CLASSICAL AND MOLECULAR EPIDEMIOLOGY OF *CAMPYLOBACTER*, IN PARTICULAR *CAMPYLOBACTER JEJUNI*, IN THE ALBERTA BEEF INDUSTRY

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ABSTRACT

This research used classical and molecular epidemiology tools to assess the potential importance of feedlot cattle as *Campylobacter* reservoirs. The project was conducted from November 2004 to September 2005 in southern Alberta.

Fresh pen-floor fecal samples were collected from commercial feedlot cattle near slaughter weight in seven feedlots. Overall, 87% of 2,776 fecal samples were culture positive for *Campylobacter* species (86% of 1,400 in winter, 88% of 1,376 in summer), and 69% of 1,486 *Campylobacter* positive isolates were identified as *Campylobacter jejuni*. After accounting for clustering within pen and feedlot, the number of days-on-feed and feedlot size were associated ($p \leq 0.05$) with *Campylobacter* species isolation rates.

Retail ground beef was collected from 60 grocery stores (four chains, three cities). None of the 1,200 packages were culture positive for *Campylobacter* species. Polymerase chain reaction (PCR) results from a subset of samples (n=142) indicated that 48% of packages were positive for *Campylobacter* DNA. By species, 14.8% (21/142), 26.8% (38/142) and 1.4% (2/142) of packages were PCR positive for *C. jejuni, C. coli* and *C. hyointestinalis* DNA, respectively. The collection period (1, 2, 3 or 4) was associated ($p \leq 0.05$) with the odds of detecting *Campylobacter* species DNA using PCR.

Oligonucleotide DNA microarrays were used as a platform for comparative genomic hybridization (CGH) analysis of 87 *C. jejuni* isolates (46 bovine, 41 human) obtained within the same geographical regions and time frame. Of the 13 CGH clusters identified based on overall comparative genomic profile similarity, nine contained

human and cattle isolates, three contained only human isolates, and one contained only cattle isolates. In addition, human clinical and feedlot cattle *C. jejuni* isolates were compared on a gene-by-gene basis and only a small number of the 1,399 genes tested were unequally distributed between the two groups ($p \leq 0.05$).

The high isolation rates of *Campylobacter* species and *C. jejuni* reported here may have implications for food safety, public health and environmental contamination. Our findings suggest that feedlot cattle and human *C. jejuni* strains are very similar and may be endemic within southern Alberta.

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DEDICATION

This manuscript is dedicated to my husband, Sean Hannon, who walked beside me on this journey.

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AB	Alberta
AFLP	amplified fragment length polymorphism
APLPH	Alberta Provincial Laboratory of Public Health
CA	California
CGH	comparative genomic hybridization
C. jejuni	*Campylobacter jejuni*
C. coli	*Campylobacter coli*
CE	"cattle enriched"
CI	confidence interval
CS	coding sequences
CY	Cyanine
DNA	deoxyribonucleic acid
GBS	Guillain-Barré syndrome
HACCP	hazard and critical control point
HE	"human enriched"
HSD	Holm step-down
lbs	pounds
mCCDA	modified cefoperazone charcoal deoxycholate agar
MA	Massachusetts
MB	Manitoba
MN	Minnesota
MFS	Miller Fisher syndrome
MLST	multi-locus sequence typing
mod.	modification
mPCR	multiplex polymerase chain reaction
NC	North Carolina
NH	New Hampshire
NI	Northern Ireland
NV	Nevada
NZ	New Zealand
ON	Ontario
ORF	open reading frame
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
pmol	picomol
put.	putative
QC	Québec
RFLP	random fragment length polymorphism
RHA	regional health authority
RR	relative risk
RTQ PCR	real-time quantitative polymerase chain reaction
SK	Saskatchewan
spp.	species

TX	Texas
UK	United Kingdom
USA	United States of America
VIDO	Vaccine and Infectious Disease Organization
WI	Wisconsin
WY	Westfall and Young correction

CHAPTER 1
OVERVIEW

1.1 Introduction

Campylobacters are ubiquitous bacteria, found in water, in soil, and in the intestinal tract of reservoir hosts (including mammals, birds and insects). Many *Campylobacter* species are zoonotic (transmissible between people and animals), and are important in both public health and food safety. Public awareness of campylobacters as human pathogens has been relatively limited compared to other enteric bacteria. In developing countries, people may become infected much earlier in life and may acquire high levels of immunity (Oberhelman and Taylor 2000), resulting in fewer clinical cases. In developed countries, lack of awareness may be because clinical signs from campylobacteriosis are less severe and mortality rates lower compared to gastrointestinal infections caused by bacteria such as *Salmonella* or *Eschericia coli* O157:H7 (PHAC 2007, Kennedy et al. 2004, Statistics Canada 2007), resulting in less media coverage. In reality however, campylobacters have been identified as the most commonly isolated bacteria in human diarrhea cases in countries in both developed and developing worlds (Friedman et al. 2000, Oberhelman and Taylor 2000), and as such these bacteria have garnered increasing respect and research dollars.

Historically, Escherich was credited as the first to describe spiral bacteria in the stool of clinical human cases in 1886 (Butzler 2004). However, campylobacters were considered veterinary pathogens until 1938 when the first human clinical report of a

milk-borne outbreak was published (Levy 1946), and the major breakthrough was the isolation of campylobacters from human feces in 1968 (Dekeyser et al. 1972). The taxonomy of campylobacters has not been straightforward. The organisms now considered campylobacters began as part of the genus *Vibrio* and were separated into their own genus in 1963 based on both biochemical and genetic differences from *Vibrio* (On 2005). Since then, different species have entered and exited the *Campylobacter* genus as new, and in particular genetic, insights have become apparent (Euzéby 1997, On 2005).

Our understanding of the epidemiology of campylobacters is incomplete and classical epidemiological questions have not been fully answered.

Epidemiological considerations

Who: Which populations and subpopulations of hosts (human, livestock, pet, insect, bird, protozoa) are becoming infected or colonized, and which strains of campylobacters are most important for human disease?

What: What pathogenic mechanisms and genotypic/phenotypic characteristics are associated with virulence, colonization, and infection?

When: Do seasonal effects exist which promote transmission or infection and, if so, are these effects important?

Where: What is the geographical distribution of campylobacters in particular hosts, and what adaptive microenvironments (e.g. biofilm) play a role in the epidemiology?

Why: Why are people becoming infected in such numbers, and can interventions be found to eliminate transmission routes and reduce infection levels?

How: How are these bacteria transmitted between hosts—contact, water, fomite, food, other?

Research into each of these questions is ongoing. Detection of campylobacters requires precise temperature, atmospheric, and nutrient conditions. Further, the

adaptability of these bacteria to real-world conditions e.g. in water, biofilm, and environmental extremes, make it difficult to implement successful interventions. The genetic diversity of strains, including hypervariable regions and plasmids, has made it challenging for researchers trying to identify virulence factors and predict clinical manifestations (e.g. Guillain Barré syndrome). While many gaps in our knowledge of these pathogens still exist, breakthroughs, particularly in molecular typing, are continuing to advance our knowledge.

Below is a general overview of the techniques used in this research and a description of the study objectives and goals.

1.2 Methods

1.2.1 Literature review

General keywords and MeSH terms (Appendix A) were used to search the following databases: Agricola, BioOne, Biosis Previews, Cab Abstracts, Canadian Newstand, EMBASE, Health and Safety Science Abstracts, Microbiology Abstracts, ProQuest Dissertaitions and Theses, PubMed, Scopus, Web of Science and the search engine Google™. The main search was from July 3-16, 2007 followed by weekly publication notifications from PubMed (Appendix A).

1.2.2 Primary research

The contamination of feedlot cattle feces and retail ground beef with campylobacters was assessed through two prevalence studies described in Chapters 3 and 4 of this manuscript. Enrichment culture, hippurate hydrolysis testing, and

polymerase chain reaction technologies were used to detect campylobacters in feces or ground beef. In addition, seasonal (winter and summer) isolation rates of *Campylobacter* species in feedlot cattle feces were also obtained. Associations between risk factors and the presence of *Campylobacter* spp. in both cattle feces and retail ground beef samples were investigated using hierarchical models.

DNA microarray technology was used to describe the genomic profiles of feedlot cattle and human clinical *Campylobacter jejuni* isolates (Chapter 5). Description was based on global clustering and gene-by-gene comparison between the two groups.

1.2.3 Ethics

Ethics approval for this research was received from both the University of Saskatchewan Biomedical Research and the University of Calgary Conjoint Health Research Ethics Boards. An ethics analysis written prior to commencing the research has been included (Appendix B).

1.3 Specific objectives

Targeted feedlot cattle feces survey

1) To estimate the prevalence of *Campylobacter* spp., in particular *C. jejuni*, in fresh fecal samples from commercial Alberta feedlot cattle near slaughter weight
2) To obtain point estimates for *Campylobacter* spp., in particular *C. jejuni*, in commercial feedlot cattle feces
3) To evaluate potential associations between risk factors and isolation rates of *Campylobacter* spp. in commercial feedlot cattle feces
4) To collect isolates for future molecular characterization (DNA microarray)

Retail ground beef survey

1) to estimate the prevalence of *Campylobacter* spp. and *C. jejuni* in retail ground beef from southern Alberta
2) To evaluate associations between potential risk factors and contamination rates of retail ground beef with *Campylobacter* spp.
3) To collect isolates for future molecular characterization (DNA microarray)

Molecular characterization of *C. jejuni* using DNA microarray

1) To describe human clinical and feedlot cattle *C. jejuni* isolates using DNA microarray technology
2) To compare isolates by source population (human, feedlot cattle) and by season
3) To assess the plausibility of cattle as a source of infection to people based on the genomic profiling

1.4 Conclusions

The purpose of this thesis was to address unanswered questions regarding the epidemiology of campylobacters, in particular *C. jejuni*, in the Alberta beef industry. This research was designed to indirectly assess the plausibility that feedlot cattle may be sources of *Campylobacter* spp., in particular *C. jejuni*, to people. The prevalence studies contribute baseline data on contamination of feedlot cattle feces and retail ground beef with these bacteria. DNA microarray, a relatively new molecular technique, was assessed as an epidemiologic tool, and used to describe *C. jejuni* isolates from feedlot cattle and people.

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CHAPTER 2
LITERATURE REVIEW

2.1 Epidemiology of campylobacters—The Classical Triad

2.1.1 Agent

The family Campylobacteriaceae is composed of Gram-negative, nonsporeforming, spiral or curved rod bacteria 0.2 to 0.8 μm wide and 0.5 to 5 μm long. Most species are motile with a single unsheathed flagellum at one or both poles (Vandamme 2000). Within the *Campylobacter* genus, 17 species have been identified including six subspecies and three biovars (Euzéby 1997). *Campylobacter coli, Campylobacter concisus, Campylobacter curvus, Campylobacter fetus, Campylobacter gracilis, Campylobacter hyointestinalis, Campylobacter jejuni, Campylobacter lanienae, Campylobacter lari, Campylobacter rectus, Campylobacter showae* and *Campylobacter sputorum* have been isolated from cattle and people (Giacoboni et al. 1993d, Humphrey et al. 2007, Logan et al. 2000, Yan et al. 2005). *C. jejuni* encompasses two subspecies: *C. jejuni* subspecies *jejuni* and *C. jejuni* subspecies *doylei*. *C. jejuni* subspecies *doylei* has not been found in animal hosts to date, does not grow well at 42°C, and cannot reduce nitrate (On 2005, Parker et al. 2007). Hereafter, *C. jejuni* refers to *C. jejuni* subspecies *jejuni*.

C. jejuni and *C. coli* are considered the most important *Campylobacter* species for public health. Based on selective media culture in developed countries, *C. jejuni* and

C. coli account for approximately 80-94% and 2-15% of human infections respectively (Moore et al. 2005, Nachamkin et al. 2000b, Rajda and Middleton 2004).

In the laboratory setting, campylobacters are usually considered fragile; susceptible to aerobic, acidic, and highly osmotic environments, dessication, and temperature extremes (Humphrey et al. 2007, Murphy et al. 2006). Campylobacters are microaerophilic, surviving optimally in atmospheres with 5-7% oxygen and approximately 10% carbon dioxide (Corry et al. 1995, Leuchtefeld et al. 1982). Thermophilic campylobacters have a narrow temperature growth range of approximately 30°C to 46°C (Humphrey et al. 2007), and thermophilic species relevant to both cattle flora and human infection include *C. coli, C. hyointestinalis C. jejuni, C. lanienae, C. lari*, and *C. sputorum*, with 97-100% of isolates from these species growing at 42°C (Humphrey et al. 2007, On 2005).

Despite their limited temperature growth range, thermophilic campylobacters can be isolated from frozen meats (Moorhead and Dykes 2002, Paulsen et al. 2005) but are killed by pasteurization and adequate cooking; factors important for managing food safety concerns. Campylobacters cannot multiply in concentrations of 2% or greater sodium chloride (Doyle and Roman 1982), do not usually survive well on surfaces (susceptible to dessication) (Fernandez 1985), and are able to persist in aquatic environments and biofilm (Buswell et al. 1998). Interestingly, the presence of food has been found to protect campylobacters usually susceptible to the low pH levels of gastric acidity (Waterman and Small 1998). The above properties indicate the complexity of these enteric pathogens, and their ability to adapt to a variety of extreme environments.

Change in the morphological structure of campylobacters from spiral to coccoid has been observed during environmental stress and in old cultures. The coccoid forms tend to be difficult to subculture and lose motility (Ng et al. 1985). These changes may allow campylobacters to survive in environmentally challenging environments in a "viable but non culturable" (VBNC) state (Oliver 2005, Rollins and Colwell 1986). However conflicting evidence as to the importance of coccoid forms, injured bacterial cells, and the VBNC form exists, with strain-to-strain variation suggested as an explanation (Jones et al. 1991). Some VBNC strains do not display coccoid morphology and were not able to colonize birds (Fearnley et al. 1994), while other injured cells have been found to resuscitate (and in some cases become virulent) after passage through animal and protozoal hosts (Axelsson-Olsson et al. 2005, Jones et al. 1991, Saha et al. 1991). Further, injured strains have also been culturable but not able to colonize avian hosts and have been referred to as "culturable but not infectious" (Hald et al. 2001). The ability of injured strains to be non-culturable and yet revert to virulence with passage through an appropriate host may be epidemiologically important for identification of transmission routes to people (e.g. food and water). While campylobacters are considered relatively susceptible to environmental extremes in the laboratory, the high levels of human infection, and the ability of these organisms to survive in less than optimal real-world conditions (e.g. survive food processing and environmental water) deserve continued research attention.

2.1.2 Host

In people

Campylobacter spp. cause bacterial gastroenteritis in people in both the developed and developing world (Oberhelman and Taylor 2000). Approximately 72-80% of *Campylobacter* infections in Canada and the United States (USA) have been the result of foodborne transmission (Mead et al. 1999, Rajda and Middleton 2004). In people, disease incubation is approximately 1-8 days (mean 3 days) after ingestion, (Skirrow and Blaser 2000) and the infective dose may be as low as 500 cells in contaminated foods (Robinson 1981).

Symptoms in people include fever, headache, muscle pain and vomiting, although diarrhea (watery and often progressing to bloody) and abdominal cramping are the most common clinical signs (Skirrow and Blaser 1995). After onset, clinical signs usually last approximately a week, although prolonged illness and relapse may occur. Rarely extraintestinal sequelae may occur including rash, hepatitis, cholecystitis, pancreatitis, cystitis, septic abortion, reactive arthritis, hemorrhagic uremic syndrome, and bacteremia (particularly in immunocompromised people) (Skirrow and Blaser 2000).

