samples until after it had been seen in the chicken samples.

Pulsed-field gel electrophoresis was carried out on all samples, and indicated that birds in growout house D were colonized first with one clone of *C. jejuni*, and then with a different clone ten days later. In growout house H, a different clone was found to have colonized the chickens than the clone isolated from the area of concrete immediately outside the growout house.

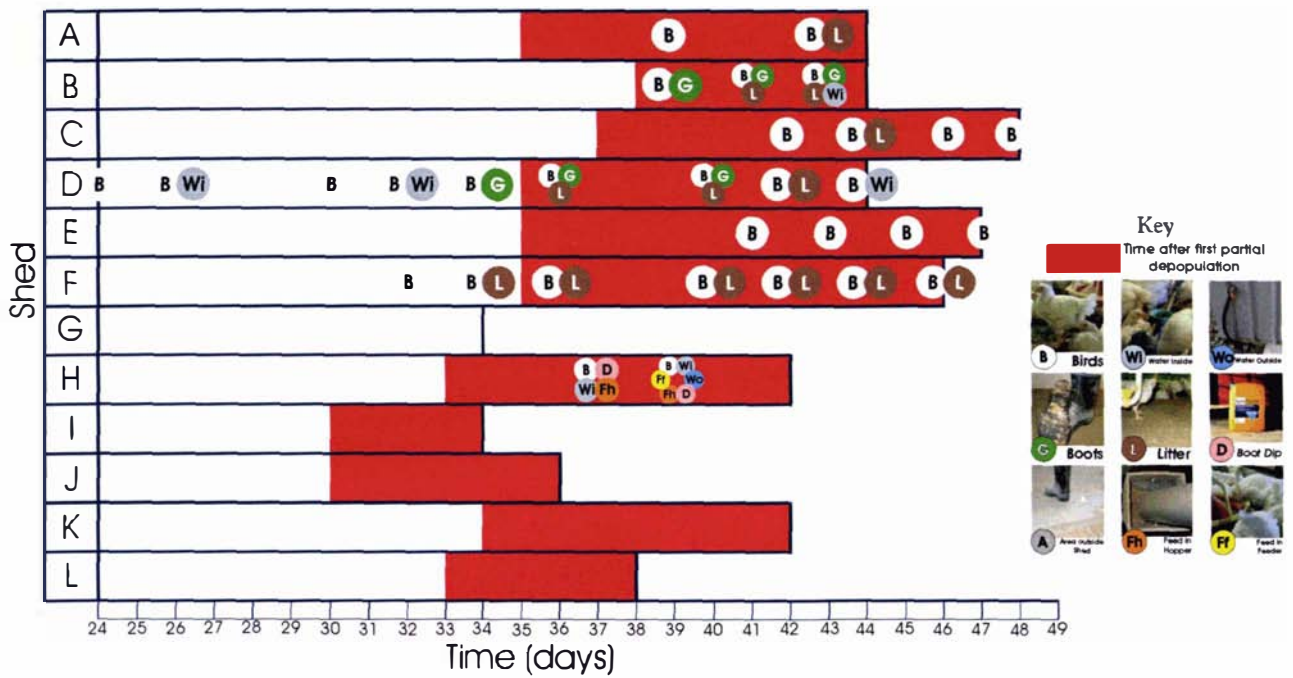


Figure 5. The result of environmental and chicken testing on alternate days after 14 days of age in 12 growout houses. Sampling began at day 14, but *Campylobacter jejuni* was not isolated until day 24, as shown.

3.3. Discussion

The risk of being colonized with *C. jejuni* appears to increase with age. As chickens from company B growout houses were processed earlier than those from company A, they were therefore probably less likely to be colonized with *C. jejuni*. The driving force for early depopulation of these birds was the market demand for smaller birds by fast-food outlets.

The inability to repeatedly isolate *C. jejuni* from the environment and chickens from growout houses that have already demonstrated colonization by *C. jejuni* is of definite concern. It indicates a low sensitivity of the testing protocol, which may cause under-representation of colonized environment elements within and around the growout house. Although the caecal content of a chicken may contain up to 10^6 CFU/ml, this is not the quantity measured by a cloacal swab. Comparison of 'caecal droppings' and 'cloacal swabs' (not caecal contents which are gathered at the slaughterhouse - these are both non-destructive methods) mention that 58% of birds were found positive using caecal droppings, and only 71% of these were positive using cloacal swab technique (19).

This demonstrates the difficulties in sampling the environment with sufficient rigour to isolate *C. jejuni* that is likely to be present, if at all, in very small numbers. The demand upon industry laboratories to comply with testing for other foodborne bacteria, and the large incubator volume and time required to test for the presence of *C. jejuni* does not allow for regular testing to be carried out at the industry laboratories where this work was conducted.

The broiler chickens and their environment were not tested prior to 21 days of age. Since there was no evidence of infection until well after sampling began (i.e. no sheds were positive on the first sampling occasion) this supports the theory that the primary route of infection is horizontal not vertical. hatchery (9, 16, 21). The possibility that one bird may enter the shed with *C. jejuni* already in low numbers in it's gut and then the colonies grow within one bird, such that they are shed and infect other birds in the flock can be disputed by once again looking at the arguments for and against vertical transmission. If indeed, this could occur, then it would stand to reason that the strains seen in broiler chickens would be similar or highly related to those seen in parent flocks. Yet evidence does not support this (10).

The proportion of flocks that were positive at their first depopulation (43%) was too similar to the proportion of flocks positive by their last depopulation (57%) to indicate whether partial depopulations increased the risk of colonization. Indeed, the partial depopulation does cause a breach of biosecurity surrounding the growout houses, but whether this has an effect on the *C. jejuni* status of the birds within the growout house is yet to be determined for New Zealand growing conditions. A study conducted recently in Denmark indicated that partial depopulations played a vital role in the increase of flock prevalence, with introduction of *C. jejuni* occurring during the first depopulation (7). Indeed, it has been demonstrated in New Zealand (17) and England (18) that the fomites that enter the growout house during the catching process are contaminated with *C. jejuni* after being washed at the processing plant. This may yet be prove to be one of the most likely introductory points for *C. jejuni* to enter the growout house.

This study did not imply an environmental origin for the colonization of birds within the growout house by *C. jejuni*. However, the possibility that the birds are colonised following horizontal transmission via a contaminated environment still remains, as the sampling plan in this study appears to have been insufficiently robust. It is also possible that an as yet unidentified environmental source could have introduced *C. jejuni* to the susceptible birds, and, being unidentified, has not been included in the sampling plan for this study.

Disappointingly, the study did not confirm the hypothesis that the contaminated environment led to the colonisation of the susceptible broiler chickens by *C. jejuni*, as in nearly all cases, the birds were colonised prior to the environmental samples. In three of the sheds, environmental contamination and broiler colonisation appeared to occur simultaneously, but in the remaining five sheds, the birds were colonised first. If there were sufficient resources to run a study similar to this again, it would be run on a larger scale, with much more environmental sampling occurring within each shed. This would increase the likelihood of finding a positive sample prior to those found in the chickens, given the likelihood that *C. jejuni* was distributed unevenly within the shed environment.

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**PREHARVEST FACTORS ASSOCIATED WITH
CAMPYLOBACTER JEJUNI COLONISATION OF
BROILER FLOCKS IN NEW ZEALAND**

Preharvest Factors associated with Campylobacter jejuni Colonisation of Broiler Flocks in New Zealand

It is only too easy to catch people's attention by doing something worse than anyone else has dared to do it before

- CLAUDE MONET

CHAPTER 4 PREHARVEST FACTORS ASSOCIATED WITH CAMPYLOBACTER JEJUNI COLONISATION OF BROILER FLOCKS IN NEW ZEALAND	115
4.0. INTRODUCTION.....	119
4.1. MATERIALS AND METHODS	121
4.1.1. Sensitivity testing.....	121
4.1.2. Flock information.....	121
4.1.3. Bacteriological examination.....	122
4.1.4. Statistical Analyses.....	123
4.2. RESULTS	124
4.2.1. Sensitivity testing.....	124
4.2.2. Descriptive results.....	124
4.3. DISCUSSION	133
4.4. REFERENCES	139

Campylobacteriosis is an important food-borne disease in industrialised nations and consumption of contaminated poultry meat is considered a major source of human infection. This study was designed to first evaluate the sensitivity of caecal sampling to determine whether a flock contained birds colonised by *Campylobacter jejuni*. The caecal contents of ten birds were collected from the evisceration room in the processing plant, pooled, and cultured for the presence of *C. jejuni*. This method was compared to a reference method cloacal sampling of 16 individual birds from the same flock. The sensitivity of caecal sampling was 50% that of cloacal sampling, though both are considered 100% specific (95% CI 93.4 – 100.0%). Secondly, all flocks were tested for *C. jejuni* colonisation to evaluate farm management, biosecurity and flock demographic factors for association with the *C. jejuni*-status. Over a period of 13 months (five seasons), 810 flocks of broilers reared commercially for two different companies in 219 growout houses on 77 farms from two companies were examined for *C. jejuni* using caecal sampling. Multivariable

logistic regression was performed with flock infection status as the dependent variable. The multivariable model initially included all putative risk factors and used backward selection of significant variables. Overall, 16.5% of flocks were positive for *C. jejuni*, with the highest proportion of positive groups observed in winter 2002 (26.7%) and the lowest in Winter 2001 (11.9%), indicating either a lack of seasonality or that a year effect was stronger than a seasonal effect.

More flocks reared for Company One were colonised by *C. jejuni* than for Company Two. Protective factors included having hard (i.e. gravel, asphalt or concrete) pathways to the growout houses, being near to another broiler farm, using the reticulated town water supply for the birds drinking water, using tunnel or crossflow shaped growout houses, using a Chore-Time™ feed delivery system within the growout house and chlorinating the water supply to the birds (only in winter).

The odds of raising flocks colonised with *C. jejuni* increased if rodents were seen on the farm, if the growout houses were constructed with a concrete nib wall, if gas heaters were used during brooding, if cattle were farmed on the property, or if workers were employed on the farm.

Sanitising the annex at least as frequently as once per run decreased the odds during summer, and tended to have a similar effect in other seasons. This study has identified factors that may be manageable and lead to a gradual reduction in the colonisation of flocks with *C. jejuni* over time.

4.0. Introduction

Campylobacter jejuni is a major cause of enteric disease in people, and annual incidence rates of campylobacteriosis in New Zealand (332.4 cases per 100,000) are higher than in other developed countries (1). Risk factors linked to sporadic cases of human campylobacteriosis in New Zealand include consuming poultry at a friend's house or at a barbecue, or eating undercooked or raw chicken. However, eating at home or eating roasted or baked chicken were negatively associated with disease risk (11, 18). The observations that contaminated broiler meat appears to be an important source of human campylobacteriosis has led to efforts to decrease product contamination rates through interventions at farms or during slaughter and processing. Cross-contamination in the processing plant is an important source of contamination of poultry carcasses (7, 28), however, as cross-contamination at this stage is difficult to control, investigative efforts have been spent investigating factors that influence *C. jejuni* colonisation of broilers on farms. Studies conducted overseas indicate that *C. jejuni* is more prevalent on fresh and frozen poultry carcasses than *C. coli*, thus *C. jejuni* was the targeted species in this study (23, 26).