People of any age may be infected, although children under two years of age (developed and developing countries) and young adults (developed countries) may have higher incidence rates (Blaser 1997, Friedman et al. 2000). Severity of clinical signs can be strain dependent and it is possible for people to be infected by several strains at the same time (Black et al. 1988, Richardson et al. 2001).

Due to underreporting issues, it is difficult to estimate human campylobacteriosis rates. Surveillance in Europe and the USA have reported rates of 3 to 167 cases per 100,000 population (1998 and 1999 data) (Friedman et al. 2000, Takkinen et al. 2003). However, rates of 900-1000 cases per 100,000 population (USA) have been suggested after factoring in estimates for underreporting (Friedman et al. 2000, Nachamkin et al. 2000a). Case fatality rates from *Campylobacter* infections have been estimated to be approximately 0.1-0.2% in the USA and Sweden (Kennedy et al. 2004, Mead et al. 1999, Ternhag et al. 2005).

Serious autoimmune disorders related to *Campylobacter* infection include Guillain-Barré syndrome (GBS), its variant Miller Fisher syndrome (MFS), and reactive arthritis (Bremell et al. 1991, Endtz et al. 2000, Nachamkin 2002). *Campylobacter* infection was first postulated as a potential cause of GBS in 1982 (Rhodes and Tattersfield 1982). Approximately 0.3 to 1 per 1000 *C. jejuni* cases lead to GBS, and *Campylobacter* infection has been identified as the most common trigger to GBS (Gilbert et al. 2005, Nachamkin et al. 2000a, Nachamkin 2002, Rees et al. 1995). A slightly higher incidence of GBS has been reported in males compared to females, and GBS can affect patients of all ages (Nachamkin et al. 1998, Rees et al. 1995). In patients with GBS, inflammation and demyelination of peripheral nerves results in acute flaccid paralysis, sensory abnormalities, symmetrical weakness of limbs and respiratory muscles, and loss of reflexes. Treatment may take weeks to months. While complete recovery is possible, up to 25% of GBS patients may require mechanical ventilation and 15-20% may have prolonged or permanent neurological deficits (Briscoe et al. 1987, Kuwahara 2004, Nachamkin et al. 1998, Winner and Evans 1993). In the developed

world, case fatality from GBS has been estimated at 2-10% and may be even higher in the developing world (Beale and Miller 1985, de Jager and Sluiter 1991, Kuwahara 2004, Nachamkin et al. 1998).

Miller Fisher syndrome has also been associated with *Campylobacter* infection in people. This syndrome is characterized by ophthalmoparesis, lack of reflexes, and incoordination without weakness (Nachamkin et al. 2000a). Cases of reactive arthritis post campylobacteriosis have also been reported with duration of recovery lasting from a few weeks to years (Bremell et al. 1991, Skirrow and Blaser 2000). These autoimmune disorders resulting from campylobacteriosis may be debilitating, with prolonged recovery times and significant associated economic burden.

In poultry

Poultry are considered a primary source of *Campylobacter* infection to people (Corry and Atabay 2001, Humphrey et al. 2007, Pearson et al. 2000). In a recent Quebec study, 38.5% of poultry carcasses surveyed (82 broiler chicken flocks, 2,414 carcasses) were positive for *Campylobacter* spp. (Arsenault et al. 2007). Poultry carcass contamination may vary from 10^2 to 10^5 *Campylobacter* cells per carcass and the infective dose in people has been estimated to be as low as 500 *Campylobacter* cells in contaminated foods (Jacobs-Reitsma 2000, Robinson 1981). An interesting study from Belgium (1999) documented a drop in campylobacteriosis corresponding to the removal of local poultry meat from shelves due to unrelated dioxin contamination. In that study, modeling estimated that local poultry meat was responsible for approximately 40% of human *Campylobacter* infections (Vellinga and Van Loock 2002). In Iceland, intensive epidemiological risk factor and intervention studies are underway to study transmission

of campylobacters into poultry flocks in that closed agricultural system (Campy-On-Ice project). Risk factors positively associated with flock or farm *Campylobacter* status were: increasing age and flock size, number of broiler houses on-site, manure spreading on-farm, the presence of vertical or vertical and horizontal ventilation shafts, producer boot disinfection procedures, and the use of geothermal water for broiler house cleaning. Protective practices included the use of treated water, the presence of other domestic livestock and storing manure on-farm (Barrios et al. 2006, Guerin et al. 2007a, Guerin et al. 2007b).

In cattle

The prevalence of *Campylobacter* spp. in cattle feces may range from 5 to 100 % (Giacoboni et al. 1993c, Hoar et al. 1999, Inglis et al. 2003, Inglis et al. 2004). *Campylobacter* spp. prevalence levels have been identified based on the type of production system: dairy cattle 6-64% (Humphrey et al. 2007), pasture cattle 5-55% (Bailey et al. 2003, Beach et al. 2002, Busato et al. 1999, Giacoboni et al. 1993, Hoar et al. 1999), feedlot cattle 20-100% (Hyatt et al. 1998, Inglis et al. 2004, Minihan 2004), Tables 2.1 and 2.2. Beef calves also tend to have high fecal prevalences of campylobacters (20-100%) (Adesiyun et al. 1992, Firehammer and Myers 1981, Giacoboni et al. 1993, Myers et al. 1984). Campylobacters and *C. jejuni* have been commonly isolated from seemingly healthy cattle (Inglis et al. 2004, Inglis et al. 2006), and *C. jejuni* has been implicated as a cause of abortion in western Canadian cattle (Van Donkersgoed et al. 1990).

Table 2.1 Previously published fecal prevalence surveys of *Campylobacter* spp. and *C. jejuni* in commercial and experimental feedlots

Area	Year	Type	C spp. or Cj	# Pos (n)[b]	% Pos	ID	Reference
Australia	1998	COM	C spp. Cj	55(100) 55(100)	55.0 55.0	C B	(Bailey et al. 2003)
Canada	2000	COM	Cj	124(200)[a]	62.2	B,CBH, mPCR	(Besser et al. 2005)[c]
Canada	2002	EXP	C spp. Cj	318(380) 144(380)	83.7 37.9	PCR PCR	(Inglis et al. 2003)
Canada	2002-2003	EXP	C spp.	63(80)	78.7	C	(Lefebvre et al. 2006)
Canada	NR	EXP	C spp. C spp. Cj Cj	268(299)[a] 60(60) 40(299)[a] 19(60)	89.6 100 13.4 31.7	PCR PCR mPCR mPCR	(Inglis et al. 2004)
Canada	NR	EXP	Cj	130(300)	43.3	C, cPCR	(Inglis et al. 2005b)
Canada	1999	COM	C spp. Cj	177(278) 130(278)	63.7 46.8	C B, cPCR	(Inglis et al. 2006)[d]
Ireland	2001-2002	COM	C spp. C spp. Cj	322(600)[a] 107(109) 215(600)[a]	54.0 98.2 35.8	C C B, PCR	(Minihan et al. 2004)
Ireland		COM	C.spp. Cj	62(109) 45(109)	57.0 41.3	C B, PCR	(Minihan 2004)[e]
US	2002-2003	COM	Cj	36(49)	73.5	B, PCR	(Lee et al. 2004)
US	2002-2003	COM	C spp. Cj	46(98)[a] 31(98)[a]	46.9 31.6	C CBH, mPCR,	(Bae et al. 2005)
US	NR	COM	C spp.	64(100)	64.0	C, ELISA	(Beach et al. 2002)[e]
US	NR	COM	C spp.	537(2672)[a]	20.1	NR	(Hyatt et al. 1998)[a]
US	NR	EXP	C spp. C spp. Cj	47(816)[a] 21(51) 44(816)[a]	5.8 41.2 5.4	C C B, PCR	(Berry et al. 2006)

[a] # samples tested; [b] n=# animals tested unless otherwise specified; [c] Final sampling; [d] at exit of feedlot (modified charcoal cefoperazone deoxycholate agar); [e] pretransit to abattoir. B: culture and biochemical testing; C: culture; C spp.: *Campylobacter* species; Cj: *Campylobacter jejuni*; CBH: colony blot hybridization; COM: commercial; ELISA: enzyme-linked immunosorbent assay; EXP: experimental; NR: not reported; PCR: polymerase chain reaction; cPCR: colony PCR; mPCR: multiplex PCR; RTQ PCR: real-time quantitative PCR.

Table 2.2 Previously published fecal prevalence surveys for *Campylobacter* spp. and *C. jejuni* in cattle intestinal contents/feces sampled in abattoirs

Area	Year	C spp. or Cj	# Pos (n)[a]	% Pos	ID	Reference
Australia	NR	Cj	9(30)[b]	30.0	B	(Grau 1988)
		Cj	3(66)[c]	4.5	B	
Canada	2004	C spp.	18(19)	95.0	PCR	(Inglis et al. 2005a)
		Cj	11(19)	57.9	RTQ-PCR	
Canada	NR	Cj	35(100)	35.0	B	(Garcia et al. 1985)
Denmark	1995-1996	C spp.	44(94)	47.0	C	(Nielsen et al. 1997)
		Cj	40(94)	42.5	B	
Ireland	NR	C spp.	60(109)[d]	55.0	C	(Minihan 2004)
		Cj	42(109)[d]	38.5	B, PCR	
		C spp.	69(109)[e]	63.0	C	
		Cj	39(109)[e]	35.8	B, PCR	
Italy	1999-2001	C spp.	27(193)	14.0	C	(Bywater et al. 2004)
Germany		C.spp.	126(191)	66.0	C	
UK		C.spp.	36(72)	50.0	C	
		Cj	141(456)	30.9	B	
Italy	2000-2001	C spp.	48(89)[f]	53.9	C	(Pezzotti et al. 2003)
		Cj	12(89)[f]	13.5	B	
Japan	1993-1997	C spp.	410(648)	63.3	C	(Ono and Yamamoto 1999)
		Cj	325(648)	50.2	B	
Japan	2003	Cj	18(78)	23.1	B, mPCR	(Saito et al. 2005)
New Zealand	NR	C spp.	0(65)	0	C	(Gill and Harris 1982)
Nth Ireland	NR	C spp.	52(210)	24.8	C[i]	(Madden et al. 2007)
		Cj	30(210)	14.3	B	
Norway	1999-2001	C spp.	241(804)[g]	30.0	C	(Johnsen et al. 2006)
		Cj	208(804)[g]	25.8	B	
Sweden	1999-2000	C spp.	NR	16.0	NR	(Blixt et al. 2001)[j]
Switzerland	2002-2003	C spp.	95(935)[h]	10.2	C	(Al-Saigh et al. 2004)
		Cj	95(935)[h]	10.2	PCR	
Turkey	2003	C spp.	35(200)	17.5	C	(Acik and Cetinkaya 2005)
		Cj	9(200)	4.5	mPCR	
UK	1993-1994	C spp.	322(360)	89.4	C	(Stanley et al. 1998c)
US	2001-2003	C spp.	48(252)	19.0	C	(Gharst et al. 2006)
		Cj	48(252)	19.0	PCR	

[a] n=# animals unless otherwise specified; [b] feedlot cattle; [c] pasture cattle; [d] post-transit sample; [e] post-lairage sample; [f] n=# herds; [g] adults and calves sampled; [h] 172/935 feedlot cattle; [i] 37°C; [j] abstract only. B: culture and biochemical testing; C: culture; C spp.: *Campylobacter* species; Cj: *Campylobacter jejuni*; NR: not reported; Nth: Northern; PCR: polymerase chain reaction; mPCR: multiplex PCR; RTQ PCR: real-time quantitative PCR.

In southern Alberta, a body of research on the importance of feedlot cattle as *Campylobacter* reservoirs continues by Inglis et al. This research group developed polymerase chain reaction (PCR) technology to directly test cattle feces for campylobacters, and nested real-time quantitative PCR (RTQ-PCR) to quantify microbial numbers in the feces (Inglis and Kalischuk 2003, Inglis and Kalischuk 2004). In several surveys using these technologies, feedlot cattle in southern Alberta were found to shed campylobacters chronically with 84-90% of samples positive for *Campylobacter* spp., 13-38% positive for *C. jejuni*, and 27% of the fecal samples contained > 10^4 g^{-1} *C. jejuni* cells (Inglis et al. 2003, Inglis et al. 2004). Further, this group established that *C. jejuni* colonizes the proximal small intestine in healthy cattle (Inglis et al. 2005a). Antimicrobial resistance patterns in southern Alberta feedlot cattle have also been described, including both prevalence and development over time. Development of antimicrobial resistance in *C. jejuni* isolates to ampicillin, azithromycin, ciprofloxacin, enrofloxacin, erythromycin, gentamicin, meropenem, and naladixic acid over time were limited, while tetracycline and doxycycline resistance tended to develop quickly and to high levels over the feeding period (Inglis et al. 2005b, Inglis et al. 2006).

Cattle contact, occupational exposure to cattle, feces, or farm animals (Eberhart-Phillips et al. 1997, Kapperud et al. 2003, Neimann et al. 2003, Studahl and Andersson 2000), barbecuing red meat, and consuming undercooked red meat (Adak et al. 1995, Neimann et al. 2003) have been identified as factors increasing human risk of *Campylobacter* infection. Further, ruminant or cattle density have also been associated with infection (Louis et al. 2005, Nygard et al. 2004). Alternatively, other studies have

found no association with cattle contact (Kapperud et al. 1992) or consuming red meat (Kapperud et al. 2003, Schonberg-Norio et al. 2004, Studahl and Andersson 2000). One study interestingly found occupational contact with livestock or feces protective (Adak et al. 1995).

A 2004 study examined antimicrobial risk of pathogens (nasal and rectal swabs) from Alberta feedlot personnel and from animal care workers at the Lethbridge Research Station. Sixty-one people were tested at the beginning of the study and 46 were retested nine months later. None of the participants were found to be shedding campylobacters. For inclusion in the study people had to be 18 years old, employed by and work at one of four feedlots or at the Lethbridge Research Station, and give informed consent (Read et al. 2004). It was not specified in the design that the employees worked directly with cattle, and it is possible that volunteer bias might apply. In addition, no serological evaluation for *Campylobacter* antibodies (indicating previous exposure) were carried out.

Other potentially important reservoirs

Campylobacters may be carried by or infect a variety of wild mammals, birds and insects (Fallacara et al. 2001, Szalanski et al. 2004, Wahlstrom et al. 2003). Companion animals are known to be chronic carriers and can be infected with multiple *Campylobacter* spp. at the same time (Hald et al. 2004, Koene et al. 2004). While the transmission of campylobacters from pets to people is of public health concern, particularly in immunocompromised people, children, and the elderly, the ability of people to transmit campylobacters to pets is also worthy of note (Damborg et al. 2004).

Campylobacters have been detected in house flies, filth flies and darkling beetles

(Adhikari et al. 2004, Szalanski et al. 2004, Templeton et al. 2006). These insects may be important reservoirs for transmission of campylobacters to poultry, cattle and other reservoir hosts. Recently amoeba were identified as *C. jejuni* reservoirs able to resuscitate nonviable strains, which may be relevant to *C. jejuni* survival in water and to the persistence of these bacteria in the environment (Axelsson-Olsson et al. 2005). It is interesting to note that in a New Zealand molecular typing study, isolates from cattle, sparrows, flies and rodents were tested by pulsed field gel electrophoresis and all had identical clonal *C. jejuni* profiles (Adhikari et al. 2004). The complex ecology of campylobacters, including the multitude of reservoir host species, illustrates the challenges in controlling and preventing these important public health pathogens.

Transmission

Transmission of *Campylobacter* spp. is through three main routes; food, water, and direct contact with infected or reservoir hosts (Humphrey et al. 2007). Consumption of undercooked meat (poultry, pork, beef, lamb, and seafood), unpasteurized milk, contaminated water, cross-contamination from raw meat, and direct contact with animals are some known sources (Clark et al. 2003, Damborg et al. 2004, Humphrey et al. 2007, McNaughton et al. 1982, Miller and Mandrell 2005, Neimann et al. 2003, Savill et al. 2001, Stanley et al. 1998b, Steele et al. 1997). Human-human transmission is possible, and while campylobacteriosis outbreaks do occur, greater than 95% of cases are thought to be due to endemic spread (Blaser 1997). Further, foreign travel can be an important human risk factor for developing campylobacteriosis and for fluoroquinolone resistance (Engberg et al. 2004, Johnson et al. 2008, Russell et al. 1993).

2.1.3 Environment

Water

While campylobacters are not able to multiply in water, these bacteria are able to persist and remain viable in aquatic environments. In 2000, a large waterborne outbreak in Walkerton, Ontario raised public awareness to *Campylobacter* and *Escherichia coli (E. coli)* as waterborne pathogens; seven people died (two from *C. jejuni*) and 2,321 people became ill from these enteric bacteria. Heavy rains washed cattle feces from nearby farms into supply wells, and inadequate chlorination of drinking water was blamed for the outbreak (O'Connor 2002). A large cohort study monitoring long term sequelae (Walkerton Health Study) has found an increased risk of chronic gastrointestinal symptoms (relative risk (RR) 2.4, 95% confidence interval (CI) 2.2-2.7) and arthritis (RR 1.4, 95% CI 1.0-2.0) in adults suffering from diarrhea at the time of the outbreak (Garg et al. 2006). This large-scale outbreak illustrates important public health consequences from contaminated water systems. Campylobacters have been isolated from marine, drinking, recreational, roof, and ground water (Hernandez et al. 1995, Savill et al. 2001, Stanley et al. 1998a), and water is a potential reservoir for transmission of environmental campylobacters.

Soil and livestock manure

The farm environment has been described as "a self-perpetuating reservoir of infection" (Jones 2001). In 2005, the number of feedlot cattle in Canada was estimated to be 3,552,500 head (CanFax 2007b). Campylobacters have been isolated from dairy, pasture, and feedlot cattle (Acik and Cetinkaya 2005, Atabay and Corry 1998, Bailey et al. 2003, Giacoboni et al. 1993, Hoar et al. 1999, Inglis et al. 2006, Stanley et al. 1998b).

Livestock manure (e.g. cattle, and swine) is composted and spread on agricultural lands. In Alberta, the application and management of manure is regulated through the Agricultural Operation Practices Act (Gov of AB 2008). In previous experiments, *C. jejuni* inoculated into stored beef manure (slurry) took greater than 112 days to decline 90% (17°C) (Kearney et al. 1993), and in experimental application of *C. jejuni* to four types of New Zealand soils, 99% of *C. jejuni* remained in the top 5 cm of the soils and survived for at least 25 days (10°C) (Ross and Donnison 2006). In order to inactivate *C. jejuni* in soil, reduce possible contamination of water sources, and prevent infection of reservoir hosts (e.g. wild birds, and insects), appropriate composting and application practices are critical. In addition, access to manure within feedlot pens by birds, insects and potentially other reservoir hosts may be unfeasible to control.

Biofilm

Biofilm is an extracellular matrix binding together microcolonies of organisms (e.g. bacteria, protozoa, and fungi) (Buswell et al. 1998, Donlan 2002). These microenvironments may protect campylobacters from detergents, antimicrobial agents and environmental stressors, leading to bacterial persistence and survival (Buswell et al. 1998, Donlan 2002, Thomas et al. 1999, Trachoo and Frank 2002). Contamination of food and water with *Campylobacter* from biofilm reservoirs in kitchens, poultry houses, and on farms may be potential transmission routes for these pathogens to people (Buswell et al. 1998, Trachoo et al. 2002).

Seasonality

Although peak times may vary with region, campylobacteriosis rates have been found to rise during the warmer months of the year (spring and summer peaks) (Frost 2001, Kovats et al. 2005, Louis et al. 2005, Nylen et al. 2002). In Alberta (1992 to 2000 data), the lowest number of campylobacteriosis cases were reported in February-March and the highest numbers in late June-early July (Kovats et al. 2005). Seasonal prevalence increases during the warmer months have also been seen in poultry production (Guerin et al. 2007b, Meldrum et al. 2004). Interestingly, prevalence studies of campylobacters in water found peaks during the colder winter months (Carter et al. 1987, Jones 2001, Obiri-Danso and Jones 1999). In cattle, research on the effect of seasonal *Campylobacter* shedding has been limited, and results may reflect the type of production system studied. In dairy cattle, seasonal peaks in *Campylobacter* shedding have been reported in spring (April) and autumn (October) in the United Kingdom (UK) including periodicity between years (Stanley et al. 1998b), and in autumn (March) in New Zealand (Meanger and Marshall 1989). An Irish study found higher fecal prevalences in feedlot cattle in January-February compared to November (Minihan et al. 2004), a USA study found a summer peak (Berry et al. 2007), and a UK study found no seasonality in feedlot cattle (Stanley et al. 1998b). These results indicate that seasonal effects on *Campylobacter* shedding in feedlot cattle are not clear cut, and that continued research in this area is warranted.

2.2 Identification and characterization of campylobacters

The ability to sequence bacterial genomes has revolutionized the taxonomy, characterization, and diagnostic tests for campylobacters. In 2000, Parkhill et al

published the first sequenced genome of *C. jejuni* (NCTC 11168) (Parkhill et al. 2000). Since then eight other *C. jejuni* subsp. *jejuni* genomes have been sequenced (Fouts et al. 2005, Hofreuter et al. 2006, Poly et al. 2007). Strain variability is an important characteristic of campylobacters. Hypervariable regions have been identified using genomic sequencing and DNA microarray technology, and some strains (e.g. *C. jejuni* 81-176) contain plasmids (Hofreuter et al. 2006, Larsen and Guerry 2005, Parkhill et al. 2000). Strain variability may influence persistence in environmental extremes, virulence, antimicrobial resistance patterns, ability to infect/cause disease or induce sequelae such as GBS or MFS in people, and survival of campylobacters in water, feces, or food (Fitzgerald et al. 2005, Park 2005). At the same time, *C. jejuni* 81116 has been found to be genetically stable for over 20 years in different environments (Manning et al. 2001). Microbial populations consisting of both campylobacters that are genetically stable and those able to adapt to external forces may explain, to some extent, the continued success of campylobacters as human and foodborne pathogens.

Traditionally, selective culture media have been used to isolate *Campylobacter* spp. More recently, a combination of techniques including selective enrichment, culture isolation from selective agar, and then biochemical, serological or genotypic methods have been used to fully describe isolates (Miller and Mandrell 2005, Nachamkin et al. 2000b, Yu et al. 2001). A variety of phenotypic and genotypic identification and typing tools have been developed, Tables 2.3 and 2.4.

The choice of molecular typing tool may be based on implementation costs, labour required, access to specialized equipment or software, portability and standardization, discriminatory ability and resolution of the technique, flexibility of

experiment design, and the time frame required for results (long-term research project compared to outbreak investigation). The number of available techniques, the lack of consensus within the research community, and the ongoing development of molecular typing tools illustrate that currently no one method is optimal for all circumstances.

Table 2.3 Phenotypic methods commonly used to identify or characterize campylobacters

Technique	Principle	Advantages and Disadvantages
Culture	Selective growth based on agar components	A: Inexpensive Indicates viability
		D: Long incubation times, precise temperature and atmospheric conditions required Training may be required for recognition of colony morphology Discriminates to genus only Viable but non culturable strains exist
Morphology e.g. Gram stain	Direct microscopy	A: Inexpensive Indication of sample purity Presumptive identification of genus
		D:Training may be required for recognition of organism shape Discriminates to genus only
Biochemical characteristics	Differentiate based on metabolic activities. Groupings resulting from a series of tests constitute a 'biotype'.	A:1.Easy to perform
		D:May be labour intensive and expensive depending on the level of resolution required May have poor reproducibility because depends on growth conditions Limited resolution unless a large number of biochemical traits analysed for each sample
Serological characteristics	Antibody reaction of a mammal to microbial antigens.	A:Well established technique
		D: Requires constant supply of antisera Can be expensive and time consuming Limited resolution as many strains non-typeable Antigen cross-reaction can lead to false positives Training may be required for consistent results
Phage typing	Lytic areas on lawn of bacterial growth on agar.	A: Easy to perform Results specific
		D:Requires constant supply of bacteriophages (time consuming and labour intensive) Not standardized Each species and subspecies requires its own specifically designed typing system Resolution limited and based on the number of phages tested.
Antibiograms	Lytic areas on a lawn of bacterial growth on agar	A: Reproducible
		D: Limited resolution as unrelated strains can have the same patterns

A: advantages; D: disadvantages.(Klena and Konkel 2005, Riley 2004a, Riley 2004b) (E Taboada personal communication)

Table 2.4 Genotypic methods commonly used to identify or characterize campylobacters

Technique	Principle	Advantages and Disadvantages
PCR	Amplification of genetic material based on specific primers, followed by electrophoresis. May be used as an amplification step for many molecular techniques, including DNA microarray, RFLP, PFGE, AFLP.	A: High sensitivity Inexpensive High throughput Can be used for speciation
		D:High risk of contamination Electrophoresis reproducibility may be poor Nonspecific primers possible Low resolution (not good for subtyping)
RFLP Commonly used for *fla* typing (RFLP-*fla*)	Comparison of banding patterns from restriction endunuclease digestion of PCR amplified polymorphic DNA fragments e.g. flagellin genes.	A:Moderate to high resolution for subtyping Easy to perform High throughput
		D:Dedicated software required for analysis of banding patterns Increased resolution requires the use of more restriction enzymes High throughput
PFGE	Comparison of banding patterns from macrorestriction endonuclease digestion of whole genomic DNA.	A:Uses whole genome to create profile Moderate resolution Can enable you to see genomic events (rearrangements, insertions, deletions
		D:Dedicated software required for analysis of banding patterns Some strains untypeable because of endogenous nuclease activity Patterns can sometimes be unstable Low throughput Requires highly specialized and expensive equipment
AFLP	PCR amplification of polymorphic DNA fragments flanked by specific restriction endonuclease sites, followed by comparison of fragments.	A:1. Uses whole genome to create profile High resolution Does not require the genetic sequence of DNA targets to be known
		D:Dedicated software required for analysis of banding patterns Requires highly specialized and expensive equipment Moderate throughput
DNA microarray	Hybridization of test DNA (fluorescently labeled) to short DNA sequences (targets) bound to solid substrate (e.g. glass slide).	A:Flexibility of design May be used on whole genomes or portions High resolution
		D: Protocols and cutoffs not standardized Gene sequences for targets must be known Low throughput Requires highly specialized and expensive equipment
MLST	Portions from 7 or 8 housekeeping genes are amplified using PCR and then sequenced to look at allelic differences within each loci	A:1. High resolution Reproducible and protocols standardized
		D:Gene sequences for targets must be known Requires highly specialized and expensive equipment Comparisons based on only a few genes Moderate throughput
Ribotyping	Hybridization of digested genomic DNA to ribosomal operon targets	A: Reproducible and reliable
		D:Low resolution Automation requires expensive equipment Moderate throughput

A: advantages; D: disadvantages; AFLP: amplified fragment length polymorphism; MLST: multi-locus sequence typing; PCR: polymerase chain reaction; PFGE: pulsed field gel electrophoresis; RFLP: restriction fragment length polymorphism.
(Klena and Konkel 2005, Riley 2004a, Riley 2004b) (E Taboada personal communication)

2.2.1 Phenotypic methods

Phenotypic methods (Gram stain, culture, biotyping, serotyping, phage typing and antibiograms) have traditionally been used to detect and characterize campylobacters (Riley 2004a). Culture of these bacteria is not straightforward. Temperature, duration of incubation, microaerophilic atmosphere, and type of enrichment and culture media are all important for successful growth of campylobacters. The most common temperature for incubation of thermophilic campylobacters is 42°C, and microaerophilic environments (10% CO_2, 5% O_2, 85% N_2) are often used to promote growth (Nachamkin et al. 2000b).

A variety of different culture techniques have been used to isolate campylobacters from cattle feces and red meat (Corry et al. 1995, Inglis et al. 2006, Whyte et al. 2004, Wong et al. 2007). Enrichment broths including Bolton, Exeter, Park and Sanders and Preston broth (Baylis et al. 2000, Corry et al. 1995, Humphrey 1994) have been developed to increase growth and resuscitate damaged cells prior to culture. Most post-enrichment media are combinations of nutrient agar with antibiotics or blood to improve selection and growth of targeted *Campylobacter* spp. Examples of post-enrichment media include cefoperazone amphoteracin teicoplanin agar, cefoperazone-vancomycin-amphoteracin agar, Karmali, modified cefoperazone charcoal deoxycholate agar (mCCDA), Preston's, and Skirrow's media (Atabay and Corry 1998, Besser et al. 2005, Bolton and Robertson 1982, Corry et al. 1995, Karmali et al. 1986).

While culture is usually inexpensive and has the advantage of indicating organism viability, the unpredictable sensitivity if conditions are not precise can be problematic, and usually 3-4 days are required to confirm detection.

After culture identification, biochemical testing to distinguish the species of thermophilic campylobacters is routine and based on preferred growth temperatures, metabolic characteristics, and antibiotic resistance patterns (Riley 2004a). For example, *C. jejuni* will grow at 37°C and 42°C but not at 25°C (Corry et al. 1995). As with other *Campylobacter* spp., *C. jejuni* can produce catalase and oxidase, but only *C. jejuni* will hydrolyze hippurate (Corry et al. 1995). A drawback of biochemical identification is that strain variation exists, leading to possible misclassifications. For example, strains of *C. jejuni* have been found to be hippuricase negative (Hébert et al. 1984). As a result, genotypic methods continue to develop to replace phenotypic identification and improve sensitivity of detection (Denis et al. 1999, Hong et al. 2004, Inglis and Kalischuk 2003, Inglis and Kalischuk 2004).

Two serotyping methods, the Penner scheme which uses passive hemagglutination techniques based on heat stable antigens to a capsular polysaccharide (Penner and Hennessy 1980), and the Lior scheme which uses bacterial agglutination techniques based on heat labile antigens (Lior et al. 1982), have been used extensively to type *Campylobacter* strains. Both methods are time consuming, technically demanding, rely on constant supply of antisera, and are hindered by the large number of untypeable strains (Wassenaar and Newell 2000). Further, strains with the same serotype have often been found to be very different genetically (Wassenaar and Newell 2000). Even so, serotyping has been used worldwide to characterize campylobacters in epidemiologic studies (Clark et al. 2003, Devane et al. 2005, Fayos et al. 1992, Kramer et al. 2000, Nielsen et al. 2005, Saito et al. 2005).