Studies conducted elsewhere have sampled chickens at the slaughterhouse for an estimation of the number of flocks that are colonized with *Campylobacter* spp. (4, 15). Others have sampled chickens on the farm (6, 20). Popular sampling techniques at the farm are caecal dropping collection, faecal dropping collection, cloacal swabs and environmental surface (drag) swabs. The most common sampling technique at the slaughterhouse is caecal sampling, emptying the viscera once it has been removed from the carcass. Transportation stress increases the growout housing of *Campylobacter* spp. in faecal material of broilers, thus it is important that sampling techniques are compared within a laboratory (36). In New Zealand, cloacal sampling broiler chickens at the farm has been used to determine the prevalence of chickens, flocks and farms (8). Results from caecal sampling at the slaughterhouse and cloacal sampling at the farm are compared.

Researchers investigating potential risk factors in other countries have identified several risk factors including: season; geographic region; pigs reared in conjunction with chickens; other poultry reared in conjunction with broilers; non-disinfected water; proximity to pigs, sheep, cattle, and other poultry; poor farm hygiene; increasing age of birds; and rodent infestation (10, 22, 30, 35). Despite the high incidence of campylobacteriosis in New Zealand, little investigation of the epidemiology of *C. jejuni* in poultry industry has occurred. A cross sectional study was undertaken to determine associations between possible risk factors on poultry farms and the probability of *C. jejuni* isolation from caecal samples taken from birds at the processing plant.

4.1. Materials and Methods

4.1.1. Sensitivity testing

Caecal sampling was conducted by staff in the evisceration room of the processing plant. The caecal contents from every third bird of the first 30 to be processed for each flocks (ten chickens) were collected into a jar. This jar was forwarded to the laboratory. A swab was used to stir and mix the pooled caecal contents in the jar into which they had been squeezed. The swab was then placed into a sterile bijou of Bolton's Enrichment Broth.

During this period, 39 flocks were also tested on-farm prior to the first partial depopulation, using the cloacal sampling method. This was taken by turning the bird upside down, supporting it with the left arm, while using the right hand to remove a sterile swab from a packet and insert into the cloaca. The bird was then gently placed upright onto the ground, and the swab broken off into a sterile bijou of Bolton's broth, which was opened and closed with the left hand. A further 52 flocks were tested at the final depopulation – using caecal and cloacal sampling again.

Bijoux containing swabs and Bolton's broth from both caecal and cloacal samples were tested as below.

4.1.2. Flock information

Data were collected between July 2001 and December 2002. All farms growing broiler chickens for two companies in the northern part of the North Island were included in the study. Farms were contracted to one of two major broiler producers in New Zealand (38 to Company One and 39 to Company Two).

A flock was defined as one growth cohort of chickens within a growout house, beginning with placement of one-day-old chicks and ending with final depopulation and transport of broilers to a processing plant. Growout houses were then cleaned and disinfected prior to placement of the next flock. All growout houses for which the first partial depopulation and the final depopulation occurred during the sampling period were sampled.

Flocks were sampled for the presence of *C. jejuni* during 5 periods spanning 13 months. These were categorized as follows: winter 2001 (July-September), summer 2002 (January-March), autumn 2002 (April-June), winter 2002 and spring 2002 (October-December).

The flocks were not randomly selected for testing; a census approach was taken. During each of the five periods, every flock that was slaughtered was tested for *C. jejuni* colonisation. The caecal contents were systematically obtained from every third bird of the first 30 of every flock. A marker was placed on the processing chain at the end of one flock (indicating the beginning of the next flock), so this method of systematic sampling was used to avoid confusion where flock sizes differed. The number of flocks at slaughter differed between sampling period and between Company. These differences are shown in Table 1.

Seventy-seven farmers growing birds for the two companies were contacted by telephone during the day and interviewed. Interviews were conducted between January and March 2002. Data collected pertained to the management, demographic and biosecurity practices maintained on a total of 219 broiler growout houses on the 77 farms (a copy of the interview form is available in Appendix A). All farm interviews were conducted by the first author, to reduce interview bias. One farmer did not comply with the study. The reason for this choice and additional information on this farm could not be obtained.

4.1.3. Bacteriological examination

Lids of vials were loosened and the vials were placed inside a 1.5L gas jar containing one 15g sachet of Campygen[®] (Oxoid Ltd. Basingstoke, Hampshire, England) and incubated at $42\pm 1^{\circ}\text{C}$ for 48-50 hours. Subsequently the bottles were agitated to resuspend particulate matter and the swab used to inoculate part of a plate of Modified *Campylobacter* Blood Free Selective Agar (mCCDA) with the suspension; sterile loops were used to streak the growth over the remainder of the plate. Plates were incubated for a further 48 hours, at $37\pm 1^{\circ}\text{C}$ using the Campygen[®] system in gas jars.

Following incubation, plates were examined for the presence of suspect colonies; if present, one colony per plate was transferred to one side of a divided Trypticase Soy Agar (TSA) plate. The other side was inoculated for confluent growth using a swab from the heavy growth area of the preceding streak. *Campylobacter* isolates were characterised further by biochemical and antimicrobial profiles. Isolates were examined for the production of oxidase and catalase and hippurate hydrolysis, and a Gram-Stain was performed.

A flock was considered positive if *C. jejuni* was isolated from the pooled sample; otherwise it was recorded as negative.

4.1.4. Statistical Analyses

Univariable and multivariable logistic regression was performed with flock infection status as dependent variable (1/0). The multivariable model initially included all putative risk factors and used backward selection of significant variables. The significance level required to enter the model was $p \leq 0.30$, and to stay $p \leq 0.15$ (Wald's test, two tailed). Variables where the OR had changed in direction between seasons in the univariate analysis were added to the single multivariate model as interaction terms. The intra-class-correlation coefficient for the correlation of flocks within farm was 0.02, which was considered low. Because the number of flocks per farm was five or smaller the VIF was also low, thus there was low correlation between flocks within a farm, such that the crude ORs and confidence limits calculated were valid without adjustment.

Odds ratios and 95% confidence intervals were generated for all explanatory variables in the final model.

4.2. Results

4.2.1. Sensitivity testing

Of the 39 growout houses containing birds tested for the presence of *Campylobacter* spp. before the first partial depopulation, four were found to contain birds colonized with *Campylobacter* spp. using the cloacal swab method. Two of those four growout houses were also determined to have contained birds colonized with *Campylobacter* spp. when caecal sampling was conducted at the slaughterhouse. The sensitivity and specificity of caecal sampling at this stage of production was calculated, using cloacal sampling as the 'gold standard'. The flock sensitivity of caecal testing appeared to be 50% of what the flock sensitivity of cloacal sampling was i.e. 50% (95% Confidence Intervals: 0.2 – 0.9) and the specificity was 1.0 (95% CI: 0.9 – 1.0).

When 52 growout houses were tested at the final depopulation, 18 and 9 growout houses contained birds colonized with *Campylobacter* spp., using the cloacal swab method and caecal sampling method respectively. The sensitivity of caecal sampling at this stage of production was 0.5 (95% CI: 0.3 – 0.7) and the specificity was 1.0 (95% CI: 0.9 – 1.0).

4.2.2. Descriptive results

During the course of the study, 923 flocks were sampled of which 810 had conformed completely to the experimental protocol (523 and 287 from Company One and Two, respectively) and were eligible for analysis (see Table 7).

In Company One, 234 flocks (44.7%) were completely depopulated at one time, and 289 (55.3%) underwent multiple depopulations. In Company Two, 104 (36.2) were depopulated at one time, the remaining 183 (63.8%) were depopulated over two or more events. Company Two was unable to complete sampling during all of the sampling period, consequently caecal testing was only carried out in three of the five sampling periods.

Over the course of the study, 16.5% of all flocks were colonised with *C. jejuni*. Company One had a higher proportion of positive flocks than Company Two (18.9% compared to 12.2%, respectively) except during winter 2001 and summer 2002, when the prevalence in Company Two was higher than that in Company One. Flock prevalence was highest in winter and spring 2002 for Company One, but winter 2001 and summer 2002 for Company Two.

During winter 2001, 11.9% of growout houses contained birds colonised with *C. jejuni*.

In summer 2002, 15.1% of flocks contained birds colonised with *C. jejuni*. Twenty one point three percent of the growout houses sampled during autumn 2002 contained birds colonised by *C. jejuni*, but all housed birds belonging to Company One. The proportion of positive flocks was highest in winter 2002 (29.7%), and again, all birds tested were being grown for Company One. In spring 2002, 16.7% of growout houses tested contained birds colonised with *C. jejuni*, and growout houses from both companies were sampled.

The highest overall prevalence was seen in winter 2002, with only results from Company One included (26.7%), however the highest prevalence when stratified by Company and depopulation status was in Company One growout houses in autumn 2002 where partial depopulations were used (31.0%).

Few variables were associated with the risk of colonisation of flocks at the univariate level, when all variables were examined across all seasons. Rearing flocks for Company Two appeared to be protective, as did supplying the birds with water from underground bores and using electric heaters during the early brooding stage of growth. Results of univariate analysis of factors associated with *Campylobacter jejuni* colonisation are summarized in Table 8.

All variables were used to create a multivariate model, to predict the outcome of all growout houses during all seasons. Three interaction terms were entered (season and annex sanitation once a run, season and partial depopulations, and season and chlorine), since these variables were associated with risk in one season but were protective in another.

The results of multivariate logistic regression analyses are presented in Table 9.

Season Months		Winter 01 June, July, August	Summer 02 January, February, March	Autumn 02 April, May	Winter 02 June, July, August	Spring 02 September, October	Total	
Company One	No Partial Depopulations	n	52	29	50	67	234	
		Positive	7	2	5	19	41	
		%	13.5	6.9	10.0	28.4	22.2	
	Partial Depopulations	n	93	37	58	68	33	289
		Positive	9	5	18	17	9	58
	%	9.7	13.5	31.0	25.0	27.3		
	% positive	11.0	10.6	21.3	26.7	24.6	18.9	
	N	145	66	108	135	69	523	
Company Two	No Partial Depopulations	n	40	30			104	
		Positive	1	4			7	
		%	2.5	13.3			5.9	
	Partial Depopulations	n	84	76			23	183
		Positive	15	15			2	32
	%	17.9	19.7			8.7		
	% positive	12.9	17.9			7.0	12.2	
	N	124	106			57	287	
% positive		11.9	15.1	21.3	26.7	16.7	16.5	
N		269	172	108	135	126	810	

Table 7. Summary of growout house sampling

Table 8. Descriptive data for each variable for all seasons. n=number of growout houses examined, No=number of growout houses in each category, C+=percentage of growout houses positive for *C. jejuni*, SE=standard error, OR=crude odds ratio, 95% CI = 95% Confidence Interval of OR

Variable	All Seasons					
	n	Frequency No	C+	OR	95% Confidence Interval	P-value
Depopulation Types						
All-out		338	14.2%	1.423	(0.971, 2.086)	0.042
Partial depopulation	810	472	19.1%			
Company						
Company 1		657	63.2%	1.535	(1.023, 2.303)	0.023
Company 2	795	138	72.5%			
Rodent Status of Farm						
Absent		657	49.6%	0.904	(0.626, 1.306)	0.329
Present	795	138	47.1%			
Washing Hands						
Do not clean		657	32.4%	0.881	(0.591, 1.314)	0.304
Clean using soap	795	138	29.7%			
Drying Hands						
Do not dry		657	19.5%	0.784	(0.478, 1.286)	0.200
Dry	795	138	15.9%			
Broiler-specific Clothes Worn						
No		657	7.6%	0.649	(0.288, 1.463)	0.195
Yes	795	138	5.1%			
Broiler-specific Boots Worn						
No		657	95.7%	3.027	(0.713, 12.859)	0.083
Yes	795	138	98.6%			
Boots Dipped						
No		657	31.1%	0.726	(0.477, 1.106)	0.080
Yes	795	138	24.6%			
Gravel/Concrete/Asphalt paths to growout house						
No		657	70.5%	1.103	(0.732, 1.660)	0.361
Yes	795	138	72.5%			
Gravel/Concrete/Asphalt paths between growout houses						
No		651	82.3%	1.514	(0.877, 2.616)	0.082
Yes	788	137	87.6%			
Neighbouring Dairy Farm						
No		657	46.1%	1.135	(0.786, 1.639)	0.280
Yes	795	138	49.3%			
Concrete Nib Walls in Growout houses						
No		672	83.3%	1.000	(0.612, 1.635)	0.543
Yes	810	138	83.3%			

Table 8. continued

Number of Growout houses						
1 – 2		657	68.5%	1.088	(0.729, 1.624)	0.380
3 or more	795	138	70.3%			
Number of Placement Days						
1 – 2		10-Oct	29.9%	1.023	(0.683, 1.531)	0.494
3 or more	784	135	30.4%			
People Living on Farm						
1 – 4		18-Oct	33.3%	1.247	(0.853, 1.823)	0.149
5 or more	795	138	38.4%			
Nearest Poultry Farm						
Other		672	6.4%	0.665	(0.277, 1.594)	0.240
Breeder	810	138	4.3%			
Nearest Poultry Farm						
Other		672	71.7%	0.842	(0.567, 1.251)	0.226
Broiler	810	138	68.1%			
Banks Litter						
No		672	36.5%	1.120	(0.769, 1.632)	0.309
Yes	810	138	39.1%			
Growganic Litter						
No		672	24.7%	0.997	(0.651, 1.524)	0.542
Yes	810	138	24.6%			
Bore Water						
No		672	72.5%	1.636	(1.034, 2.590)	0.020
Yes	810	138	81.2%			
Town Water						
No		672	12.4%	0.931	(0.527, 1.645)	0.468
Yes	810	138	11.6%			
Water Treated with Chlorine						
No		672	9.4%	1.005	(0.537, 1.883)	0.546
Yes	810	138	9.4%			
Treat Before Growout house						
No		672	57.1%	1.240	(0.851, 1.808)	0.152
Yes	810	138	62.3%			
Insecticide Once A Run						
No		672	36.6%	0.758	(0.51, 1.125)	0.099
Yes	810	138	30.4%			
Insecticide Never						
No		672	33.2%	1.144	(0.78, 1.677)	0.276
Yes	810	138	36.2%			
RipCord Insecticide Used						
No		672	47.3%	0.991	(0.687, 1.431)	0.519
Yes	810	138	47.1%			
Annex Sanitised Once A Run						
No		672	31.1%	0.782	(0.517, 1.182)	0.143
Yes	810	138	26.1%			

Table 8. continued

Annex Sanitised Never						
No		672	23.8%	1.400	(0.935, 2.096)	0.065
Yes	810	138	30.4%			
Water Birds Seen on Farm						
No		672	50.9%	1.053	(0.73, 1.519)	0.428
Yes	810	138	52.2%			
Game Birds Seen on Farm						
No		672	48.4%	1.068	(0.74, 1.540)	0.398
Yes	810	138	50.0%			
Scavenging Birds Seen on Farm						
No		672	80.5%	1.429	(0.857, 2.381)	0.103
Yes	810	138	85.5%			
Any Other Farm Animals						
No		672	71.1%	0.928	(0.622, 1.383)	0.392
Yes	810	138	69.6%			
Number of Birds in Growout house						
1 – 24,999		672	42.7%	0.862	(0.593, 1.254)	0.249
25,000 or more	810	138	39.1%			
Nipple Drinkers Used						
No		672	23.2%	0.919	(0.59, 1.430)	0.401
Yes	810	138	21.7%			
Bell Drinkers Used						
No		672	35.3%	0.948	(0.644, 1.395)	0.434
Yes	810	138	34.1%			
Plywood Growout house Walls						
No		672	51.3%	0.729	(0.504, 1.054)	0.056
Yes	810	138	43.5%			
Growout houses run North to South						
No		672	46.7%	1.318	(0.913, 1.903)	0.083
Yes	810	138	53.6%			
Electric Heaters Used						
No		672	35.7%	1.468	(1.014, 2.127)	0.027
Yes	810	138	44.9%			
Gas Heaters Used						
No		672	41.2%	0.737	(0.502, 1.081)	0.070
Yes	810	138	34.1%			
Tunnel Shape						
No		672	8.3%	0.588	(0.262, 1.319)	0.127
Yes	810	138	5.1%			
Crossflow Shape						
No		672	65.8%	1.321	(0.882, 1.977)	0.104
Yes	810	138	71.7%			
Number of Heaters						
1 – 2		672	6.7%	1.207	(0.608, 2.397)	0.351
3 or more	810	138	8.0%			

Table 8. continued

Any Cattle on Farm						
No		672	62.8%	0.950	(0.652, 1.385)	0.431
Yes	810	138	61.6%			
Any Dairy Cattle						
No		672	14.9%	0.698	(0.392, 1.242)	0.135
Yes	810	138	10.9%			
Birds Per Feeder						
1 – 59		672	68.9%	1.495	(0.975, 2.293)	0.038
60 or more	810	138	76.8%			
Birds Per Drinker						
1 – 29		672	64.1%	1.156	(0.783, 1.706)	0.265
30 or more	810	138	67.4%			
Tonnes of Grain Storage						
1 – 16		Oct-01	76.5%	1.443	(0.897, 2.322)	0.078
17 or more	802	137	82.5%			
Area (m²)						
400 – 1599		667	9.3%	0.762	(0.381, 1.527)	0.280
1600 or more	805	138	7.2%			
Volume (m³)						
Less than 4999		644	42.9%	1.018	(0.699, 1.482)	0.501
5000 or more	778	134	43.3%			
Dust Masks Worn in Growout house						
No		657	37.0%	1.062	(0.728, 1.550)	0.412
Yes	795	138	38.4%			
Gloves Worn in Growout house						
No		657	10.5%	0.738	(0.38, 1.435)	0.233
Yes	795	138	8.0%			
Workers on Farm						
No		657	32.9%	0.924	(0.622, 1.372)	0.388
Yes	795	138	31.2%			
Water Bacteria Level Checked						
No		657	17.8%	0.692	(0.406, 1.181)	0.107
Yes	795	138	13.0%			
Feed Early Includes Trays						
No		657	10.7%	1.505	(0.889, 2.548)	0.086
Yes	795	138	15.2%			
Choretime Feeders Used						
No		657	45.5%	1.197	(0.829, 1.729)	0.193
Yes	795	138	50.0%			
Household Cleaners Used						
No		672	1.0%	0.693	(0.085, 5.682)	0.593
Yes	810	138	0.7%			

Table 9. Results of multivariate logistic regression analysis of significant risk factors associated with colonisation by *Campylobacter jejuni*

Variable	B	S.E. (B)	OR	Lower 95% CI	Upper 95% CI	p-value (Wald)
Intercept	-0.486	0.984	0.615	0.089	4.230	0.621
Growing birds for Company Two	-1.435	0.353	0.238	0.119	0.475	<.0001
Rodents seen on farm	0.829	0.344	2.291	1.167	4.498	0.016
Hands washed entering growout house	0.666	0.311	1.946	1.058	3.581	0.032
Broiler-specific Clothes Worn	1.388	0.556	4.008	1.348	11.916	0.013
Gravel/Concrete/Asphalt paths to growout house	-1.270	0.399	0.281	0.129	0.614	0.002
Concrete nib walls in growout houses	1.546	0.518	4.695	1.700	12.968	0.003
Three or more growout houses on farm	-0.719	0.398	0.487	0.223	1.063	0.071
Three or more days of placement	0.694	0.352	2.001	1.004	3.987	0.049
Nearest poultry farm is a broiler	-1.597	0.456	0.203	0.083	0.496	0.001
Drinking water from town supply	-2.387	0.729	0.092	0.022	0.384	0.001
Insecticide never used	-1.056	0.430	0.348	0.150	0.808	0.014
Scavenging birds seen on property	-0.862	0.408	0.422	0.190	0.940	0.035
Growout houses lie north to south	0.770	0.326	2.159	1.140	4.088	0.018
Gas heaters used during brooding	1.674	0.530	5.333	1.887	15.077	0.002
Growout house is tunnel shaped	-1.896	0.625	0.150	0.044	0.511	0.002
Growout house is crossflow shaped	-1.440	0.414	0.237	0.105	0.534	0.001
Three or more heaters used during brooding	1.877	0.605	6.533	1.997	21.368	0.002
Cattle kept on property	0.977	0.416	2.656	1.176	5.999	0.019
Growout house area $\geq 1600\text{m}^2$	-1.141	0.576	0.320	0.103	0.987	0.048
Workers employed on farm	0.958	0.426	2.607	1.132	6.004	0.024
Chore-Time™ feed distribution used	-1.713	0.505	0.180	0.067	0.485	0.001
INTERACTION TERMS						
Annex sanitised X winter			2.190	1.034	4.640	
Annex sanitised X spring			0.877	.0241	3.187	
Annex sanitised X summer			0.131	0.026	0.668	
Annex sanitised X autumn			1.012	0.481	2.130	
Chlorine used to treat water X winter			0.114	0.019	0.671	
Chlorine used to treat water X spring			0.982	0.153	6.310	
Chlorine used to treat water X summer			0.254	0.014	4.752	
Chlorine used to treat water X autumn			0.266	0.055	1.279	
Partial depopulation X winter			0.668	0.360	1.239	
Partial depopulation X spring			0.973	0.317	2.990	
Partial depopulation X summer			2.863	0.876	9.357	
Partial depopulation X summer			1.484	0.862	2.554	

When all sampling and interview results were combined, flocks reared for Company Two were less likely to be colonised. Other protective factors included having hard (i.e. gravel, asphalt or concrete) pathways to the growout houses, having another broiler farm nearby, using the reticulated town water supply for the birds drinking water, using tunnel or crossflow shaped growout houses, chlorinating water during the winter, sanitising the annex once during a run, and using a Chore-Time™ feed delivery system within the growout house.