2.2.2 Genotypic methods

A variety of molecular techniques have been developed to characterize strains of *Campylobacter* based on genetic composition. Sequencing and the amplification of genetic material has aided progress in the development of molecular techniques. Polymerase chain reaction is relatively inexpensive, with good sensitivity and specificity if contamination is minimized and if careful genetic sequence design is used (Riley 2004b). Polymerase chain reaction amplifies genetic material based on specific primers, and has been used to identify genes of interest (e.g. virulence genes) and to detect *Campylobacter* spp. in food, water, and feces (Bang et al. 2003, Datta et al. 2003, Devane et al. 2005, Inglis et al. 2003). Modifications to PCR (e.g. nested PCR, multiplex PCR or real-time PCR) have enabled researchers to distinguish between closely related species (e.g. *C. jejuni* and *C. coli)* and to quantitatively estimate the number of organisms in samples (Inglis and Kalischuk 2004, Josefsen et al. 2004, Klena et al. 2004, Sails et al. 2003, Waage et al. 1999). Genetic sequences must be available for primer construction, and PCR does not differentiate between viable, damaged, or dead organisms.

Polymerase chain reaction has also been incorporated into techniques such as amplified fragment length polymorphism (AFLP) and PCR restriction fragment length polymorphism (PCR-RFLP). *Fla*-typing (PCR-RFLP of flagellin genes) has been used to successfully characterize *Campylobacter* strains, and to group epidemiologically related (outbreak) strains (Clark et al. 2003, Nielsen et al. 2000, Petersen and Newell 2001). Amplified fragment length polymorphism (AFLP), a technique which utilizes the entire genome, can be highly discriminatory (Desai et al. 2001, Duim et al. 1999,

Manning et al. 2001, Schouls et al. 2003), but may be time consuming and requires specific equipment.

Multilocus sequence typing (MLST) is based on sequencing and amplification of housekeeping genes, and is replacing pulsed-field gel electrophoresis (PFGE) as the "gold standard" in *Campylobacter* typing (Djordjevic et al. 2007). Multilocus sequence typing is automated, data are reproducible, protocols have been standardized, and the method has been successfully applied to epidemiological investigation and to the population biology of campylobacters (Colles et al. 2003, Dingle et al. 2001, Dingle et al. 2005, Karenlampi et al. 2007, Manning et al. 2003). However, MLSTs discriminatory power may depend on the genes chosen and comparisons are based on relatively few genes.

Over time, molecular typing techniques have described *Campylobacter* strain similarities and differences in a variety of ecological niches, host species, food, and environmental sources (Klena and Konkel 2005, Riley 2004a), and the evolution of these methods must continue if the epidemiology of campylobacters is to be fully understood.

2.2.3 DNA microarray

DNA microarray technology involves binding specific gene sequences to solid substrate (e.g. glass slide) using complementary base pair hybridization. On spotted arrays, currently the most common because of study design flexibility, each spot on the substrate may represent thousands of replicates of a particular genetic sequence (Taboada et al. 2007). Digested genomic DNA from test strains will bind competitively to the bound sequences if sufficient sequence similarity exists. Further, because so many

replicates of the sequence are bound to one spot, whole genome comparison of a reference strain to a test strain is possible. The bound genetic sequences (open reading frames, ORFs) may be synthesized oligonucleotides usually 20-70 base pairs in length (oligonucleotide arrays), or gene-specific DNA fragments (average 1000 base pairs) which are amplified using PCR (cDNA amplicon arrays) (Dorrell et al. 2005a, Taboada et al. 2007).

Oligonucleotide arrays require genetic sequences to be known (and are therefore not able to detect novel genes), do not require amplification steps, often have less cross-hybridization than amplicon arrays, and may be able to detect single nucleotide polymorphisms with specific experimental designs (Dorrell et al. 2005b, Pearson et al. 2003, Taboada et al. 2007). Amplicon arrays may be cheaper to produce than oligonucleotide arrays, however the sequences often exhibit more cross-hybridization and care must be taken to avoid contamination during PCR amplification (Dorrell et al. 2005b, Taboada et al. 2007).

Array hybridizations are usually quantified through the use of fluorescent (e.g. Cyanine dye) labeling of DNA (Draghici 2003). Scanning at appropriate wavelengths for the label allows the intensity of each spot to be quantified in pixel units. Intensity cut-offs are then assigned to designate each gene as present or absent/divergent. DNA microarray may be used for both global clustering (whole genome evaluation) or for specific gene expression analysis. Microarray technology may also be used for detection of *Campylobacter* spp. in mixed microbial populations, for distinguishing between similar *Campylobacter* spp. (e.g. *C. jejuni* and *C. coli*), and for investigating strain diversity and evolutionary relationships (Champion et al. 2005,

Gaynor et al. 2004, Leonard II et al. 2003, On et al. 2006, Pearson et al. 2003, Sergeev et al. 2004, Taboada et al. 2004, Volokhov et al. 2003, Yoo et al. 2004). Array methods may be labour intensive and expensive, with lack of protocol standardization between laboratories. However, the use of robotics and automation are addressing these disadvantages. There is also little agreement as to how best to analyze results, which may include thousands of genes on one slide. The sequencing of *Campylobacter* strains has greatly accelerated our ability to investigate the epidemiology of campylobacters in the real world, and because DNA microarrays can assess entire microbial genomes (thousands of genes on one chip) this methodology has one of the highest discriminatory powers of any genotypic methodology to date.

2.3 Rationale

2.3.1 The beef industry in Alberta

Canada's beef industry is the largest source of farm cash receipts of any single agricultural commodity (2005 data), and Canada is the third largest exporter of beef and cattle at 10% of the world's exports (2006 data) (CanFax 2007b, Statistics Canada 2006). In 2005, Alberta cattle were worth three billion dollars in farm cash receipts, 38.4% of the province's total (Statistics Canada 2006). In that same year, Canadians consumed 51.4 lbs of beef per capita (retail weight) (CanFax 2007b).

Alberta had 2,370,800 cattle on feed in 2005; 67% of the national total (CanFax 2007b). As of January 1, 2007, 197 sites fed 1000 head or more. Thirty-five of those feedlots had one-time capacities of 10,000 head or more, and 12 operations fed 20,000 or more cattle (representing 37% of the provincial total capacity) (CanFax 2007a).

Feedlots are distributed through the central corridor of the province, with the majority of sites in the southern portion of the province (CanFax 2007a).

In 2005 there were six federally inspected cattle slaughter plants in Alberta (AAFC 2005), all of which are required to meet federal government (Canadian Food Inspection Agency) guidelines on the use of hazard and critical control points (HACCP) during slaughter and processing. Provincially inspected slaughterhouses also exist and are responsible for approximately 5% of cattle slaughtered (CFIA 2003).

On May 20, 2003 the Canada/USA border was closed to the movement of live cattle because bovine spongiform encephalopathy was identified in a mature cow from Alberta. Movement of live cattle under 30 months of age from Canada to the USA did not resume until July 18, 2005 (Weerahawa et al. 2007).

The beef industry is extremely important to both the provincial and the national economies and to Canadian consumers in general. Further, high levels of *Campylobacter* spp. shedding by cattle may be relevant to public health, food safety and the environment. Continued research into the epidemiology of campylobacters in cattle, with emphasis on understanding transmission, reservoirs, and persistence of these bacteria in food and the environment, could help to reduce cattle shedding and the possibility of pathogen transmission in the future.

2.3.2 The importance of campylobacters to public health

Incidence of campylobacters in Alberta

Campylobacter spp. are a major cause of food and waterborne disease in people worldwide (Miller and Mandrell 2005). In Alberta, each human case of campylobacteriosis is reportable at the provincial level, and all cases are followed up by

the Alberta Medical Officer of Health in each regional health authority. In Alberta, stool culturing for campylobacteriosis is recommended by physicians if the patient has had diarrhea for five days or more (with no prior antibiotic use) or in cases with bloody diarrhea (ACPGP 1997). Further, a confirmed case is based on laboratory isolation of *Campylobacter* spp. from an appropriate clinical specimen, whether or not the patient has clinical symptoms (Gov of AB 2005). In Alberta in 2005, campylobacteriosis was reported at a rate of 36.1 cases per 100,000 people, a rate much higher than either *E. coli* O157:H7 infection (5.8/100,000) or salmonellosis (19.5/100,000) over the same time period (PHAC 2007, Statistics Canada 2007). Because transmission of campylobacters to people is commonly through food sources, food handling and kitchen guidelines to prevent cross-contamination of foods and to ensure the adequate cooking of meat are currently available to the public (CFIA 2007, Health Canada 2006). However improved education and public awareness are essential to reduce the incidence of campylobacteriosis in people.

Burden of disease

Campylobacteriosis has significant economic impacts on the public health system. The true number of *Campylobacter* cases in people is difficult to estimate because underreporting of enteric disease is known to occur. This may be because symptoms are not severe enough for patients to access the healthcare system, because physicians may not always request a stool culture for identification, because diagnostic methods lack the sensitivity to find all cases, or because public health officials are not made aware of all clinical cases. Further, outbreaks may not be recognized because of the long incubation period, the wide host reservoir range, and the potential for cross-

contamination between foods (Flint et al. 2004, Frost 2001, Mead et al. 1999, Miller and Mandrell 2005). In a UK study, an estimated 8.7 out of 1000 people were infected with *Campylobacter* annually. Of these, only 4.1 per 1000 population were likely to consult a physician and only 1.7 of 1000 would be confirmed positive by a laboratory (Frost 2001, Wheeler et al. 1999). An Ontario study showed that for every reported case of gastrointestinal illness in Ontario, a median of 285 community cases went unreported (Majowicz et al. 2005).

Because of the numbers of people affected and the potentially serious sequelae, *Campylobacter* infection can be a significant burden to health care systems. Several Canadian studies have looked at the incidence of GBS in Canada (Hauck et al. 2007, McLean et al. 1994). An administrative data study in Alberta found a mean incidence of 1.6 per 100,000 over 11 years (GBS from all causes). Guillain-Barré syndrome development in patients has resulted in recurrent hospital admissions, extended hospital stays, ventilator support and patient death (Hauck et al. 2007). A USA study estimated a total annual cost due to *Campylobacter* infection in people to be 1.5-8 billion dollars (1995 USA dollars), including both the costs of campylobacteriosis and of related GBS, a significant economic burden on the health system (Buzby et al. 1997).

Antimicrobial resistance

Research into antimicrobial use in animal production and increasing levels of antimicrobial resistance in people has been given high research priority. It has been found that *C. jejuni* and *C. coli* in people are usually resistant to cephalosporins (except a few broad-spectrum formulations) penicillins, rifampin, sulfamethoxazole, trimethoprim, and vancomycin (Nachamkin et al. 2000b). While macrolides are the

treatment of choice for most complicated cases in people, resistance rates for erythromycin range from 0-11% for *C. jejuni* and 0-68.4% for *C. coli*, and resistance rates for clindamycin are variable (Nachamkin et al. 2000b, Reina et al. 1994). Fluoroquinolones have good activity against *Campylobacter* spp. *in vivo*, although resistant strains have been isolated from patients both prior to and after treatment (Nachamkin et al. 2000b).

In people, increases in quinolone resistance over time have been dramatic. In a study by Sanchez et al., over a five year period *C. jejuni* resistance to ciprofloxacin increased from 0% (n=23) to 51.3% (n=73) (Sanchez et al. 1994). The introduction of enrofloxacin into veterinary practice has been blamed for the increase in quinolone resistance in human populations, with both livestock and companion animals suggested as potential sources (Anderson et al. 2001, Endtz et al. 1991, Engberg et al. 2001). In Canada, enroflaxacin is currently approved for use in beef cattle (not dairy cattle or veal calves) and in dogs and cats; this drug was withdrawn from use in poultry in 1997 (Engberg et al. 2001, Health Canada 2004).

Active surveillance of *Campylobacter* antimicrobial resistance profiles in both human and veterinary medicine has increased as a means of describing trends over time and assessing interventions. The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) actively surveys campylobacters in retail meat products and on-farm antimicrobial resistance patterns (Gov of Canada 2006a, Gov of Canada 2006b). Ongoing research into the mechanisms of resistance and the identification of resistance genes using molecular techniques will continue to increase

our understanding of *Campylobacter* infection and treatment, and the role of antibiotic use in people and animals.

2.3.3 Food safety

Foodborne transmission is responsible for approximately three quarters of all cases of campylobacteriosis in North America (Mead et al. 1999, Rajda and Middleton 2004). Poultry products are generally considered the primary source of *Campylobacter* infection in people (Humphrey et al. 2007). In Canada, people have become infected through drinking unpasteurized milk or contaminated water, and through eating undercooked or cross-contaminated meat (Clark et al. 2003, McNaughton et al. 1982, Miller and Mandrell 2005). An extensive list of outbreaks due to *Campylobacter* spp. in food and water sources (worldwide) has previously been published (Miller and Mandrell 2005).

Campylobacter prevalence surveys in retail meat have been conducted as an assessment of disease risk through the food chain. Retail meats such as poultry, beef, pork and lamb have been assessed as sources of infection from host reservoir contamination, and as possible cross-contamination sources (Datta et al. 2003, Kramer et al. 2000, Pezzotti et al. 2003, Zhao et al. 2001). The prevalence of *Campylobacter* spp. in beef, retail and abattoir surveys (not including carcass swabs), has been reported to be 0-32%, Tables 2.5 and 2.6. In a Canadian study, Lammerding et al found 17.1% of 269 abattoir beef samples to carry *Campylobacter* spp. (Lammerding et al. 1988). More recently, a retail poultry and beef survey in Edmonton, Alberta found *Campylobacter* prevalences of 62% (n=100) in poultry meat and 0% (n=100) in ground beef (Bohaychuk et al. 2006).

Table 2.5 Previously published surveys of the prevalence of *Campylobacter* spp. and *C. jejuni* in fresh, uncooked, retail beef samples

Area	Year	C spp. or Cj	Pos(n)	% Pos	Method	Beef Type (Notes)	Reference
Austria	NR	C spp.	2(84)	2.4	C	RM	(Mayrhofer et al. 2005)
		Cj	1(84)	1.2	B	RM	
Canada	2001	C spp.	0(100)	0	C	G	(Bohaychuk et al. 2006)
Denmark	2001-2002	C spp.	0(777)	0	C	RM	(Nielsen et al. 2005)
England	1998	C spp.	52(96)	54.2	C	L	(Kramer et al. 2000)
		Cj	47(96)	49.0	B	L	
England	1984-1986	C spp.	30(127)	23.6	C	RM	(Fricker and Park 1989)
		Cj	30(127)	23.6	B	RM	
England	NR	C spp.	0(1)	0.0	C	S	(Bolton et al. 2002)
		Cj	5(5)	100	B,PCR-ELISA	L	
Ireland	2001-2002	C spp.	7(221)	3.2	C	RM	(Whyte et al. 2004)
		Cj	6(221)	2.7	B	RM	
Ireland	NR	C spp.	4(20)	20.0	C	G	(Cloak et al. 2001)
Italy	2000-2001	C spp.	2(151)[H]	1.3	C	RM	(Pezzotti et al. 2003)
	2000-2001	Cj	2(151)[H]	1.3	B	RM	
Japan	1984-1985	C spp.	2(120)	1.7	C	G	(Fukushima et al. 1987)
		Cj	2(120)	1.7	B	G	
Japan	1993-1998	C spp.	0(58)	0	C	RM, I	(Ono and Yamamoto 1999)
		C spp.	0(54)	0	C	RM, D	
Kenya	NR	C spp.	1(50)	2.0	C	RM	(Osano and Arimi 1999)
		Cj	1(50)	2.0	B	RM	
Korea	2001-2006	C spp.	3(250)	1.2	C	RM (R,W,TR)	(Hong et al. 2007)
	2001-2002	Cj	0(50)	0	B,PCR	RM	
	2003-2004	Cj	1(100)	1.0	B,PCR	RM	
	2005-2006	Cj	2(100)	2.0	B,PCR	RM	
NZ	2003-2004	C spp.	8(230)	3.5	C	G,RM	(Wong et al. 2007)
		Cj	8(230)	3.5	C,PCR	G,RM	
NI	NR	C spp.	0(50)	0	C	RM not G	(Madden et al. 1998)
UK	1997	C spp.	10(1015)	1.0	C	G (BT)	(Little and de Louvois 1998)
USA	1983-1984	Cj	17(360)	4.7	B	RM	(Stern et al. 1985)
		Cj	13(360)	3.6	B	G	
USA	1982-1983	C spp.	1(230)	0.4	C	RM	(Harris et al. 1986)
USA	1999-2000	C spp.	1(210)	0.5	C	S	(Zhao et al. 2001)
		Cj	1(210)	0.5	B,PCR	S	
USA	2002-2005	C spp.	1(2073)	0.05	C	G	(Gov of USA 2005)
		Cj	1(2073)	0.05	B,PCR	G	

B: culture and biochemical testing; BT: butcher; C: culture; C spp.: *Campylobacter* species; Cj: *C. jejuni*; D: domestic; RM: red meat (beef); G: ground beef; GA: gall bladder; I: imported; L: liver; N: neck; NI: Northern Ireland; NZ: New Zealand; PCR: polymerase chain reaction; PCR-ELISA: PCR enzyme-linked immunosorbent assay; S: steak; W: warehouse; TR: traditional market; UK: United Kingdom; USA: United States.