The odds of raising birds colonised with *C. jejuni* increase if rodents were seen on the farm, if broiler specific clothes were worn within growout houses, if the growout houses were constructed with a concrete nib wall, if the axis of the growout house lay north to south, if gas heaters were used, if cattle were also raised on the property, or if workers were employed on farm.

There were two factors associated with risk of colonisation in particular seasons only: Chlorinating the birds water supply during winter (protective) or sanitising the annex once per run (protective during summer, risk during winter).

4.3. Discussion

Comparisons of chicken sampling techniques are scarce; the studies that have been conducted have predominantly been driven by the retail market such that food sample methods are tested and compared (21, 25), and none have been carried out in New Zealand, looking at broilers. It is not fully understood how the method of sampling may affect results, although a diversity of *Campylobacter* genotypes has been observed in oviduct and faecal isolates collected from breeder flocks in the USA (16). The effect of enrichment has not been fully examined either, though it has been observed that enrichment of chicken caecal samples results in a decreased rate of detection (24). The few studies that have examined the differences between sampling methods rather than the differences between media or laboratory conditions have concluded that caecal droppings are preferable to faecal droppings, which are more sensitive than cloacal swabs for determining the presence of *Campylobacter* spp. (33). Comparisons of sampling techniques for detecting *Arrobacter butzleri* in chickens indicated that environmental surface (drag) swabs were the most sensitive method for determining the presence of the bacterium in a growout house (12).

Time of sampling had no effect on the positive predictive value of the caecal sampling and since the two tests behaved the same at both times of sampling, the results were pooled. The results indicated that if caecal sampling yielded a positive result, then it is almost certain that the flock would have tested positive had it been tested with the cloacal sampling method. This is not to say that the flock was necessarily positive on the farm, especially given the effect of transport stress, as described previously. It is possible that the rate of coprophagy may increase during the holding time at the slaughterhouse, prior to slaughter, when the broiler chickens are under stress and have not had access to feed (19).

The negative predictive value of the pooled-caecal test, was 86% (95% CI 76-93%). This indicates that a large proportion of the growout houses tested using the caecal sampling method would also have been negative for the presence of *Campylobacter* spp. using the cloacal sampling method. Sensitivity of pooled-caecal tests is 50% (29-72%) when compared with individual cloacal sampling of 16 birds. However, the specificity remained 100% (93-100%). These calculations should be interpreted with care, however, as this study indicates that half as many growout houses contained birds colonized with *Campylobacter* spp. when the caecal sampling method was used in comparison to the cloacal method, so the prevalence may have been underestimated when calculated using the caecal method.

Imperfect diagnostic testing might have resulted in misclassification of flocks. False-positive results are unlikely, as the isolates were speciated. However, false negatives are

possible given the low sensitivity of the caecal method. Thus, the proportion of *Campylobacter*-colonised flocks should be interpreted with care.

The overall prevalence estimate (17% - true prevalence 33%) is comparable to prevalence estimates from overseas (2, 4, 17, 35) despite different sample collection and isolation methods being used for each. If the differences seen were real, and not an artefact of the different sampling and/or identification methods, then parameters such as number of animals per farm, climate conditions and distance between farms may influence the infection rate.

The overall low prevalence of flocks in the commercial broiler farms tested within this study does not fully explain the high rate of human Campylobacteriosis seen in New Zealand. If the results from this study are applicable across the industry within the country, there is a large gap of knowledge between the 'on-farm' prevalence of colonisation and the 'human' prevalence of disease. The processing plant is commonly considered a 'multiplier' of *Campylobacter* spp. whereby few flocks colonised with *Campylobacter* spp. may enter the plant, but many carcasses or carcass portions may be contaminated throughout processing, and may still be contaminated when they leave the processing plant. The poultry meat product leaving the processing plant is often sold to a wholesaler, or to supermarkets that then package the product. This means there were several opportunities for cross-contamination to occur after the initial processing of the carcass. Consequently, concurrent studies involving all levels of processing will be required before a control point may be defined in the poultry to human food chain in New Zealand.

The question of seasonality of *Campylobacter* infection of animals is important due to the marked seasonality of human infections reported from temperate countries, with incidence highest in summer (27). Several authors have reported seasonality of *C. jejuni* infection of poultry flocks, with higher risk occurring in the spring/summer periods (10, 30, 35) and (3). However, significantly higher odds of *Campylobacter* infection in autumn have been reported in broilers (22) and in wild gulls (9). Evidence of seasonality is also apparent from studies of ruminants (32). In our study, the proportion of flocks colonised with *C. jejuni* tended to be highest – and lowest - in winter, thus the effect was not statistically significant ($p=0.102$). No seasonal effect was reported in this study, but a year effect was seen. However, it is intriguing to note that seasonality of human campylobacteriosis in New Zealand appears to be less pronounced and more extended into autumn than in European countries (27). This may be due to a longer period of 'summer-like' weather that has begun to extend into months traditionally associated with autumnal weather in New Zealand. It may also result from weather and atmospheric similarities throughout the sampling period within the

sampling space. All farms tested were in the north of the north island of New Zealand, and as New Zealand has a temperate climate, dramatic climate changes as seen overseas (Scandinavia, Quebec etc) are not experienced. This means that the results seen in this study cannot be applied to farms in the South Island, since climate differences may have an effect. Such differences are seen in the red-meat animal rearing practices (R. Cook, personal communication). This study shows different rates in the two winter seasons of subsequent years (21 vs. 11%) thus there is no clear seasonality.

This study was a screening process, to observe and identify possible risk factors for investigation for further studies. Due to uncertain validity, discussion is limited to only those significant factors that appear biologically plausible. As the sensitivity and specificity of the diagnostic method used is the same regardless of the exposure status of the birds to each risk factor, misclassification that may have occurred is likely to be non-differential. Consequently, odds ratios that reported may be underestimates.

When the nearest poultry farm to the farm in question was also a broiler farm, the risk of rearing birds colonised with *C. jejuni* was decreased. This may have been due to an increased awareness of the need to maintain biosecurity measures for staff and visitors to the growout houses, as farmers from the two farms would be aware of the risk of disease transmission, and may be more vigilant than otherwise.

The inclusion of concrete, gravel or asphalt paths to the growout houses also protected the birds. *Campylobacter* spp. typically die in dry environments, and a hard surface is less likely to turn into mud when wet. Mud is comprised of particulate matter suspended in water; a complex and diverse environmental matrix. It is often filled with particles of sand, salt, soil, decaying plant tissue, bacteria and is often moist; an ideal environment for the survival of *Campylobacter* cells. Mud - due to its clumping nature - would form a protective environment from the effect of UV light and desiccation, under which *Campylobacter* cells will die. Dust, on the other hand, is dry, and *Campylobacter* cells would not survive in these conditions. The introduction of mud that potentially harbours *Campylobacter*, to the growout house increases the bird's risk of exposure to *Campylobacter* spp. Consequently concrete, gravel or asphalt paths to and from the growout house entrances are a means of protecting the birds from *Campylobacter* colonisation.

Providing the birds with drinking water from the town reticulation system was also associated with decreased risk of colonisation, perhaps a result of the more stringent water control measures taken with town water intended for consumption by humans than are usually applied to bore or river water for animal needs. Chlorinating the water, in winter more so than other seasons, was also associated with a decreased risk of colonisation.

Campylobacter spp. die when exposed to chlorine. However, the build up of biofilms in the pipelines that carry water to the individual drinkers within the growout house may affect the Free Available Chlorine that is available in the drinking water to which the birds have access. If this is the case, then it seems likely that chlorinating the drinking water will not affect *Campylobacter* carriage in the chickens, as there is no free available chlorine left in the water by the time it reaches the chickens. The validity of this risk factor was subsequently assessed. The reported effects of treating water have been variable among previous studies (4, 22) and (14), and part of this variation might be attributable to reliability of chlorination procedures used on farms.

Tunnel and crossflow growout houses are the most popular shape growout houses, with 542 flocks (66.8%) being reared in a crossflow growout house, and 7.8% in a tunnel shaped growout house. The other alternative growout house shape is a 'ridge extraction' where vents are only in the roof. The crossflow and tunnel shaped growout houses are reputed to have a lower relative humidity, to the point where some farmers spray a fine mist of water over the birds to maintain a comfortable humidity level (D. Rippin, personal communication). *Campylobacter* spp. do not survive in dry environments, so the crossflow and tunnel shaped growout houses may be too dry for them to survive, therefore the broilers are less likely to be exposed and colonised.

The risk of rearing birds colonised with *C. jejuni* could also be minimised by using Chore-Time™ feed distribution system. This variable is highly correlated with birds per feeder, such that it may be confounding the model. It stands to reason that the fewer birds eating from the same feeder, there are fewer opportunities for bird-to-bird contact. Also, Chore-Time™ feeders are set at a level above the floor that minimises the amount of debris, including faecal material, that is likely to enter the feeder, and the design of the feeder includes a grill that orientates the birds to the feed to prevent unnecessary raking of the feed. This in turn limits the amount of feed that lands in the litter, which may limit the degree of pecking at the floor that occurs.

It is also possible that the concrete nib wall structure, built to approximately 100mm separating the base concrete pad from the wall material, is a means by which litter material contaminated with *C. jejuni* may linger after a thorough clean, and present an opportunity for colonisation of the naturally inquisitive birds once the new flock enters the growout house, however, a higher degree of similar strain types would be expected within subsequent flocks if this was the case. This was not seen. It is also possible that the presence of the wall does not allow the area where the wall directly meets the floor to dry adequately following the cleaning, such that whichever *C. jejuni* strains are brought into the

growout house during the clean-out stage remain in a damp environment, contaminating the environment before the birds are introduced to the growout house.

The risk of colonisation by *C. jejuni* was increased by the presence of rodents on the farm. Though farmers were not asked to specify whether rats or mice were commonly seen, the presence of mice on the farm has been associated with increased risk in Sweden (4) and Norway (22), and they have also been shown to be long-term excretors of *Campylobacter* spp. (5).

When a farm employs additional workers to manage the workload, the potential for colonisation by *C. jejuni* appears to increase. Workers other than the farm owner may not be as motivated to maintain stringent biosecurity measures, and thus may increase the risk of the birds being colonised.

Rearing cattle on the same farm property as poultry increased the risk of having birds colonised by *C. jejuni*. Studies have shown that similar genotypes of *C. jejuni* are present in the cattle and the human population (31), while others have indicated that the same serotype is found in Danish humans, cattle and poultry (26). In a Dutch study, genotyping analysis suggested that poultry had become colonised following horizontal transmission of *Campylobacter* spp. from cattle on the same farm (34). This study provides sufficient evidence to suggest that cattle should not be farmed on the same property as the poultry, though perhaps if separate farm labourers managed the enterprises and seldom met or used common land, then perhaps the risk would not be so high.

Gas heaters are used during the brooding stages in some growout houses. These are usually large “unflued” LPG (liquid petroleum gas) heaters (no specific chimney), which are known to expel a lot of water vapour as the LPG is burnt. The humidity may increase the likelihood of opportunistic *Campylobacter* cells that may have entered the growout house surviving long enough to colonise the birds.

There appears to be a trend towards a protection from *C. jejuni* colonisation by sanitising the annex at least as frequently as once per run, though this appeared to be a risk factor during winter, but protective during summer (with non-significant results showing a protective influence during autumn and spring). The annex environment during winter is often very damp and muddy, as boots are worn into the annex (but changed before entering the growout house). Cleaning the annex, despite using chemical disinfectants, may actually create pockets of dampness in which *Campylobacter* cells can survive, and can be transmitted into the growout house via fomites. The fact that this variable was associated with decreased risk of colonisation during summer, may be a spurious result. The annex

during summer is likely to be dry, an environment in which *Campylobacter* spp. does not thrive.

Some other factors that may have been anticipated to be risk factors based on previous studies, but were not found to be significant in our study. Other studies indicate that hygiene behaviour, cleaning and disinfection of the growout house environment and the drinking water status to be important risk factors in the control of *C. jejuni* in the poultry growout house (13, 14, 22) and (30).

These results suggest that *C. jejuni* colonisation may be associated with multiple causal factors that may vary between seasons and indicate that it may be possible to reduce flock level risk of *C. jejuni* colonisation by attention to farm management issues. Reduction in flock risk is likely to have a beneficial flow on effect on contamination of carcasses during processing and on reducing human health risk.

Most of the commercial broiler flocks in New Zealand are grown specifically for two companies. Consequently, farm management techniques are likely to be the same throughout. This implies that the farm management type variables that have an effect on the *Campylobacter* carriage of the broiler chickens are likely to be valid for farms other than those examined explicitly in this study. On the other hand, those variables that are likely to be affected by weather may influence the carriage of *Campylobacter* in different ways between the North and South islands of the country.

The study has shown that there is no single intervention strategy to decrease the likelihood of *Campylobacter jejuni* colonisation in the flock, but that the colonisation of chickens occurs due to multiple factors some of which have an effect in different seasons. The description of particular risk factors associated with increased risk of colonisation with *C. jejuni* will allow protective management practices to be suggested to the broiler-growing industry in New Zealand.

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**CHLORINE IN COMMERCIAL BROILER
DRINKING WATER**

Chlorine in Commercial Broiler Drinking Water

Animals are such agreeable friends - they ask no questions, they pass no criticisms.

GEORGE ELIOT

CHAPTER 5 CHLORINE IN COMMERCIAL BROILER DRINKING WATER

.....	145
5.0. INTRODUCTION.....	148
5.1. MATERIALS AND METHODS	149
5.1.1. <i>Growout houses.</i>	149
5.1.2. <i>Chlorine Concentration.</i>	149
5.2. RESULTS	151
5.2.1. <i>Temporal Differences in FAC Concentration.</i>	151
5.2.2. <i>Spatial Differences in FAC Concentration.</i>	152
5.3. DISCUSSION	153

Free Available Chlorine (FAC) concentrations in drinking water supplied to broiler chickens grown commercially in New Zealand were monitored over 11 farms in two companies. Different sites within a growout house were examined at different times of the day to determine spatial and temporal differences in FAC concentrations.

Taps provided water with significantly higher FAC concentrations than did drinkers. There were no significant differences between the concentrations of FAC taken from various drinkers around the growout house. There were differences in the variation of measurements taken from the same drinker within a growout house at different times of the day, with variation increasing in the afternoon.

No growout houses provided an average FAC content of 2ppm, the suggested standard in one company. Three growout houses consistently met the chlorine concentration suggested by the New Zealand Drinking Water Standards of 0.2ppm.

5.0. Introduction

Foodborne diseases are among the most common zoonoses occurring in industrialised countries, and New Zealand has the highest reported annual incidence of human campylobacteriosis (222.4 cases per 100,000 capita in 2001 (3)) when compared with European countries and the United States of America. Efforts to reduce the incidence, including education of the public about food preparation and storage, appear to have had little impact. Although raw milk (4), contaminated water (14, 30) and tuna salad (26) have been associated with outbreaks of campylobacteriosis, 'sporadic' cases are considered to account for most of the public health burden of this disease (12, 15). The relative importance of potential sources of infection of sporadic cases is not well understood. Several risk factors have been implicated in studies in New Zealand and overseas including consumption of raw water, raw milk, and contact with household animals (11, 13). However, mishandling of raw poultry and consumption of undercooked poultry have been regularly identified as risk factors for infections (2, 12, 13, 22). Consequently, there has been considerable research into the epidemiology and control of *Campylobacter* infection of poultry in both the production (17, 27, 28) and processing (18, 20, 27) environments.

Contaminated drinking water has been associated with large outbreaks of human campylobacteriosis (9), and is considered an important route of transmission among broiler chickens (25). Chlorine is an effective disinfectant against *Campylobacter* (29) and both chlorination and acidification of drinking water have been recommended as potential control measures in broiler production (5, 16) and processing (21). The amount of HOCl and OCl⁻ in water is referred to as the free available chlorine (FAC). Chlorine reacts with ammonia in organic compounds found within water, and forms chloramines. These are known as combined chlorine (CC) which also have microbicidal effects, but are much slower acting than FAC (6). In New Zealand, chlorination policies differ between major poultry companies, one company aims to provide growing broilers with chlorinated water at a target FAC concentration of 2ppm (mg/L), ten times higher than the concentration allowed in potable water for human consumption (24).

This study was conducted to measure the concentration of chlorine being delivered in water to broilers raised under commercial conditions in New Zealand.

5.1. Materials and Methods

5.1.1. Growout houses.

Six growout houses from Company One and five from Company Two were tested in this study. The eleven growout houses housed broilers at a range of ages and were sampled to determine free available chlorine (FAC) concentrations in drinking water at different times of the day and in several locations within growout houses. Ten growout houses were sampled to detect a statistically significant 50% reduction of FAC concentration from the targeted 2ppm (95% confidence level, 80% power, alpha of 0.05, and standard deviations of either 0.5 or 0.75 ppm).

A pilot study involving one broiler growout house was performed to investigate the effect of sampling time and location on FAC. Water samples were collected at three different times throughout the day (10.30 am, 1:00 pm and 3:00 pm) and at multiple locations from different water lines. Locations were chosen to investigate variation both between and within water lines. These results indicated subsequent sampling should occur between 9:30 and 11:00 am to minimise variation in measured FAC due to sampling time.

The farms were supplied with water from a single bore or a well on site. Each growout house had a single header tank, in which the drinking water was stored. The water was divided between four or five pipes (lines) throughout the growout house. Eight growout houses used cup drinkers (five Plasson™ and three Swish™), two used nipple drinkers (both Ziggedy™) and one used a bell drinker. Individual drinkers were placed at uniform distances along each line where water was dispensed on demand.

Within each growout house, water samples were taken from seven to ten different locations with at least one sample taken from every drinker line and five samples taken from one drinker line to observe for a potential drop in FAC concentration along one line as the distance from the header tank increased. Where possible, a water sample was also taken from a tap at the end of a drinker line, as close to the header tank as physically possible (within 5m).

5.1.2. Chlorine Concentration.

The DPD Ferrous Titrimetric (DPDFT) method is applicable to determining total chlorine in polluted water. (1) Under ideal conditions this method will detect 18µg Chlorine as Cl₂/L (i.e. 18ppb). A chlorometer, cuvettes and N,N-diethyl-p-phenylenediamine (DPD)

tablets were used within the growout house to perform the DPDFT (1). Cuvettes were rinsed at the end of each day with 99% H₂SO₄ and then with distilled water. The Palintest 1000 Chlorometer™ was validated using a burette system to titrate a concentration of chlorine in a prepared solution.

Fifty millilitres of water was collected from each drinker. This was allowed to settle for 2 min, to decrease turbidity. A sample of 10ml was removed and tested by crushing a DPD tablet into the water, measuring the concentration of FAC by measuring the amount of light absorbed by the sample at 510nm.

Over three weeks, eleven growout houses were tested in a similar fashion with birds of different ages (five-35 days), six growout houses from one company and five from another company. One growout house from Company One that did not chlorinate drinking water was included in the study as a negative control (Growout house F) (7).

The duplicate FAC concentrations were averaged at each sampling site and the sampling sites averaged within each growout house. Box plots were used to indicate the variation in values obtained within each growout house and between growout houses (Figure 3). A one-way analysis of variance was used to determine the effect of growout house and site within growout house on drinking water FAC concentration. The Levene statistic accepted variances of FAC concentration measurements between growout houses were equal. The FAC concentrations from tap samples were excluded from the analysis as the broilers do not have direct access to this water. In addition FAC concentrations from the growout house that did not add any chlorine to the drinking water were also excluded from the analysis. Values of $P < 0.05$ were deemed significant. Analyses were performed using SPSS version 11 for Windows (SPSS Inc. Headquarters, 233 S. Wacker Drive, 11th floor Chicago, Illinois 60606) and NCSS2000 for Windows (NCSS Statistical Software, 329 North 1000 East, Kaysville, Utah 84037).

5.2. Results

5.2.1. Temporal Differences in FAC Concentration.

Three sites along one line (one closest to the header tank, one furthest from the header tank, and one in between) and two sites (each from the middle of other drinker lines) in one growout house were monitored three times in one day. There was no significant difference between different sites within a growout house ($p=0.75$). Chlorine concentration did not significantly increase with time (Figure 6).

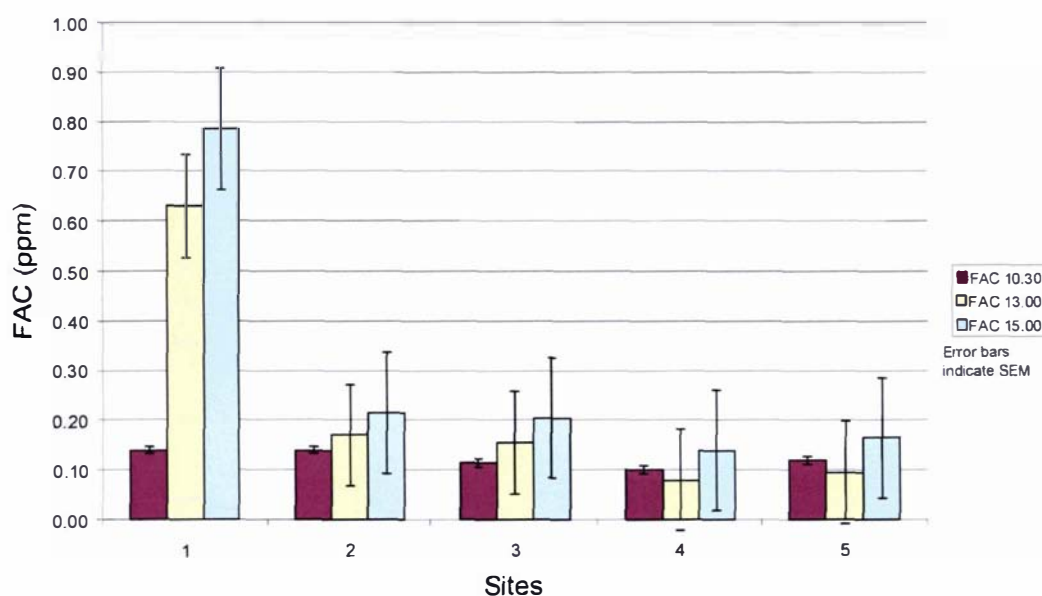


Figure 6. Average Free Available Chlorine (FAC) concentration at 5 sites in one growout house at different times of the day (Error bars indicate 95% CI)

There was less variation in the morning readings compared to the afternoon readings (standard error of the mean for morning: 0.008, compared to 0.102 and 0.121 for the two afternoon readings). The coefficient of variation was used as a comparative measure of variation between sampling times. In the morning reading, the coefficient of variation was 13.8%, whereas at 1pm it was 101.3% and at 3pm 90.1%. Free available chlorine concentrations were the least variable at the first sampling in the morning. To avoid potential variability associated with time of sampling, all samples in the remaining ten growout houses were collected as close to 10:30 am as possible.