Table 2.6 Previously published surveys of the prevalence of *Campylobacter* spp. and *C. jejuni* in beef abattoirs samples

[a] geographical area inferred from country of residence of first author;
[b] denominator is # *Campylobacter* isolates tested.
A: abattoir; B: culture and biochemical testing; BT: butcher; C: culture; CA: calves; C spp.: *Campylobacter* species; Ch: chilled; Cj: *C. jejuni*; CS: carcass swab; D: deboned; F: feedlot cattle; G: ground beef; GA: gall bladder; L: liver; MM: minced meat (mostly beef); N: neck; NI: Northern Ireland; NZ: New Zealand; P: pasture cattle; S: steak; Sk: skinned; R: retail; RM: red meat (beef); US: United States; W: warehouse; Wa: washed; TR: traditional markets.

Area	Year	C spp. or Cj	Pos (n)	% Pos	ID	Beef Type (Notes)	Reference
Australia	2005	C spp.	0(151)	0.0	C,PCR	G,RM (R,W,TR)	(Bosilevac et al. 2007)
NZ	2005	C spp.	1(216)	0.5	C,PCR	G,RM	
Uruguay	2005	C spp.	1(250)	0.4	C,PCR	G,RM (I)	
		Cj	1(250)	0.4	C,PCR, mPCR	G,RM(I)	
USA	2005	C spp.	5(393)	1.3	C,PCR	G,RM (I)	
		Cj	5(393)	1.3	C,PCR, mPCR	G,RM (I)	
Australia	2000-2001	Cj	60(65)	92.3	B	CS (CA)	(Grau 1988)
		Cj	11(44)	25.0	B	CS (F)	
		Cj	3(70)	4.3	B	CS (P)	
Belgium	1996	C spp.	6(62)	9.7	C	CS	(Korsak et al. 1998)
Belgium	1997	C spp.	3(60)	5.0	C	RM	(Ghafir et al. 2007)
	1997	C spp.	2(60)	3.3	C	CS	
	1997	C spp.	0(67)	0.0	C	G	
	1997	C spp.	19(60)	31.7	C	L	
	2000-2001	C spp.	5(786)	0.6	C	G (R,A)	
	2000-2001	Cj	5(786)	0.6	B,PCR	G (R,A)	
Canada	NR	Cj	12(100)	12.0	B	L	(Garcia et al. 1985)
		Cj	33(100)	33.0	B	GA	
Canada	1983-1984	C spp.	46(269)	17.1	C	N	(Lammerding et al. 1988)
	1983-1984	Cj[b]	58(76)[b]	76.3	B	N,GA	
	1985-1986	C spp.	42(329)	12.8	C	N	
	1985-1986	Cj[b]	71(74)[b]	95.9	B	N,GA	
[a]England	NR	C spp.	37(117)	31.6	C	CS	(Bolton et al. 1982a)
England	1979	C spp.	21(2015)	1.0	C	G (R,A)	(Turnbull, Rose 1982)
England	NR	C spp.	3(135)	2.2	C	MM (R,BT,A)	(Bolton et al. 1985)
		Cj	3(135)	2.2	B	MM	
Ireland	NR	C spp.	0(109)	0	C	CS	(Minihan 2004)
Japan	NR	C.spp.	0(52)	0	C	RM (R,A)	(Tokumaru et al. 1990)
NZ	NR	C spp.	7(30)	23.3	C	CS (CA Sk)	(Gill and Harris 1982)
		C spp.	6(30)	20.0	C	CS (CA Wa)	
		C spp.	3(30)	10.0	C	CS (CA Ch)	
		C spp.	4(30)	13.3	C	CS (D)	
NI	NR	C spp.	0(100)	0.0	C	N	(Madden et al. 2001)
NI	NR	C spp.	0(100)	0.0	C	N	(Madden et al. 1998)
Poland	NR	C spp.	1(114)	0.9	C	CS	(Kwiatek et al. 1990)
		Cj	0(114)	0	C	CS	
Sweden	1999-2000	C spp.	NR	<3.0	NR	CW	(Blixt et al. 2001)
USA	1992-1993	C spp.	84(2064)	4.0	C	CS	(Gov of US 1994)
USA	1993-1994	C spp.	24(2109)	1.1	C	CS	(Gov of US 1996a)
USA	1993-1994	C spp.	1(562)	0.2	C	G	(Gov of US 1996b)
USA	NR	C spp.	2(100)	2.0	C	CS (F)	(Beach et al. 2002)
	NR	C spp.	0(96)	0.0	C	CS (P)	
USA	2002-2003	Cj	18(19)	94.7	B,PCR	CS	(Lee et al. 2004)

2.4 Conclusions

Public awareness of food safety issues continues to increase, and further research in this area is essential to maintaining consumer confidence. The potential impact of campylobacters on food safety, on clinical disease in human populations, and on the economic burden to health care systems requires continued investigation. Further research into the adaptive mechanisms, genetic characteristics, and the epidemiology of these bacteria, including the importance of animal and environmental reservoirs, is critical if appropriate prevention and control measures are to be implemented to protect public health.

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CHAPTER 3
PREVALENCE AND RISK FACTOR INVESTIGATION OF *CAMPYLOBACTER* SPECIES AND *CAMPYLOBACTER JEJUNI* IN FEEDOT CATTLE FECES

3.1 Introduction

Thermophilic *Campylobacter* species are important targets for veterinary and public health research because of their zoonotic potential, large range of reservoir hosts, and environmental persistence (e.g. survivability in water). *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli (C. coli*) cause the majority of human cases of illness, with *C. jejuni* responsible for 80-85% of these (Moore et al. 2005). Transmission to people may be through the ingestion of contaminated food or undercooked meat, water, or raw milk (Humphrey et al. 2007).

Thermophilic *Campylobacter* species are commonly isolated from food producing animals including poultry, cattle, swine, and sheep (Bywater et al. 2004, Humphrey et al. 2007), with poultry generally recognized as the primary source of infection for people (Humphrey et al. 2007). Cattle may be sources of infection for people (Clark et al. 2003, Garcia et al. 1985, Humphrey et al. 2007) and potentially important reservoirs for environmental spread to water (Clark et al. 2003) and to other hosts (e.g. wild birds, insects, rodents, and poultry (Adhikari et al. 2004, Ziprin et al. 2003). In 2000, a large waterborne outbreak of enteric disease (*Campylobacter* spp. and *Escherichia coli* O157:H7) in Walkerton, Ontario affected 2,321 people (two deaths from *C. jejuni*) and raised public awareness to cattle as enteric pathogen reservoirs

(O'Connor 2002). Canadians have been infected with *Campylobacter* spp. from direct contact with cattle and through the consumption of raw milk (Dilworth et al. 1988, McNaughton et al. 1982). In Alberta, campylobacteriosis is the most common notifiable bacterial enteric disease with 36.1 cases per 100,000 people in 2005 (PHAC 2007, Statistics Canada 2007).

In 2005, the province of Alberta had 2,370,800 cattle on feed; 67% of the national total (CanFax 2007b). As of January 1, 2007, 197 feedlots fed 1,000 head or more. Thirty-five of these feedlots had one-time capacities of 10,000 head or more, and 12 operations had one time capacities of 20,000 head or more (representing 37% of the provincial total capacity) (CanFax 2007a).

In cattle, *C. jejuni* colonizes the proximal small intestine and may be found in digesta throughout the intestinal tract (Inglis et al. 2005a). The prevalence of *Campylobacter* spp. in dairy cattle has been reported to be 6-64% (Humphrey et al. 2007), and North American fecal studies report the prevalence of *Campylobacter* spp. to be 5-49% in pasture cattle (Bae et al. 2005, Beach et al. 2002, Hoar et al. 1999). In North American feedlots, fecal *Campylobacter* spp. prevalences range from 46-100% using culture or polymerase chain reaction (PCR) methodology (Bae et al. 2005, Inglis et al. 2003, Inglis et al. 2004, Inglis et al. 2006, Lefebvre et al. 2006), and 32-74% for *C. jejuni* using biochemical or molecular identification techniques (Bae et al. 2005, Besser et al. 2005, Inglis et al. 2003, Inglis et al. 2004, Inglis et al. 2005b, Inglis et al. 2006, Lee et al. 2004). Because of the possibility of transmission of campylobacters to other cattle, poultry, other livestock, companion animals, birds, insects or the environment, research on campylobacters in cattle continues to be supported.

Few risk factors for *Campylobacter* spp. shedding in beef cattle have been identified (Hoar et al. 2001, Minihan et al. 2004). In an Irish longitudinal study, shedding of *Campylobacter* spp. in feedlot cattle was associated with the pen involved, environmental contamination of pen dividing bars and water troughs, and the month of sampling (Minihan et al. 2004). Distinct seasonal trends in *Campylobacter* spp. prevalence have been reported in human and poultry studies (Humphrey et al. 2007), although the exact timing of the peaks varies among countries (Kovats et al. 2005). In cattle, the effect of season on *Campylobacter* spp. shedding has not been as definitive. Seasonal peaks have been identified in dairy cattle including a United Kingdom study in which seasonal differences in prevalence of *Campylobacter* spp. were found in dairy but not beef cattle (Stanley et al. 1998). The generally higher fecal *Campylobacter* spp. prevalences reported in feedlot cattle compared to dairy or pasture surveys (Bae et al. 2005, Hoar et al. 1999, Humphrey et al. 2007) also suggest that differences may exist between beef production industries, and that targeted research into risk factors in feedlot cattle is required. Further, production system differences between North America, Europe and other continents suggest that geographically targeted studies should be conducted.

This chapter reports the results of a fecal survey from feedlot cattle near the end of the feeding period. The goals of this project were 1) to obtain isolation rates of *Campylobacter* spp. and *C. jejuni* in cattle feces prior to the animals entering the food chain, 2) to obtain isolation rates of *Campylobacter* spp. and *C. jejuni* in cattle feces in summer and winter as indicators of seasonal fluctuation, 3) to conduct preliminary risk factor analyses based on *Campylobacter* spp. isolation rates in feces after adjusting for

clustering at pen and feedlot levels, and 4) to collect *C. jejuni* isolates for molecular characterization (Chapter 5).

3.2 Materials and methods

3.2.1 Sample size calculation

For a survey using simple random sampling, 22 samples would have been necessary to measure a 68% expected prevalence of *C. jejuni* (Minihan et al. 2004) with 20% precision and 95% confidence (Epi-Info, version 3.01, CDC, USA, 2003). After applying an inflation factor formula (Dohoo et al. 2003) to account for clustering of the expected frequency of *Campylobacter* within pens, the survey required seven feedlots, assuming an intraclass correlation coefficient (ICC) of 0.3, an unadjusted sample size of 22, sampling of 40 pens per feedlot, and one sample per pen. An ICC for clustering of *C. jejuni* in cattle feces was not available from previous publications, and the choice of 0.3 was a slightly more conservative estimate than previously published ICCs for non-enteric cattle conditions (McDermott and Schukken 1994). To assess within-pen variability, it was decided to sample 10 fecal pats per pen for a total of 2,800 fecal samples. Ethics approval for this project was received from both the University of Saskatchewan Biomedical Research and the University of Calgary Conjoint Health Research Ethics Boards.

3.2.2 Study animals and sampling protocol

The sampling target was feedlot beef cattle near slaughter weight. Seven commercial Alberta feedlots agreed to participate. Four feedlots had one-time capacities of 10,000-19,999 animals, two had capacities between 20,000 and 39,999 head, and one

had a capacity of ≥ 40,000 cattle. Animals sampled in this study were crossbred steers or heifers typical of the beef herds in western Canada (British Columbia, Alberta, Saskatchewan and Manitoba). Cattle were kept in open-air, dirt floor pens with 20% porosity wood fences, and the number of animals per pen ranged from 14-538.

Upon arrival to feedlots, cattle were processed as per standard practices of the feedlot based on the age class, gender, weight, and health risk category of the animal. This may have included recording body weight, individual animal identification (e.g. ear tagging), administration of growth implants, castration of bulls, induction of abortion in pregnant heifers, vaccination for protection against agents including infectious bovine rhinotracheitis, parainfluenza-3 virus, bovine viral diarrhea virus, bovine respiratory syncytial virus, *Mannheimia haemolytica*, *Clostridia* spp. and/or *Histophilus somni*, and parasiticide treatment (e.g. topical avermectin). In addition, injectable metaphylactic antimicrobials may have been administered at processing depending on the assigned health risk category of each group of animals. Individual animal injectable antimicrobial use data were not collected for this study.

Within the feedlot industry, "on feed" refers to cattle confined within feedlot pens and fed *ad libitum* high concentrate "finishing" rations to maximize growth prior to slaughter. In this study cattle finishing diets varied between feedlots, but generally contained approximately 80% carbohydrate sources (barley or other cereal grains), approximately 18% roughage sources (cereal or corn silage), and approximately 2% vitamin and mineral supplements (all as-fed). Ionophores and antimicrobials (monensin, oxytetracycline, chlortetracycline, tylosin) fed for seven or more days during the feeding

period were documented by pen, and the average number of days study cattle were on feed was 150 (range 38 to 462 days).

For each sampling date, feedlot personnel at each site identified the 20 pens closest to slaughter based on expected shipping dates. In this study, 85% of pens were sampled within 47 days of slaughter (range 22-120 days). Average pen weight was supplied by feedlots. Pen size (area) was supplied by feedlots or approximated at the time of sampling for calculation of pen density (# animals/m^2). Each feedlot was visited twice, once in winter (Jan 17 – Feb 1, 2005) and once in summer (Aug 22 – Sept 13, 2005). Geographically, feedlots were located within four regional health authorities: Chinook (4 sites), Palliser (1 site), Calgary (1 site) and East Central (1 site).