5.2.2. Spatial Differences in FAC Concentration.

Mean growout house FAC concentrations in drinking water were consistently below the 2ppm target recommended by Company Two. There was also considerable variation within each growout house. The tap concentrations were consistently higher than those seen in the drinkers, more than 2.8 standard deviations from the mean. Tap results were therefore excluded from analyses of drinker FAC concentrations since they did not represent water available for consumption by birds and the results appeared to be outliers with respect to samples taken from bird drinkers.

Free available chlorine concentrations were different between growout houses ($p < 0.001$), FAC concentration was below 1ppm in all samples tested from drinkers, and mean FAC concentrations exceeding 0.2ppm were found in only three of the growout houses tested (Figure 7).

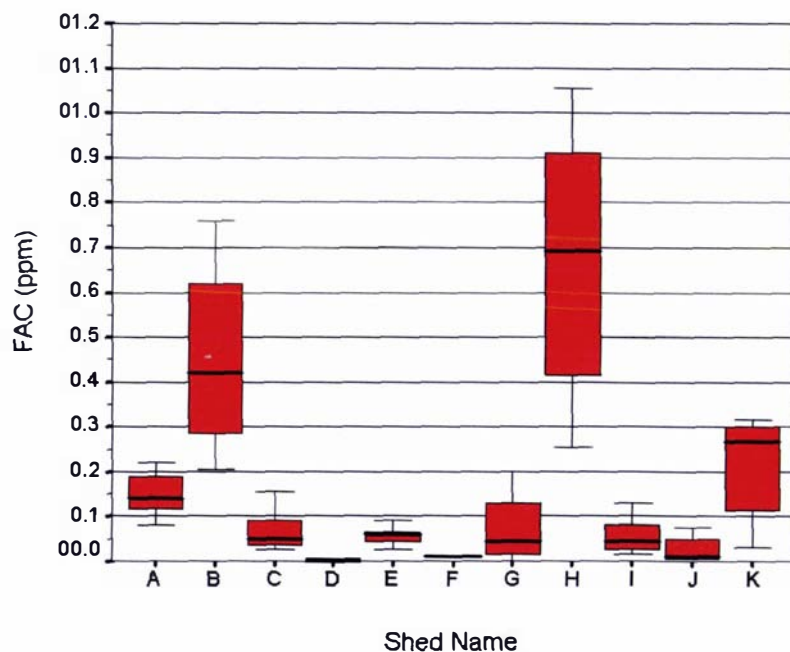


Figure 7. Box Plot of Free Available Chlorine (FAC) measurements within each growout house. The horizontal line in each bar indicates mean FAC measurement.

5.3. Discussion

Five of the eleven growout houses tested were attempting to reach a company target concentration of 2ppm FAC in drinking water. Five growout houses were attempting to provide 'some' chlorine to the broilers, and the remaining growout house was providing unchlorinated water. None of the ten chlorinating growout houses tested reached the 2ppm target concentration in any sample. Three of the ten chlorinating growout houses achieved the FAC concentration of 0.2ppm advised for human drinking water (24).

There did not appear to be any effect of distance from the header tank on the FAC concentration in the drinkers. Greater variation was observed between the measurements of FAC concentrations taken during the afternoon compared to the morning measurements. This may be due to an increased usage of drinkers in the afternoon, as light and temperature in the growout houses increase slightly. The increase in drinker use may 'pull down' more water from the header tank, resulting in fluctuations of FAC concentrations in drinkers. The timing of lighting schedules may also affect this; broilers drink more while the lights are bright, which occurred in the afternoon.

A number of factors may explain the inability to achieve the targeted FAC, including incoming water quality (presence of inorganic and organic solids), drinker type, delivery system (metal or plastic) and whether sufficient chlorine solids were added to the water. Two growout houses used nipple drinkers, eight used cup drinkers and one used a bell drinker system. While the difference in FAC concentration between different drinker types was insignificant ($p=0.163$) it is interesting to note that there was a trend for a higher mean for the nipple drinkers (0.26ppm) than for the cup drinkers (0.17ppm). This supports results from other studies where nipple drinkers reportedly maintain higher levels of FAC in drinking water than cups, and cup drinkers maintain higher levels than trough/bell drinkers (23).

In this study the drinker lines had higher concentrations of free available chlorine than did the drinkers. Often, the main water supply joined the line 1m away from the tap. There may have been a build up of water within this 1m space and the decreased water demand in that part of the line close to the tap may have affected the FAC measurement at the tap. Biofilms may also have been present in the drinker lines which would reduce the FAC present in the broilers drinking water (19). Care should be taken when interpreting FAC concentrations measured from taps within growout houses as this provides an overestimate of chlorine concentration and may not represent that being delivered to the birds.

Chlorine destroys bacteria and various micro-organisms by interfering in the functioning of the organism. Organism death results from a chemical reaction of HOCl with the enzyme system triosephosphate dehydrogenase – essential for digesting glucose (6). The disadvantages of using chlorine as a disinfectant are its corrosiveness, its inactivation by organic matter, and its tendency to decompose (10). FAC concentrations from 0.05 to 5.0ppm will kill a range of vegetative bacteria within 15 seconds to 5 min. In good quality water, less than 0.5ppm is adequate, whereas as much as 20ppm may be necessary in heavily contaminated water. For drinking water, FAC concentrations of 0.2-0.4ppm is considered sufficient to give satisfactory action after satisfying the chlorine demand of water (8, 24).

This study has shown that broiler chickens on commercial farms in New Zealand are rarely supplied with similar free available chlorine concentrations as humans. It also demonstrates the differences seen when taps are used to measure FAC concentration instead of the drinkers. This has validated the exclusion of drinking water chlorination as a risk factor for the transmission of *Campylobacter jejuni* within a broiler flock, by demonstrating insufficient chlorination of drinking water provided to the commercial flocks included in this study. This information should prove useful to the managers of broiler farms, and will encourage more accurate measurement of the FAC concentration in water available to commercial broiler chickens.

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GENERAL DISCUSSION

General Discussion

All this will not be finished in the first hundred days. Nor will it be finished in the first thousand days, nor in the life of this administration, nor even perhaps in our lifetime on this planet. But let us begin.

JOHN F KENNEDY

The field of research into the relationship between *Campylobacter* spp. and the broiler chicken has grown rapidly over a short period of time, and a wide geographical space. The first recognition of the possibility that humans may become infected by consuming contaminated poultry products was published in 1974 (218), since then, many thousands of dollars have been utilised in an attempt to determine how broilers are infected by *Campylobacter* spp., what routes the bacteria take to enter the broiler population and whether or not these routes can be disrupted to reduce the risk of human exposure to the bacteria, and consequently, the risk of human disease.

The scientific community researching *Campylobacter* in broiler chickens is divided almost equally into those who believe vertical transmission occurs, and those who think it highly unlikely. Many flocks of broilers that remain uncolonised by *Campylobacter* spp. have been the progeny of breeder flocks that were colonised by the bacteria, demonstrating the low probability of vertical transmission in the field (246). The fact that broilers are seldom found to be colonised (shedding) by *Campylobacter* spp. until 14 days of age or older consolidates this theory, though it has been demonstrated under test conditions that 50% of broilers inoculated at one day of age will shed the bacteria within 24 hours (4). There is some evidence for vertical transmission: under laboratory conditions, eggs can be penetrated by *C. jejuni* (50, 102, 158), and some researchers propose that the lack of strain diversity exhibited by *C. jejuni* in broiler chickens indicates that vertical transmission occurs, perhaps more often than horizontal transmission or contamination (176). This is a fundamental assumption preceding the objective that broiler chickens can be grown free of colonisation by *C. jejuni*. The importance of distinguishing between potential routes is paramount, and as the debate continues, evidence accumulates in support of horizontal transmission.

If horizontal transmission or contamination is the primary route by which a poultry flock is exposed to *C. jejuni*, then a closer inspection of the growing process is required. There

are many different periods of growth in the relatively short life span of the broiler; different feeding schedules, light patterns, environments, not to mention the stress that broilers experience as each of these regimes or environments changes. The hatchery, considered a reservoir for *C. jejuni* has been examined closely by many scientists, yet is seldom implicated in transmission, thereby failing to meet the definition of a 'reservoir' (89, 103, 119). In addition, fomites associated with chick delivery have been rigorously sampled, yet *Campylobacter* spp. were not isolated (79). It is not intended that this collection of studies be used to distinguish between - or quantify the likelihood of - horizontal and vertical transmission. Instead, the underlying premise supporting this research is that horizontal transmission or contamination occurs is the primary means by which New Zealand broilers are exposed to *C. jejuni*, and that the birds are rapidly colonised subsequent to the initial introduction of the bacteria. The downstream objective this research supports the theory that measures taken to delay or prevent colonisation will have a flow-on effect through the poultry production process; potentially reducing the number of carcasses contaminated with *Campylobacter* spp. cells in the human food supply and decreasing the human risk of exposure to the organism.

Prior to this research, no routine assessment had occurred to examine the prevalence of colonised broilers, colonised flocks of broilers or farms used to house colonised flocks of broilers in New Zealand. As such, prevalence estimates were required immediately, as the effect of future preventative measures these estimates.

Commercial broiler flocks in New Zealand are raised differently to those overseas. In the UK (Newell, personal communication), some flocks are raised on wire mesh floors. This has the advantage that droppings pass through the floor and are less likely to be pecked. Also, it decreases the moisture level of the underfoot material for the chickens, *Campylobacter* spp. prefer damp environments. In Quebec (Nadeau, personal communication), sheds are not placed next to one another on a farm, but are stacked vertically above one another. Chicks are placed in from one direction and are removed from the other end of the collective sheds, but the farmer has very little interaction with the birds during the rearing period. In the US (Line, personal communication) birds are reared on litter that has been used by the previous flock, so the all-in-all-out manner of shed clearing is not attributable to the US situation. In New Zealand, farmers conduct at least two 'walk-throughs' per day to ensure the health and safety of the birds, remove the carcasses of those that have died, and monitor weights to ensure good growth. At the end of a run, following the removal of all of the birds, all sheds in this study were emptied entirely, most were thoroughly cleaned (including feeder and drinker lines) and left to

'stand down' for a minimum of two days before the new flock was introduced. These differences in broiler rearing practices indicate that the proportion of broilers and flocks of broilers that were colonised with *C. jejuni* may also differ between countries.