The first 10 fresh pen-floor fecal pats (steaming or observed defecation) were sampled in each pen using Starswab II (Starplex Scientific Inc, Etobicoke, ON, Canada) charcoal transport media swabs. Efforts were made to avoid sampling multiple pats from the same animal. Each swab was inserted into five different locations within each fecal pat with care to avoid gross environmental contamination, and re-inserted into the charcoal transport media. Ten swabs from each pen were placed into a medium Ziploc bag (SC Johnson, Racine, WI, USA), the bags were placed into an insulated foil bag (KeepCool-GenericKC9, Winnipeg, MB, Canada) with four frozen gel packs (Ice-Pak/Hot-Pak, Montreal, QC, Canada), with care to avoid placing the ice packs onto the swab tips. The insulated bag was then packaged into a cardboard box and shipped via courier (labeled as per International Air Transport Association regulations) to the Vaccine and Infectious Disease Organization (VIDO) in Saskatoon, SK, Canada. A Hobo H08 Pro temperature monitor (Onset Computer Corporation, Pocasset, MA, USA)

was included in each of the 14 shipments. Swabs were processed within approximately 24 hours of collection, with the exception of one shipment which was processed at 48 hours due to weather related transport delay. Transport temperature ranges were monitored from two hours after closure to two hours before the shipment was opened.

3.2.3 Culture methodology

Each charcoal swab was streaked onto Karmali selective agar (Oxoid, CM935 with supplement SR0167E, Nepean, ON, Canada) and incubated microaerobically (85% N_2, 10% CO_2, 5% O_2) at 42°C for 48 hours. Each incubation batch included a lab strain *C. jejuni* plate as positive control. One colony considered positive for *Campylobacter* spp. from each sample (based on growth, color and morphology of the colony, and color of the cell mass) was selected and streaked onto a Karmali agar plate and incubated at 42°C (85% N_2,10% CO_2, 5% O_2) for a further 48 hours.

3.2.4 Hippurate hydrolysis testing

Hippurate hydrolysis testing was used to differentiate *Campylobacter* colonies and identify isolates as *C. jejuni*. For the winter collection, two *Campylobacter* spp. positive isolates from most pens (277 samples from 140 pens) and for the summer collection almost all *Campylobacter* positive isolates (1,209 of 1,210) were tested for hippurate hydrolysis. A loopful of bacterial cells was emulsified in 100µL of 1% aqueous sodium hippurate (Sigma-Aldrich H529, Oakville, ON, Canada) in a single well of a 96 well titre plate. After two hours incubation at room temperature, 50 µL of ninhydrin solution (3.5% ninhydrin in a 1:1 mixture of acetone and butanol) was slowly added to each well and incubated at 37°C for 20-30 minutes. Purple color change

indicated a positive reaction, whereas clear to yellow indicated a negative sample. Positive isolates were tentatively identified as *C. jejuni* and were frozen at -70°C in 25% glycerol for later molecular characterization.

3.2.5 Polymerase chain reaction (PCR) sensitivity analysis

A subsample of study genomic DNA (104 isolates) was examined using multiplex PCR as a means of assessing the accuracy of culture and hippurate techniques. Isolates were randomly selected (using random numbers in Microsoft Office Excel 2007, Microsoft Corporation) from all hippurate positive isolates after stratification by feedlot and season. Confirmation of the isolates as *C. jejuni* was required for future molecular genotyping (Chapter 5). Published PCR primers for *Campylobacter* spp. (23S rRNA), *C. jejuni (hipO)*, and *C. coli (glyA)* were used, with initial denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, with two extension steps at 30 seconds and seven minutes at 72°C (Wang et al. 2002). Positive *C. jejuni* and *C. coli* controls were included in each PCR.

3.2.6 Data analysis

Of the 2,800 fecal samples collected, 22 of the culture plates were overgrown with mold/bacteria and could not be read, and data were missing for two samples. The missing/unreadable data were distributed across 14 pens, and were excluded from analyses. All prevalence 95% confidence intervals (CI) were calculated using the binomial exact specification (Intercooled STATA 9.2, StataCorp LP, College Station, TX, USA).

Factors affecting whether or not a fecal sample was positive for *Campylobacter* spp. using culture methodology were examined using hierarchical models with a binomial distribution and logit link function. The model was specified using restricted iterative generalized least square estimation and using second order penalized quasi-likelihood estimates (MLwiN version 2.0, Centre for Multilevel Modeling, Institute of Education, London, UK) (Dohoo et al. 2003). The strength of the association between outcome and exposure was reported as an odds ratio with 95% confidence intervals.

In the first stage of the analysis, prevalence differences among feedlots were investigated in a model with feedlot identifier as the only fixed effect. The potential lack of independence or clustering of samples within pens was accounted for by using a random effect for pen.

In the second stage of the analysis, the importance of a series of feedlot- and pen-level risk factors for the occurrence of *Campylobacter* spp. was assessed after accounting for potential clustering of observations using random effects for both pen and feedlot (Dohoo et al. 2003). Continuous variables, including number of days-on-feed ($\leq$ 149, 150-299, or $\geq$ 300) for each pen, number of head per pen ($\leq$99, 100-199, 200-299, $\geq$ 300), pen density ($<$ 0.1 or $\geq$ 0.1 animals/m^2), average pen weight ($\leq$ 499, 500-599, $\geq$ 600 kg or missing), and feedlot size (10,000-19,999, 20,000-39,999, $\geq$ 40,000 head), were categorized to assess the linearity of association between each factor and the log odds of the occurrence of *Campylobacter* spp. Categorical variables explored in analysis included pen feed treatment for seven or more days during the feedlot stay (no treatment, monensin/ tylosin, monensin/chlortetracycline/tylosin, or

monensin/ oxytetracycline), geographical location (regional health authority), pen gender (heifer, steer, or mixed), and season of sampling (winter or summer).

Risk factors were each sequentially examined in the null model containing only the random effects for pen and feedlot, and considered for inclusion in the final model if they were associated with the outcome in unconditional analysis at $p \leq 0.25$. Manual backwards stepwise analysis was used to achieve a final model containing statistically significant risk factors ($p \leq 0.05$) or variables that acted as important confounders (accounting for the variable resulted in >20% change in the measure of association). After establishing the final summary main-effect model, biologically reasonable first-order interaction terms were added, assessed for their association with the outcome, and reported if $p \leq 0.05$.

Model fit was evaluated by examining residuals and the impact of outliers. Variance components for both the final and null (constant only) models were approximated using latent variable calculation which fix error variance at $\pi^2/3$ (Dohoo et al. 2003). The herd level variance components were calculated as herd variance divided by total variance, and the pen level variance components were calculated using pen variance divided by total variance.

3.3 Results

3.3.1 Prevalence of *Campylobacter* spp. and *C. jejuni* in feces and feedlot pens

The fecal prevalence of *Campylobacter* spp. in feedlot cattle from large commercial feedlots ranged from 76-95% by culture (Table 3.1) with an overall study prevalence of 87% (95% CI 86-88, 2,420 of 2,776 samples positive). In addition, 1,020 of 1,486 (69%, 95% CI 66-71) *Campylobacter* positive isolates were identified as

C. jejuni using hippurate hydrolysis testing, Table 3.1. All of the 280 pens sampled were positive for *Campylobacter* spp. based on culture and 279 of 280 pens were positive for *C. jejuni* based on hippurate hydrolysis testing. In a model accounting for clustering of observations within pen, the prevalence of *Campylobacter* spp. positive samples was significantly different among feedlots (Wald χ^2 77.97, df 6, $p < 0.001$).

Table 3.1 Feedlot cattle fecal samples positive for *Campylobacter* spp. by culture and for *C .jejuni* (from *Campylobacter* spp. positive isolates) using hippurate hydrolysis testing

	Campylobacter species			*Campylobacter jejuni*		
Feedlot	Pos (n)	% Pos	95% CI[d]	Pos (n)	% Pos	95% CI[d]
A	365 (396)	92	89-95	163 (223)	73	67-79
B	362 (400)	91	87-93	148 (222)	67	60-73
C	381 (400)	95	93-97	144 (224)	64	58-71
D	350 (400)	88	84-91	156 (216)	72	66-78
E	366 (400)	92	88-94	159 (226)	70	64-76
F	297 (393)	76	71-80	137 (183)	74	67-80
G	299 (387)	77	73-81	114 (192)	59	52-66
Total	2420 (2776) [ab]	87	86-88	1020 (1486)[c]	69	66-71

[a] sample not readable due to mold overgrowth (n=22); [b] missing data (n=2); [c] missing data (n=4); [d] binomial exact confidence interval.
Pos: positive.

3.3.2 Feces and pen prevalences in summer and winter

Of the 1,400 winter samples collected, 1,210 were culture positive for *Campylobacter* spp. (86%, 95% CI 85-88), and 1,210 of 1,376 summer samples were positive (88%, 95% CI 86-90). Among feedlots, *Campylobacter* spp. prevalences ranged from 73-99% and 74-93% for winter and summer respectively, Table 3.2. In addition, 177 of 277 (64%, 95% CI 57-70) winter and 843 of 1,209 (70%, 95% CI 67-72) summer

Campylobacter spp. positive isolates were identified as *C. jejuni* based on culture and hippurate hydrolysis testing. In summer, the prevalence of *C. jejuni* was estimated to be 61% (843of 1,376 fecal samples). Table 3.3 reports the number of *Campylobacter* isolates identified as *C. jejuni* in each of the seven feedlots. In a model adjusting only for clustering within pen and feedlot, season was not associated with whether or not a sample was culture positive for *Campylobacter* spp. ($p = 0.40$). Transport temperatures ranged from -5.8°C to 17.5°C in the seven winter shipments and 5.4°C to 22.8°C in the seven summer shipments.

Table 3.2 Feedlot cattle fecal samples positive for *Campylobacter* spp. based on culture (Winter/Summer)

	Winter			Summer		
Feedlot	Pos(n)	% Pos	95% CI[c]	Pos(n)	% Pos	95% CI[c]
A	182 (200)	91	86-95	183 (196)	93	89-96
B	180 (200)	90	85-94	182 (200)	91	86-95
C	197 (200)	99	96-100	184 (200)	92	87-95
D	172 (200)	86	81-90	178 (200)	89	84-93
E	180 (200)	90	85-94	186 (200)	93	89-96
F	154 (200)	77	71-83	143 (193)	74	67-80
G	145 (200)	73	66-79	154 (187)	82	76-88
Total	1,210 (1,400)	86	85-88	1,210 (1,376)[ab]	88	86-90

[a] sample not readable due to mold overgrowth (n=22); [b] missing data (n=2); [c] binomial exact confidence interval. Pos: positive

Table 3.3 *Campylobacter* spp. positive feedlot cattle feces isolates identified as *Campylobacter jejuni* using hippurate hydrolysis testing (Winter/Summer)

	Winter			Summer		
Feedlot	Pos (n)	% Pos	95% CI[c]	Pos (n)	% Pos	95% CI[c]
A	24 (40)	60	43-75	139 (183)	76	43-75
B	24 (40)	60	43-75	124 (182)	68	69-82
C	28 (40)	70	53-83	116 (184)	63	56-70
D	25 (38)	66	49-80	131 (178)	74	66-80
E	24 (40)	60	43-75	135 (186)	73	66-79
F	28 (40)	70	53-83	109 (143)	76	68-82
G	24 (39)	62	45-77	90 (153)	59	51-67
Total	177 (277)[a]	64	58-70	843 (1,209)[b]	70	67-72

[a] missing data (n=3); [b] missing data (n=1); [c] binomial exact confidence interval.
Pos: positive

3.3.3 PCR of a subsample of *C. jejuni* isolates

In preparation for molecular characterization, 104 isolates determined to be *C. jejuni* based on culture and hippurate hydrolysis testing were evaluated using PCR. Isolates were selected randomly (Excel 2007, Microsoft Corporation) from all hippurate positive isolates (n=1,486) after stratification by feedlot and by season. One hundred of 104 samples (96%) were identified as *Campylobacter* spp. Of those 100, 68 samples contained *C. jejuni* DNA only, six contained *C. coli* DNA only, 17 contained both *C. jejuni* and *C. coli* DNA, and nine contained *Campylobacter* spp. DNA not identified as either *C. jejuni* or *C. coli*.

3.3.4 Factors associated with *Campylobacter* spp. culture status of fecal samples

In a series of initial models accounting only for clustering within pen and feedlot, days-on-feed, feed treatment, feedlot size and regional health authority were

unconditionally associated ($p \leq 0.25$) with the odds of a positive *Campylobacter* spp. culture, Table 3.4.

Table 3.4 Unconditional analyses of risk factors for whether a sample was positive for *Campylobacter* spp. by culture (adjusting for clustering within pen and feedlot), n=2,776.

Variable	Level	# of samples	% of samples *Campylobacter* positive	Univariable *p* value
Density (animal/m^2)	< 0.1[a]	2,456	86.9	0.899
	≥ 0.1	320	89.1	
Days-on-feed	≤ 149[a]	1,830	89.5	0.004
	150-299	856	82.8	
	≥ 300	90	82.2	
Feed treatment[c]	No treatment[a]	35	71.4	< 0.001
	Monensin/tyl	1,176	91.6	
	Monensin/chlortet/ tyl	765	77.1	
	Monensin/oxytet	800	91.0	
Feedlot size (head capacity)	10,000-19,999[a]	1,600	91.2	< 0.001
	20,000-39,999	780	76.4	
	≥ 40,000	396	92.2	
# Head per pen	≤ 99[a]	612	85.8	0.890
	100-199	896	86.7	
	200-299	879	86.9	
	≥ 300	389	91.0	
RHA where feedlot located	5[a]	393	75.6	0.006
	3	387	77.3	
	2	396	91.2	
	1	1,600	92.2	
Season of sampling	Winter[a]	1,400	86.4	0.401
	Summer	1,376	87.9	
Gender	Steer[a]	1,828	88.7	0.342
	Heifer	859	85.2	
	Mixed	89	74.2	
Weight (kg)	≤ 499[a]	150	79.3	0.619
	500-599	941	86.9	
	≥ 600	1,285	86.8	
	Missing[b]	400	92.0	

[a] reference category; [b] data missing from two feedlots for the summer sampling (40 pens); [c] feed treatments for seven days or more during the feeding period.
d: days; chlortet: chlortetracycline; oxytet: oxytetracycline; RHA: regional health authority; tyl: tylosin.

Table 3.5 Final multivariable model (accounting for clustering within pen and feedlot) showing the association between risk factors and whether a fecal sample was positive for *Campylobacter* spp. by culture (2,420 of 2,776 positive, 280 pens, seven feedlots)

Pen-level risk factors		OR[b]	95% CI	Specific *p* values	Overall *p* value
Days on feed					0.005
	≤ 149 days on feed[a]				
	150-299 days on feed	0.63	0.46-0.86	0.003	
	≥ 300 days on feed	0.47	0.22-1.02	0.054	
Feedlot size (head capacity)					< 0.001
	10,000-19,999[a]				
	20,000 to 39,999	0.29	0.15-0.53	< 0.001	
	≥ 40,000	0.93	0.39-2.21	0.862	

[a] reference category; [b] for ease of interpretation odds ratios have been inverted (e.g. 1/0.65=1.54) in results and discussion text; CI: confidence interval; OR: odds ratio;

In the final multivariable model accounting for clustering within pen and feedlot, both days-on-feed, and feedlot size were associated ($p \leq 0.05$) with the odds of positive culture, Table 3.5. No evidence of interaction or confounding was identified. The pen-level variance in the null mixed model was estimated to be 0.22 (22%) and the feedlot-level variance was 0.12 (12%). In the final mixed model, the pen-level variance reduced to 0.13 (13%), and the feedlot-level variance reduced to 0.03 (3%).