The calculated true flock prevalence in the study population in the North Island of New Zealand (33%) appears to be comparable to that found in other industrialised countries, where 18-76% of flocks contained birds colonized by *C. jejuni* at the time of testing (see Table 10).

Country	Year	Place of Sampling	Sample tested	Percentage Positive	(n)	Reference
US (California)	1983	Processing Plant	Ready to Market Skin	68%	552	(259)
			Caeca	60-100%	552	
Finland	1988	Processing Plant	Caeca	24%	490	(7)
		Farm	Faeces	1.70%	600	
Poland	1990	Processing Plant	Skin	80.30%	839	(130)
England	1993	Processing Plant	Flocks (caeca)	76%	49	(99)
Norway	1993	Processing Plant (transport crates)	Flocks (cloacal swabs)	18%	176	(117)
Sweden	1996	Processing Plant	Flocks (caeca)	27%	287	(20)
US (California)	2001	Processing Plant	Intestines	94%	202	(106)
			Skin	78%	202	
			Crop	48%	202	
England	2002	Retail	Anywhere	83%	241	(112)
			Inside or Outside of Packaging	56%	241	
Northern Ireland	2002	Retail	Fresh Birds	94%	107	(154)
			Frozen Birds	77%	44	
			Ready-to-eat Products	0%	2030	

Table 10. Prevalence estimates in poultry birds or meat around the world, and the methods used to obtain them

Of all similar research conducted overseas, the only almost-comparable study to this research is the study carried out in Norway in 1993, where cloacal swabs were collected from birds in their transport crates after arrival at the processing plant (117). Transport in crates has been shown to increase the number of birds colonised with *Campylobacter* spp. at the processing plant (216). Since the on-farm flock prevalence is 'similar' to that seen in Sweden yet our human rates are dissimilar, it seems highly likely that 'something else' influences the number of human cases in New Zealand.

A flock prevalence estimate of 76%, as seen in England (99) implies a need for control points at the farm level such as increasing and maintaining biosecurity etc. These control points may have an effect such that the risk of human exposure to *Campylobacter* spp via contaminated poultry meat is quantifiably decreased. However, with the moderately low flock prevalence seen in New Zealand, farm control points are not likely to have a large effect on the human disease rates. While the initial foray to estimate the flock prevalence of

commercial broiler flocks in New Zealand prior to the first depopulation yielded a moderately low prevalence (33%) as described above, the following longitudinal study suggested that as many as 58% of flocks were colonised by *C. jejuni* by the time of the final depopulation. The broilers themselves, their food supply and store, their water supply and store, the litter upon which they stood, the boot dip at the entrance to the growout house, the boots used within the growout house and the immediate environment outside of the growout house were tested simultaneously for the presence of *C. jejuni* from 21 days to the final depopulation. This study did not disclose whether or not partial depopulations increased the risk of *C. jejuni* colonisation, though some studies have suggested it does (83). In all growout houses, the broilers were either colonized prior to - or at the same time as - the environment in which they resided. Though the investigators certainly made every attempt to prevent the introduction of *C. jejuni* to the shed containing the birds, there remains a small chance that it occurred during one of these incursions.

This study did not define the prevalence of colonised broilers within the flock, but showed that a contaminated environment occurs at the same time as the colonisation of the broiler chickens by *C. jejuni*. Consequently, it does not repeat work done by others that looked more closely at the food and water within the shed (10, 20, 105, 136, 192, 233), but nor can it fully endorse the findings of Kapperud et al. and Pearson et al. in 1993, showing that contamination of the drinking water followed rather than preceded flock infection (117, 174). This study reinforced the theory that broilers are colonised prior to isolation of the bacteria from the litter, thus suggests that litter becomes contaminated and is not a source of the organism, as first proposed by Gregory et al. in 1997 (79). In this study, *C. jejuni* was successfully isolated from the broilers, then the litter, followed by the gumboots. This supports the theory as described by the same researchers that the organism permeates the birds' immediate environment, including workers' boots (79). The longitudinal study therefore did not present a new answer to the question of whether *C. jejuni* colonises the broilers or contaminates the environment first yet it supports theories proposed by colleagues overseas.

During the course of the prevalence and longitudinal studies, PFGE was conducted on all isolates of *C. jejuni* that survived transportation and freezing. The protocol used differs from the current New Zealand protocol developed for the National Microbial Typing Database; the ladders are different (lambda compared with *Salmonella* Branderup) as are the switch and run times for the procedure. Consequently, the isolates that were typed during this study are not comparable to human and animal isolates collected and typed from the rest of the country during the same time period. During the prevalence study, more than

one strain can have colonised birds within a shed at the same time. Geographically disparate sheds from the two separate companies, contained birds colonised with the same strain of *C. jejuni* which suggested a common source or exposure. Several strains were isolated during the studies, confirming the ubiquitous nature of the bacterium in New Zealand. If horizontal transmission is the predominant method by which broiler chickens are exposed, then exposure events are likely to occur more than once. This would mean that several different strains from the environment would have the opportunity to colonise the chickens. Indeed, this explains how two different strains can be isolated from broiler chickens in the same shed.

The environmental isolates and the bird isolates collected during the longitudinal study from the same shed were indistinguishable clones of *C. jejuni* i.e. they were the same strain. There were differences between sheds containing birds owned by the same company, and each shed was on a different farm, which supports the conclusion that *C. jejuni* strains are ubiquitous in the environment.

Moreover, it has been demonstrated that a *Campylobacter*-negative flock can be grown in the same growout house immediately following the removal of a *Campylobacter*-positive flock (210).

Despite the publication of overseas risk factor studies conducted to observe and quantify associations between risk factors and colonisation by *C. jejuni* (20, 117), the cross-sectional study included in this piece of research was undertaken. It was deemed important to determine whether the differences between the growing conditions of broilers worldwide and those in New Zealand, as previously outlined, altered which risk factors affected New Zealand broilers. Thus, the risk factor study was designed in such a way to document and quantify risks as they change in importance between seasons, given overseas results implying season as a risk (15, 53, 104, 117, 256).

Risk factors identified in this study point to the possible role of improved biosecurity procedures on farm for reducing the risk of *C. jejuni* introduction to the broilers and supports some of the risk factors identified in overseas studies within the farm biosecurity framework (20, 63, 74, 117, 188, 231). In New Zealand, similar to Great Britain, partial depopulations were not associated with flock colonisation during any season (74) rather than being associated with flock colonisation in all seasons in Sweden (20). This factor was not examined in Norway (117) nor in France (188). That partial depopulations are not positively associated with increased risk of infection seems paradoxical, given the results of a recent (2002) study conducted in New Zealand demonstrating that the equipment used to

depopulated a growout house, (including the truck beds, truck wheels, drivers' boots, catchers' boots, pallets, crates, and forklift wheels) were contaminated with *C. jejuni* cells prior to leaving the processing plant *en route* to a shed (186). A similar result was observed in Great Britain in the same year (216). Chlorinating the drinking water supply was protective in New Zealand, especially in winter, but this is a risk factor seldom examined in overseas studies, so our result cannot be compared to that of other researchers. Other protective factors included shed and farm construction factors, to the extent that recommendations can be made as to how sheds should be constructed on new farms in the future, and how they could be run to minimise the risk of broiler exposure to *C. jejuni*.

Biosecurity includes management practices that decrease the risk of flock exposure to unwanted colonisation, i.e. colonisation by *Campylobacter* spp.. Several general biosecurity measures must be taken; including keeping equipment and materials from individual flocks apart, and keeping unfamiliar people who may carry disease away from the broilers. Vermin and insects should be controlled. Large flocks are generally housed in large sheds on large farms (i.e. a large farm may have 6 large sheds). To maintain low operation costs, machinery from one shed may be utilised in other sheds (such as 'catching pens' etc). To fit a time constraint, an individual conducting a walk through of several sheds consecutively may be less rigorous in adhering to the biosecurity guidelines pertaining to clothing and fomites. Thus transmission of *Campylobacter* spp. from one shed to another can occur.

At the beginning of the flock's lifetime, it is possible that several placements will need to be made to fill the stock for that shed (i.e. three truck loads). Sometimes these placements will occur on separate days due to the demands on the hatcheries and layer flocks. When the doors open for the second placement, some chicks may leave the shed briefly, thereby coming into contact with the external shed environment, which has been proven to have been contaminated with a strain of *C. jejuni*. This creates an opportunity for *C. jejuni* to colonise at least one chicken.

The validity of the farmer interview data was examined, one specific variable was validated: chlorination of the flock drinking water. The study demonstrated that the industry guidelines for the rearing of broiler chickens are unlikely to be met by a large proportion of the growout houses. It is extrapolated that only 45 (20%) of all growout houses involved in this piece of research were likely to be meeting industry guidelines for appropriate levels of drinking water disinfection. Indeed there are many guidelines set by the industry as to appropriate fomite control during the growout stage, yet on several occasions this researcher witnessed the entrance of farm personnel into the growout house without first changing their clothes, dipping their boots or washing their hands. So although several

studies worldwide have demonstrated that enhanced farm biosecurity via farm management alterations may cause a reduction of *Campylobacter* positive flocks sent to the processing plant, enforcing those biosecurity measures will be a formidable task.

Following the results of European case-control studies, some researchers have ploughed ahead and observed the efficacy of the intervention measures implicated by these studies. In 2001, Gibbens *et al.* demonstrated that the risk of thermophilic *Campylobacter* spp. infection of broiler flocks under commercial circumstances in Great Britain was reduced by 50% in intervention flocks following the application of specific hygiene and disease security measures. Of these, the most important hygiene measures were twice-weekly replenishment of boot dip disinfectant and thorough cleaning of the houses between flocks (74). The practice of boot dipping was not seen to be a protective factor in New Zealand in the multivariate model built. Other researchers have focussed on different methods to decrease the likelihood of horizontal transmission or contamination, such as using probiotics to reduce the colonisation and frequency of faecal shedding of *C. jejuni* by American broilers (155) and acidifying the litter used in American commercial poultry production farms (138). Yet more have focussed more on cross-contamination prevention within the processing plant, in order to reduce the number of carcasses that leave the plant contaminated with *C. jejuni* (149, 171). In addition, considerable effort has been dedicated to the theory of competitive exclusion; infecting the chickens with microflora so that *Campylobacter* spp. cannot inhabit the intestinal tract of the broiler chickens (227, 228).

The major limitation of the prevalence study is the number of birds we were able to test. Due to logistic and financial constraints, it was impossible to test a greater number of birds, sheds or farms. Although a prevalence estimate was obtained, the confidence intervals are very wide. The moderately low prevalence dogged the remaining studies - as a large number of sheds had to be included in each sampling plan for each study, to ensure positive sheds were included. The large number of sheds required meant that each flock could not be more rigorously sampled, as would have been preferable. If this study were conducted again, with new molecular methods (121) that can detect the presence of *Campylobacter* spp. in poultry faeces within 4 hours of sampling, then it is conceivable the studies would have been more rigorous, and the prevalence estimates may be different but will definitely be more robust, only affected by the intra-class correlation. The bird prevalence estimate would also benefit from these new molecular identification techniques, as a positive shed could quickly be identified, and intensively sampled to provide more data for the bird prevalence estimation closer to the depopulation event after the maximum period of risk.