After adjusting for feedlot size, the odds of a sample (within the same pen and feedlot) testing positive for *Campylobacter* spp. was 1.59 times greater among animals that had been in the feedlot for less than 150 days than it was for animals in the feedlot for 150-299 days ($p = 0.003$) and 2.13 times greater in animals on feed for less than 150 days than it was for those animals in the feedlot for more than 300 days ($p = 0.054$). After accounting for days on feed, the odds of yielding a positive test for *Campylobacter*

spp. was 3.45 times greater ($p < 0.001$) in pens from smaller feedlots (10,000-19,999 head) compared to pens from medium sized feedlots (20,000 to 39,999 head), and not statistically different (OR 1.08 $p = 0.862$) when pens from smaller feedlots (10,000-19,999 head) were compared to pens from large feedlots (≥ 40,000 head), Table 3.5.

3.4 Discussion

The current study was designed to sample cattle near slaughter weight from large commercial Alberta feedlots. In previous *Campylobacter* research in Canadian feedlot cattle, animals may have been maintained in experimental feedlots (Inglis et al. 2003, Inglis et al. 2004, Inglis et al. 2005b, Lefebvre et al. 2006), which may not be representative of animal populations in commercial feedlots. Commercial Alberta feedlot cattle fecal *Campylobacter* studies have been published; however, these usually involved a smaller number of feedlots (one to four) and differing study designs (Besser et al. 2005, Inglis et al. 2006). This study targeted cattle near the end of the feedlot stay, and was timed to establish *Campylobacter* isolation rates in cattle feces just prior to animals entering the food chain. Our results suggest that in these large commercial feedlots a large proportion of individual animals and pens of cattle are shedding *Campylobacter* spp. by the time they are shipped for slaughter.

The fecal prevalences of *Campylobacter* spp. in slaughter-age animals from this study may be relevant to both food safety and public health in Canada. The overall culture prevalence of *Campylobacter* spp. in cattle feces reported here (87%, 95% CI 86-88) is similar to smaller PCR surveys in Alberta (Inglis et al. 2003, Inglis et al. 2004). However, our study is the highest published estimate of viable (culturable) *Campylobacter* spp. based on cattle feces from large commercial feedlots in Alberta.

High fecal *Campylobacter* prevalences may have direct health implications for beef and slaughter industry workers. Further, cattle feces may be an important source of *Campylobacter* contamination within slaughter plants; potentially relevant to consumer exposure to this pathogen in retail beef.

The *C. jejuni* cattle feces prevalence in summer (61%), was higher than recently published estimates from Alberta (Besser et al. 2005, Inglis et al. 2003, Inglis et al. 2004, Inglis et al. 2005a, Inglis et al. 2005b, Inglis et al. 2006), Ireland (Minihan et al. 2004) and Australia (Bailey et al. 2003) and lower than a recent North American estimate (Lee et al. 2004). Because *C. jejuni* is implicated in most cases of human campylobacteriosis, the high shedding of this bacterium in Alberta feedlot cattle feces is worthy of continued epidemiological research. Species of thermophilic campylobacters other than *C. jejuni* (including *C. coli*, *C. hyointestinalis* and C. *lanienae*) have been identified in cattle and may have implications for public health (Humphrey et al. 2007, Inglis et al. 2003, Inglis et al. 2004, Logan et al. 2000). These other bacterial species were not specifically identified in our study but would be included in our general *Campylobacter* spp. prevalence estimates.

The risk to people from *Campylobacter* spp. or *C. jejuni* from cattle is not fully known. Campylobacters are usually considered relatively fragile organisms, susceptible to dessication, pH, temperature, osmotic, and oxidative stressors (Murphy et al. 2006). However, the ability of these microbes to persist in a viable but non culturable state, in biofilm, in untreated water, and during chilling or freezing (Buswell et al. 1998, Clark et al. 2003, Moorhead and Dykes 2002, Murphy et al. 2006) contributes to their importance as foodborne and environmental pathogens.

Fecal samples in this study were collected using swabs instead of grams of feces. Collecting more fecal matter may have improved *Campylobacter* recovery with culture, resulting in higher prevalence levels. However, the swab transport media used in our study worked well in protecting campylobacters during transit. Only two shipments dipped below the 0°C mark with minimum temperatures of -5.8°C and-0.2°C. The lower of these was the shipment delayed in transport, and the culture findings for that shipment (Feedlot A winter) were similar to others obtained in the study. Our high recovery of culturable *Campylobacter* spp. isolates supports the use of fresh pen-floor swabs as an efficient, economically feasible and non-invasive sampling technique for feedlot cattle feces.

In general, campylobacters are not thought to survive for extended periods in fecal pats on the ground due to exposure to air, drying and temperature extremes. Hoar et al found a 90% difference in the prevalence of *Campylobacter* spp. when comparing rectal samples to ground fecal pats (5.0 and 0.5% positive respectively) (Hoar et al. 1999), and this was the reason why only "steaming" pats were sampled in this study. Research has shown that the distribution of *Escherichia coli* may not be uniform within a fecal pat (Pearce et al. 2004), therefore each swab was inserted into each pen-floor pat in five different locations.

Culture and hippurate hydrolysis techniques were used for identification of *Campylobacter* spp. and *C. jejuni.* Only one colony was selected per culture plate for *Campylobacter* spp. identification. This may have resulted in reduced prevalences based on the selection of the colony. For the initial winter collection, approximately two samples per pen were tested for hippurate hydrolysis. For the summer collection it was

decided to expand testing to include all of the summer *Campylobacter* spp. positive isolates, which increased the accuracy (narrowed the confidence intervals) for the summer prevalence estimates.

As an assessment of the accuracy of culture and biochemical identification, a within-study sensitivity analysis was conducted using PCR (n=104) to confirm isolates as *C. jejuni* for future molecular assays. At the genus level, the *Campylobacter* spp. culture results and PCR were very similar with only a 4% difference between the two. The PCR did indicate that hippurate hydrolysis testing may have overestimated the prevalence of *C. jejuni* in cattle feces. False positives and false negative results have been reported in the literature using this hippurate hydrolysis testing, and some *C. jejuni* strains have been found to be hippuricase negative (Nakari et al. 2008), illustrating the challenges of this phenotypic assessment. One of the goals of this study was to accumulate *C. jejuni* isolates for future molecular characterization. As a result, weak hippurate hydrolysis reactions were considered positive, which may have contributed to the discrepancy between the two techniques. While PCR can be very sensitive and specific with appropriate genetic sequence design (Nakari et al. 2008), it is unable to indicate organism viability, generally of interest in food safety, and unable to provide viable cultures which may be stored for future research.

Campylobacter spp. prevalence differed significantly among feedlots. Differences between feedlots may be due to factors such as geography, environment (temperature, precipitation), management (treatment protocols, cattle purchasing preferences), reservoir prevalence (flies, birds) or other unidentified factors. Feedlot size was a statistically significant predictor (overall p value ≤ 0.05) of *Campylobacter*

isolation rates; however the inclusion of only one feedlot with a capacity of ≥ 40,000 head may have contributed to the non significant *p* value for this category. The effect of feedlot size on *Campylobacter* isolation rates most likely reflects management differences between feedlots that were not evaluated in this study.

Feedlots entered this study based on willingness to participate (eight approached, one declined). It is possible that the non-random selection of feedlots and the use of feedlots that were willing to participate could have affected the results. Feedlots willing to participate might be different from other feedlots in the province based on size, management practices or use of veterinary services. Research into reasons for differences among feedlots should be pursued, and future risk factor studies should include a larger number of feedlots (randomly selected) to minimize potential bias and increase power in the study.

Seasonal effects were assessed in this study using winter and summer point estimates, and as such generalization of these estimates to seasonal trends should be cautious. Specifically designed longitudinal studies are required to appropriately identify trends. However based on our seasonal estimates, the proportion of samples that were culture positive for *Campylobacter* spp. was not significantly different between the winter and summer sampling periods. Previously published studies have shown that feedlot cattle shed *Campylobacter* spp. chronically (Besser et al. 2005, Inglis et al. 2004, Minihan et al. 2004), suggesting that within the feedlot it may be difficult for animals to clear these bacteria from the intestinal tract. This may be due to constant exposure to fresh feces (on pen boards, feed troughs, water troughs (Minihan et al. 2004), high stocking densities in feedlot pens, presence of biofilm within pens, or stabilized

Campylobacter populations related to finishing diets. Cattle have a relatively long lifespan compared to poultry and, as campylobacters seem able to adapt successfully to the ruminant digestive system, it may not be surprising that our summer and winter prevalence estimates were similar. Further, the persistence of *Campylobacter* spp. within reservoirs such as water sources, wild birds and flies may continue the animal-host-environment cycle.

This was a preliminary investigation of pen and feedlot level risk factors for shedding of *Campylobacter* spp. and *C. jejuni* in Alberta feedlot cattle, and is a platform (study design and results) from which more comprehensive and specific investigations may follow. *Campylobacter* spp. isolation rates were lower in pens of animals that had been in the feedlot for longer periods of time. This finding may reflect animal or bacterial physiological factors, the effects of antimicrobials in feed, or the use of growth promotants (e.g. ionophores). Although some longitudinal studies report chronic and rising carriage of *Campylobacter spp.* over time (Besser et al. 2005, Inglis et al. 2004, Inglis et al. 2006, Minihan et al. 2004), Lefebvre et al. found a decreasing prevalence of *Campylobacter* spp. in a longitudinal study on the use of growth promotants in Canadian feedlot cattle (Lefebvre et al. 2006).

As this research was designed at the pen-level, individual animal information on injectable antimicrobial use was not collected, and it is possible that treatments at the individual animal level affected shedding of campylobacters and *C. jejuni*. In addition, dosages of antimicrobials in feed were not accounted for in analyses, and it is possible that the broad classifications used to compare feed treatments in this study may have

masked true medicated feed differences. Future study designs should incorporate individual animal data and specific feed dosages into feedlot cattle risk factor analyses.

The estimates of pen level and feedlot level proportions of variation in *Campylobacter* spp. sample status reported here may be used in future research for sample size calculations in multistage designs. The results suggest that the amount of clustering within feedlot is small, and explained to a large extent by the variables in the risk factor model. Most of the clustering was found at the pen-level, and while some of this was explained by variables in the final model, inclusion of a greater number of pen-level risk factors is warranted in the future.

3.5 Conclusions

Beef cattle in Alberta are an integral part of the landscape and economy, and exist in close proximity to people in many rural areas. The high prevalence levels found in this study suggest that a large proportion of feedlot cattle near slaughter weight may be shedding campylobacters, and that seasonal effects may be small. These findings may have important implications for food safety, public health, and environmental transmission of campylobacters in the province, and further investigations will be required to fully understand the role of cattle in the epidemiology of campylobacters in Alberta.

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CHAPTER 4
PREVALENCE AND RISK FACTOR INVESTIGATION OF *CAMPYLOBACTER* SPECIES AND *CAMPYLOBACTER JEJUNI* IN RETAIL GROUND BEEF

4.1 Introduction

In Alberta, campylobacteriosis is the most common bacterial enteric illness, with 36.1 cases per 100,000 people reported in 2005 (PHAC 2007, Statistics Canada 2007). *Campylobacter jejuni* (*C. jejuni*) is the most frequently isolated species in human disease, responsible for approximately 85% of all human *Campylobacter* infections (Moore et al. 2005). While consumption of contaminated poultry meat is generally considered the primary source of infection for people (Humphrey et al. 2007), molecular typing has found strain similarities between human and other domestic animal isolates including cattle and swine (Clark et al. 2003, Johnsen et al. 2006, Manning et al. 2003, Nielsen et al. 2005).

The beef cattle industry is very important to the economies of both Alberta and Canada. In 2005, the Canadian beef industry was the largest source of farm cash receipts from a single agricultural commodity (Statistics Canada 2006). In that same year, 2.5 million cattle were slaughtered in federal or provincially regulated slaughter plants in Alberta, 63% of the national total, and Canadian per capita consumption of beef was 51 lbs (CanFax 2007b). Recently, the prevalence of culturable *Campylobacte*r spp. and *C. jejuni* in Alberta feedlot cattle feces near the end of the feeding period was found to be extremely high (87% and 61% respectively) (Besser et al. 2005, Inglis et al. 2004),

see Chapter 2. However, research into the prevalence of *Campylobacter* spp. in retail ground beef in Alberta has been limited. In 2006, a Canadian survey of retail ground beef reported no positive samples from the 100 packages tested (Bohaychuk et al. 2006). The prevalence of *Campylobacter* spp. in retail ground beef has ranged from 0-20% worldwide (Bohaychuk et al. 2006, Bolton et al. 1985b, Bosilevac et al. 2007, Cloak et al. 2001, Fukushima et al. 1987, Ghafir et al. 2007, Little and de Louvois 1998, Turnbull and Rose 1982, Wong et al. 2007). Because of the high prevalence of *Campylobacter* spp. in feedlot cattle and the clinical importance of this pathogen in people, this survey was developed as an initial assessment of the potential public health importance of retail ground beef as a source of *Campylobacter* in Alberta.

The goals of this project were to assess the prevalence *Campylobacter* spp. and *C. jejuni*, and to investigate risk factors potentially associated with the presence of *Campylobacter* spp. in retail ground beef. This chapter reports results of a survey of 1,200 packages of ground beef from 60 retail grocers of four major chains in three cities in southern Alberta.

4.2 Materials and methods

4.2.1 Sample size calculation

For a survey using simple random sampling, 179 packages of ground beef would have been necessary to measure a 3% expected prevalence of *C. jejuni* (Whyte et al. 2004) with 2.5% precision and 95% confidence (Epi-Info, version 3.01, CDC, USA, 2003). After applying an inflation factor formula (Dohoo et al. 2003) to account for clustering of the expected frequency of *Campylobacter* within retail stores, the survey required 1,200 packages from 60 stores (assuming an intraclass correlation coefficient

(ICC) of 0.3, an unadjusted sample size of 179, and collection of 20 packages per store). An ICC describing clustering of *C. jejuni* within source was not available from previous publications; the choice of 0.3 was slightly more conservative than previously published ICCs for non-enteric cattle conditions (McDermott and Schukken 1994). Ethics approval for this project was received from both the University of Saskatchewan Biomedical Research and the University of Calgary Conjoint Health Research Ethics Boards.

4.2.2 Sampling protocol.

The goal of sampling was to identify grocery chains likely to supply the largest sales volume of ground beef to consumers. Four chains with the highest number of retail stores from three cities in southern Alberta were identified, and a sampling frame of individual stores was compiled from telephone book white and yellow pages (chain name and pharmacy headings) and internet searches (chain name). Stratified random sampling (by city and by chain within city) ensured that meat samples were taken from all chains in all cities. Fifteen stores were sampled from chain 1, 22 from chain 2, 16 from chain 3 and seven from chain 4. Forty-six stores were sampled in city 1, six stores in city 2 and eight stores in city 3. Five packages per store per collection were randomly sampled from the 60 stores using a hand-held randomization program (Handy Randy, Stevens Creek Software, Cupertino, CA, USA), for a total of 1,200 retail packages of regular or lean ground beef. Three hundred packages were purchased during each of four collection periods: two winter (Nov 21-23, 2004, and Jan 9-11, 2005) and two summer (May 30, 31, June 1, 2005 and July 18-20, 2005). After purchase, each package of ground beef was placed into a pre-labeled Ziploc bag (SC Johnson, Racine, WI,

USA) and then packed into a cooler (The Coleman Company Inc., 5286B, Wichita, KS, USA) with six ice packs (Ice-Pak/Hot-Pak, Montreal, QC, Canada). A Hobo H08 Pro temperature monitor (Onset Computer Corporation, Pocasset, MA, USA) was included in one cooler from each of the 12 meat shipments. Each cooler was sealed and shipped to the Vaccine and Infectious Disease Organization (VIDO, Saskatoon, SK, Canada) by bus (Greyhound Transport Canada Corporation) overnight. Ground beef packages were processed within approximately 24 hours of collection. Transport temperature ranges were evaluated from two hours after closure to two hours before cooler was opened.