The longitudinal study was conducted to observe a contaminated environment prior to colonised birds. This would have indicated the predominant horizontal transmission route by which *C. jejuni* was entering the broiler environment. While we proved that the entire environment becomes contaminated, it appeared to occur either at the same time or after the broilers were colonised. Again, the small sample size does not allow us to say with certainty that the environment is not contaminated prior to the colonisation of the chickens. All we can conclude is that once the chickens are colonised with *C. jejuni*, the environment is quickly contaminated as well.

The cloacal sampling procedure used in both the prevalence and longitudinal study was not robust. Samples collected following a positive sampling event occasionally produced a negative result. This generates doubt around the results shown to date. During the risk factor study, financial and logistic resources were insufficient to continue sampling sheds using cloacal sampling on the farm, such that a new method was trialled: pooling caecal contents that had been collected at the eviscerating stage of the processing plant. When tested against the cloacal method used previously, the caecal method appeared to be half as sensitive again. Twice the number of sheds were positive when sampled using the cloacal method as were positive when using the pooled caecal sampling method. However, the volume of cloacal testing was too great to be conducted for a long period of time as was necessary during the risk factor study. Misclassification was considered to be unbiased such that results seen would be valid, though the OR's calculated using the results would be an underestimate.

Perhaps the most straightforward and conclusive study was the last one, testing the levels of FAC in the drinking water in the sheds. The water was not tested for the presence of *Campylobacter* spp., but evaluated chemically on site using a hand held spectrophotometer set to measure absorbance at 510nm (a chlorometer). Results obtained on the first day were analysed to pinpoint the ideal time of day at which to evaluate the sheds. Though the sampling procedure did not allow a direct correlation to be made between the FAC in the water and the likelihood of the water being the route of *Campylobacter* spp. transmission. It was surprising to note that the FAC in the drinking water was insufficiently concentrated to kill *Campylobacter* spp. cells. Research into post-processing reduction of *Campylobacter jejuni* is occurring elsewhere. Freezing contaminated poultry meat has been a successful intervention, reducing the number of people ill with campylobacteriosis in Iceland (231). It is still acknowledged that if the reduction of flocks colonized by *C. jejuni* occurs on the farm prior to processing, the risk of *C. jejuni*-contaminated poultry products entering the food chain should be reduced even further.

It is hoped that the research presented in this thesis will produce offspring research, perhaps carried out by the industry. Suggestions are: a comparison of the epidemiology of *C. jejuni* in broiler flocks between the farms studied in this thesis and: organic farms, farms in the south island, and farms of other companies. Currently, the industry does not have the logistical or technical capacity to carry this out, and it is hoped that this thesis will provide industry stimulus to engender such capabilities. One difficulty faced during these studies was the die-off of *C. jejuni* cells in transport. Laboratories in the field are ill equipped to store *C. jejuni* isolates for a long period of time, lacking -70°C freezers and appropriate storage broths. Transport swabs worked if the isolates were freshly streaked, but required a recipient ready in Palmerston North. It is recommended that the field laboratories invest in storage capacity, both for future work, and to assure safety of the product being processed. Employing staff with the technical capacity for molecular techniques, purchasing equipment to support these new methods and outlining an agreement with a reference centre to strain-type a representative sample of poultry strains each year for the National Microbial Typing Database would enhance our national ability to link the risk of human exposure to *Campylobacter* spp. with the strains observed in the poultry population.

Before this can happen, however, a standardised sampling procedure must be devised – such that difference seen between enterprises, companies and islands are not obscured by the application of different sampling techniques. It would also appear that farm management alterations are unlikely to have a large effect on the moderately low shed prevalence estimate presented in this research. Consequently, it is advisable to concentrate efforts on minimising cross-contamination within the processing plant, as the size of this effect on the human campylobacteriosis rates may be more appreciable.

Though horizontal transmission is generally considered the most significant cause of *C. jejuni* infection in broiler flocks; there is a paucity of reproducible observations, confirmed by genotyping, whereby strains in the environment have subsequently colonized broiler flocks. The collection of such observations is significantly hindered as *Campylobacter* spp. are ubiquitous in the environment, and *C. jejuni* can colonize transient populations of wild animals or birds that may never be sampled during the growout period of a colonized flock. It is hoped that the development of worldwide networks of genotyping data (e.g. PulseNet Asia-Pacific etc.) will prove instrumental in assisting the development of agent-based intervention methods (i.e. vaccines, competitive exclusion etc.).

It would be of great value to the public health community to determine the exact proportion of cases of campylobacteriosis that are caused by the consumption of

poultry meat, although the intricacies inherent in human case-control studies (e.g. recall bias etc.) will undoubtedly influence future attempts as they have those in the past. Furthermore, which sectors of the public are most at risk from infection by *C. jejuni* is not well understood. There are some indications that rural populations have a higher rate of campylobacteriosis than urban populations, but why this is so is as yet undetermined. As well as defining the population most at risk, it is important to quantify the risk of exposure in comparison to the risk of disease when considering poultry meat as a human source. It is reasonable to assume that urban dwellers have a higher risk of exposure to chicken skin, for instance, than to the faeces of dairy cattle, but these differences must be quantified in a risk management framework.

The extent of *Campylobacter jejuni* colonisation of commercial broiler flocks grown by two companies in the north island of New Zealand has been established by this piece of research, and it is possible that parts of this study may be replicated to determine whether the conditions elsewhere in New Zealand are similar. The information from this thesis can be used by the Poultry Industry Association of New Zealand to develop management practices, ascertain the efficacy of the enforcement of these practices and hopefully reduce the number of flocks that enter the food chain colonized by *C. jejuni*.

Campylobacteriosis is not a problem that will be easy to solve. The reduction of *C. jejuni* in the food chain will involve a myriad of intervention strategies, all of which must be owned by the stakeholders most involved. Such ownership may increase the likelihood of their success, especially as several are likely to involve enhanced biosecurity standards that are difficult to sustain in the farm environment. The challenge for the future is to reduce the overall health burden of campylobacteriosis in New Zealand via all means possible, which will include reducing the introduction of *C. jejuni* into the food chain via poultry meat.

**REFERENCES FOR LITERATURE REVIEW AND
GENERAL DISCUSSION**

References for Literature Review and General Discussion

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APPENDIX A

INTERVIEW FORMS FOR

POULTRY FARMERS

Chicken Questionnaire

1) IDENTIFICATION OF FARM

Name: _____

Company Name: _____

Address: _____

Phone/Fax: _____

Best time to Ring: _____

Flock Size (Today)

Shed	A	B	C	D	E	F	G	H
Number of Females								
Number of Males								

How many days does it take to place all of the sheds? _____

(days)

(a) On-site Enterprises

Are there any other enterprises on the farm (please tick) and indicate the proximity (m) to the broiler houses.

Sheep _____ Beef _____ Deer _____

Dairy _____ Crop _____ Wild animals _____

Other domestic grazing _____

(b) On-site Details

How many people live on the farm? _____

How many people work on the farm and live off? _____

How many sheds (poultry) are on the farm? _____

How far to the next poultry farm? (kms) _____

What type of farm is the nearest poultry farm?

- Great Grandparent Grandparent Parent (breeder)
Broiler Turkey Egg
Don't know

2) MANAGEMENT AND HYGIENE

(a) Litter Material

- Wood Shaving Paper Mixed

Please name the origin of the litter: _____

Is the dirty litter kept for any length of time near the shed?

- Yes
No

(b) Shed Construction

Is there a concrete nib wall?

- Yes
No

Please indicate the predominant shed material

- Wood Sandwich Panel Wood Fibre
Brick Concrete Other _____
r _____

(c) Shed dimensions

Please describe the dimensions of the shed

h _____ w _____ l _____ (m)

(d) Heating and Ventilation

Please provide details of the heating and ventilation system used

Type of heaters used

Number of heaters used

Type of fans in use

Number of fans in use

Shed shape (tunnel/conventional)



3) NUTRITION & HYGIENE

(a) Drinker management

Number of birds per drinker

Distance of drinking track per bird (cm)

Type of drinker?

- Nipple Nipple with Splash Cups
tray
Trough Bell Other
r _____

(b) Origin of Water

From where does the water in the sheds come?

- Town Supply Spring River
Borehole Lake Stream

Other _____

(c) Water Treatment

With what is the water treated?

Nothing

Treatment

Where does this treatment take place?

Before water enters tank

Before water enters shed

If the water is chemically sanitised, how often is the level checked?

Never Once a Year Once a Flock Once a month

Once a fortnight Once a week Several Times a week

(d) Microbiology

How often is the water checked for bacteria?

- Never Once a Year Once a Flock Once a month
Once a fortnight Once a week Several Times a week

(e) Feed Management

Number of birds per feeder

Distance of feed track per bird (cm)

How is feed provided in early breeding?

- Paper
Feed pans

What type of feeder is used after this?

- Pan
Dutchman

(f) Please provide details of feed storage system.

Number of silos per shed

Is feed accessible to rodents/birds

- Yes
No

Is the hopper open or closed?

- Open
Closed

Is the feed delivery system enclosed all the way to delivery?

- Yes
No



4) BIOSECURITY

(a) Pest Control

What method of insect control do you currently employ?

How often do you use insecticide?

Insecticide used

Application method (spray, soak etc)

Did you insecticide before this run

Yes

No

Have you seen litter beetle or any other insect in this run?

Yes If 'yes', what insect did you see? _____

No

Have there been any signs of rodent activity around or in the shed in this run?

No

Yes

If 'yes', can you identify which rodent/s these were?

(b) Entrance

What is the procedure to enter the shed where the birds are? Please tick if yes and describe e.g. Overalls, Disinfectant used, Soap used, Disposable or Material Towel, Gardening)

Change of Clothes	<input type="checkbox"/>	_____
Change of Boots	<input type="checkbox"/>	_____
Boots dipped	<input type="checkbox"/>	_____
Hands Washed	<input type="checkbox"/>	_____
Hands dried	<input type="checkbox"/>	_____
Gloves Worn	<input type="checkbox"/>	_____
Other	<input type="checkbox"/>	_____

Are the paths to and between the sheds hard (concrete/gravel) or soft (dirt)?

To		Between	
Hard	<input type="checkbox"/>	Hard	<input type="checkbox"/>
Soft	<input type="checkbox"/>	Soft	<input type="checkbox"/>

How often is the annex sanitised (days) and what with (Brand name)?
 every _____ (days) _____TM

Birds

How often are the following birds seen within 100m of the sheds?

Species	Never	Rarely <i>Approx. once a year</i>	Sometimes <i>Approx. once a month</i>	Frequently <i>Approx. once a week</i>
Seed eaters (<i>Mynas, Starlings, Sparrows</i>)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Game Birds (<i>Pheasants, Quail</i>)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Scavengers (<i>Gulls, Magpies, Pukeko, Blackbirds</i>)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Waterfowl (<i>Ducks, Geese, Swans</i>)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Other _____	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

(c) If there is anything else on your farm that you feel may contribute to the presence of Campylobacter in the broilers, please let us know here.

(d) If there is anything further you wish to see being done by the company that owns the broilers, please let use know here.