Employees knowledgeable about in-store meat practices were identified by phone inquiry or observed directly working with meat, and were asked questions regarding their meat department practices. Information on the cutting and packaging of raw poultry, the type of meat used to produce the ground beef (coarse tubes, market trim or both) and whether the ground beef contained meat that has previously been frozen were collected.

4.2.3 Experimental inoculation of retail ground beef as sensitivity analysis.

Prior to the study collections, packages of ground beef were purchased and inoculated with fresh laboratory strain of *C. jejuni* (NCTC 11168) and then cultured using the same technique as the study (see below). Retail ground beef was mixed with *C. jejuni* at concentrations of $1x10^4$, $1x10^3$, $1x10^2$, and $1x10^1$ cfu/g. Five packages of retail ground beef were tested at each concentration, and the experiment was repeated on two separate occasions. Each incubation of test plates included both a negative control plate and a laboratory strain *C. jejuni* plate as positive control. These experiments were

conducted to document our ability to consistently recover *C. jejuni* from ground beef using our culture protocol.

4.2.4 Detection of campylobacters using enrichment culture.

For each package of retail ground beef, the plastic wrap over the middle was sliced with a sterile scalpel blade. A deep core sample of 25 g (24-26 g) of raw ground beef was removed with a sterile spoon. Each sample was placed into a 7 by 12 in. 55oz Whirl Pak bag (82007-726, VWR International, Mississauga, ON, Canada) containing 100 ml of a Bolton broth (# CM0983 Oxoid Ltd., Basingstoke, UK) and 5% horse blood mixture and stomached for 30 seconds (Stomacher Lab Blender 400). The homogenate was then incubated (85% N_2, 10% CO_2, 5% O_2) for 44 hours at 42°C and then streaked onto Karmali selective agar (Oxoid, CM935 with supplement SR0167E, Nepean, ON, Canada) and microaerobically (85% N_2, 10% CO_2, 5% O_2) incubated at 42°C for 48-72 hours. Each culture plate was then examined visually for colonies characteristic of *Campylobacter* spp. (based on growth, color and morphology of the colony, and color of the cell mass). Each incubation included a laboratory strain *C. jejuni* plate as positive control.

4.2.5 Detection of campylobacters by polymerase chain reaction (PCR).

At the same time as samples were taken for culture, ground beef from approximately 10% of the 1,200 packages was collected for PCR (52 of 60 stores represented). A labeled plastic vial (# 9556002 Canadawide Scientific, Ottawa, ON, Canada) was filled with approximately 1 g of retail ground beef from each of 142 packages and frozen at -70°C. These samples were sent on ice to the Agriculture and

Agri-Food Canada laboratory in Lethbridge, AB, Canada for PCR testing. Briefly, a subsample of ground beef (1 g) was thawed and placed in a BagPage 100 filtered blending bag (EW-36840-58; Canadawide Scientific Ltd, Ottawa, ON, Canada) containing 9 ml of Columbia broth (Becton, Dickinson and Company, Sparks, NV, USA), and the sample was homogenized for 120 seconds at high setting using a Stomacher 80 blender (Seward Ltd., West Sussex, UK). The homogenate was then removed, centrifuged at 1,750 x g for 10 minutes and the supernatant containing *Campylobacter* cells was collected. To concentrate *Campylobacter* cells, the supernatant was centrifuged at 24,050 x g for 10 minutes, and the supernatant removed and discarded. The pellet was re-suspended in 1 ml of Columbia broth, 200 µl aliquots were placed in 2 ml tubes, and samples stored at -20^{o}C until processed. Just before extraction, an internal amplification control (IAC; 10 µl containing 700 copies/µl) was added to each tube (Inglis and Kalischuk 2003b), and DNA was extracted using the DNAeasy Tissue Kit (Qiagen, Missassauga, Canada) according to the manufacturer's protocol. Direct PCR was applied for *Campylobacter* genus, IAC, *C. jejuni*, *C. coli*, *C. fetus*, *C. hyointestinalis*, and *C. lanienae* (Inglis and Kalischuk 2003a). In addition, nested PCR to detect *C. concisus* and *C. upsaliensis* was applied (Inglis *et al.* unpublished).

4.2.6 Data analysis

Descriptive analyses were done in SPSS (version 15.0; SPSS, Chicago, US). A second commercial software package (MLwiN version 2.02; Centre for Multilevel Modeling, Institute of Education, London, UK) was used for the hierarchical model analysis. The hierarchical models were specified with a logit link, binomial distribution, restricted iterative generalized least square and second order penalized quasi-likelihood

nonlinear estimation (Dohoo et al. 2003). The outcome was whether or not a ground beef sample was positive for *Campylobacter* spp. DNA. Variables included "poultry cutting" (whether or not poultry was cut or packaged in the meat department), "trim type" (what source of ground beef was used in the grinding; coarse grind tubes, market trim or a combination), "city" (1, 2 or 3), "collection" (collection period 1, 2, 3,4), "package type" (lean or regular ground beef), and "weight" (kg, the only continuous variable). The scale of the "weight" variable was explored and categorize into "weight_c" (package less than 0.5 kg, package 0.5 to 0.999 kg, or package 1.0 kg or greater) to evaluate model linearity assumptions. Random effects (e.g. chain or store levels) were kept in the model if more than one variable at that level was entered as a fixed effect, if the amount of variability explained at that level was greater than 10%, or if the level was believed to be important to the data structure *a priori.*

4.3 Results

4.3.1 Experimental inoculation.

Of the 40 ground beef samples inoculated, only one sample at $1x10^2$ cfu/g did not yield *C. jejuni.* All other samples and the positive control plates were positive for *C. jejuni*, indicating that *C. jejuni* could be consistently isolated from ground beef using the study protocol, while none of the negative control plates grew *Campylobacter* spp.

4.3.2 Prevalence survey using culture.

All 60 stores reported that they did a final grind of beef in-store, that the beef was of Canadian origin, and that the source beef for grinding came from local (Alberta) slaughter plants or processors. Twenty-seven stores used coarse ground tubes, 17 stores

used market trim, and 16 stores used a combination of both for their second in-store grind. Forty stores did not package or cut raw poultry in the department, 19 stores reported cutting or packaging some poultry products (e.g. wings) and for one store data were unavailable. Fifty-six stores used fresh meat only, while in four stores the retail ground beef may have included previously frozen portions. Of the 1,200 packages of retail ground beef, 726 were lean and 474 were regular ground beef. Twenty-eight packages were labeled as a "discount". By weight, 121 packages were less than 0.5 kg, 1,030 packages were between 0.5 kg and 0.999 kg, and 49 packages were greater than or equal to 1.0 kg. Transport temperatures ranged from 3.31°C to 9.03°C in the six summer shipments and -2.44°C to 9.42°C in the six winter shipments.

Campylobacter species were not isolated from any of the 1,200 packages of retail ground beef.

4.3.3 PCR detection of campylobacters.

Of the 142 samples tested using PCR, 65 (46%) were positive for DNA of *Campylobacter* spp. origin while 77 were negative, Table 4.1.

Table 4.1 *Campylobacter* spp. in retail ground beef (n=142) based on PCR

Identification	Positive (%)
Genus*:*	
Campylobacter spp.	65 (45.8)
Species [a,b]*:*	
C. jejuni only	20 (14.1)
C. coli only	35 (24.6)
C. jejuni and *C. coli*	1 (0.7)
C. coli and *C. hyointestinalis*	2 (1.4)

[a] seven isolates could not be identified to the species level; [b] zero samples tested positive for DNA of *C. fetus, C. lanienae, C. concisus* or *C. upsaliensis.*

Two of the 142 samples tested using PCR could not be linked to store or chain. The remaining 140 ground beef samples represented 52 different stores. Twelve stores had greater than one meat sample tested from the same collection period. Of these 12 stores, only four stores had greater than one meat sample positive for DNA of *Campylobacter* spp. origin. Ten of these 12 stores had either four or five samples from the same collection period tested with PCR, and the most any store had positive for DNA of *Campylobacter* spp. origin was two samples.

4.3.4 Factors associated with PCR detection of *Campylobacter* spp.

Of the 142 ground beef samples submitted for PCR testing, two samples could not be linked to store or chain and were omitted from all analyses. For one sample, data were missing for whether or not the source store cut poultry. This sample was included in risk factor analysis, and designated 'missing' in the "poultry" analysis.

Chain did not explain an important part of the variance in the null model (chain level variance 0.000, standard error 0.000) and was not included as a random effect in the final analysis. After accounting for clustering within the store of origin, only the package type and the collection period variables were selected for consideration in the development of a final model ($p \leq 0.25$), Table 4.2. None of the other risk factors considered (chain, city, inclusion of frozen portions, on-site poultry cutting practices, kinds of trim in the ground beef or package weight) were associated with the odds of detecting campylobacters by PCR, Table 4.2.

Table 4.2 Unconditional analyses of risk factors for whether a sample was positive for *Campylobacter* spp. by direct PCR (n=140)

Variable	Level	# of packages	% packages *C.*spp. positive at each level	*p* value
Chain	1[a]	28	42.9	0.936
	2	45	46.7	
	3	47	51.1	
	4	20	35.0	
City	1[a]	109	45.0	0.891
	2	9	55.6	
	3	22	45.5	
Collection period	1[a]	30	30.0	< 0.001
	2	30	66.7	
	3	31	80.6	
	4	49	20.4	
Frozen portions	No[a]	124	47.6	0.459
	Yes	16	31.3	
Package type	Lean[a]	86	40.7	0.157
	Regular	54	53.7	
Poultry cutting[b]	No[a]	94	48.9	0.937
	Yes	40	45.0	
Trim type	Coarse grind tube[a]	56	41.1	0.876
	Market trim	50	50.0	
	Both	34	47.1	
Weight_c	≥1.0 kg	10	30.0	0.343
	0.5-0.999 kg	113	48.7	
	≤0.499 kg[a]	17	35.3	

[a] Reference category; [b] Data unavailable for one store (six packages).
C. spp.: *Campylobacter* species.

When package type (regular or lean) and collection period (1: Nov 21-23, 2004, 2: Jan 9-11, 2005, 3: May 30, 31, June 1, 2005, and 4: July 18-20, 2005) were examined together, only the collection period was significantly associated ($p \leq 0.05$) with the odds of detecting *Campylobacter* spp. by PCR. The odds of a retail ground beef package testing positive for *Campylobacter* spp. DNA was 5.6 times greater if the package was

from collection 2 compared to collection 1 (OR 5.6, 95% CI 1.8-17.5). Further, a package had 12 times greater odds of testing positive for *Campylobacter* spp. DNA if it was from collection 3 compared to collection 1 (OR 12.0, 95% CI 3.5-42.0). Ground beef from collection 4 was not statistically different from collection 1 (OR 0.6, 95% CI 0.2-2.0).

4.4 Discussion

The samples from this large retail ground beef survey represented four different chains and three cities in southern Alberta. Random selection of packages in stores, multiple collection periods, and limiting the number of packages purchased per store were used to try and avoid oversampling the same meat batches. In 2005, source beef for ground beef likely came from the six federally inspected slaughter plants in Alberta (AAFC 2005), or from provincially inspected facilities. Because retail chains likely purchased meat from the same plants or processors, it was expected that variation within chain would be small. As a result, only five packages of ground beef were purchased from each store at each collection time.

We initially hypothesized that cross-contamination of surfaces and equipment from raw poultry cutting and packaging might lead to ground beef contamination. However, 67% of stores received raw poultry products already packaged for retail sale, and the model did not find a statistically significant relationship between poultry cutting and the presence of *Campylobacter* spp.; shifting focus to slaughterhouse environments for possible contamination sources.

It is possible that nonrandom selection of ground beef for PCR testing might have influenced PCR prevalence findings, even though 52 of 60 stores were represented in this sample. Further, hierarchical models were likely hampered by the small sample size (n=142) tested with PCR. However, individual collection periods were associated with the presence of *Campylobacter* spp. Collections 1 and 2 were during the winter while collections 3 and 4 were in the summer. The results did not indicate a seasonal difference as one winter and one summer collection period were significantly different from the others. However, these findings do indicate that differences may exist in *Campylobacter* spp. contamination at slaughter plant or retail store levels. Descriptive analyses found that from the five packages collected at the same store on the same day, one package might be positive and the others negative. This may reflect differing package contamination levels, within package *Campylobacter* distribution (as only 1 g of ground beef was collected from the centre of each package), or possible dilution effects from the PCR process. Further, variables within the control of the meat department or slaughter plants and processors (e.g. carcass cleanliness, hygiene practices, cross-contamination through fomite transmission) may contribute to variability between collections.

It can be difficult to compare laboratory protocols with other published research because consensus and standardization of culturing methodology for campylobacters has not been achieved, because many incubation and temperature protocols, culture media, and antimicrobial supplements are available, and because viable but non culturable *Campylobacter* strains may exist (Corry et al. 1995, Habib et al. 2008, Wonglumsom et

al. 2001). Based on the spiked meat experiment, under laboratory conditions the culture technique used in this study was able to isolate *C. jejuni* at 1 x 10^1 cfu/g in samples; a level below the estimated dose required for human infection (Black et al. 1988, Humphrey et al. 2007). Further, under the culture conditions examined here none of the 1,200 packages of ground beef were positive for viable *Campylobacter* spp., an encouraging finding for public health in Alberta.

Hazard and critical control points (HACCP) have been identified and programs implemented in all federally registered beef slaughter plants in Canada (CFIA 2007b). Protocols at slaughter, including hide-on-carcass, lactic acid, hot water, and carcass washes, chilling, and the ability to remove potentially contaminating components

(e.g. hides and intestinal tracts) quickly and intact may all contribute to bacterial numbers below detectable levels in the meat. However specifically designed slaughter house sampling would be required to quantify pathogen levels within those environments and in meat products on-site.

The very low prevalence of culturable *Campylobacter* levels in retail ground beef observed in this study is similar to other North American ground beef surveys. Campylobacters were identified in only 1 of 2073 packages of ground beef using culture in the USA from 2002-2005 (Gov of USA 2006), and a smaller Alberta survey found zero of 100 packages positive (Bohaychuk et al. 2006). However it is possible that the laboratory sensitivity of the culture method used here may not have been high enough to pick up very low numbers of organisms. Further, if campylobacters were sufficiently stressed, it is possible the method was not able to resuscitate these pathogens sufficiently

for growth with culture. Three of the meat shipments dipped below the 0°C mark during shipping, however campylobacters have been isolated from ground beef frozen at -18°C for 90 days (Grigoriadis et al. 1997), and culture recovery in our study did not vary between summer and winter samplings.

Some researchers propose a preincubation step at 37°C to try and improve recovery and growth of campylobacters at culture (Humphrey 1994), which was not used here. However other published research has used similar temperature regimens to ours and had success in recovering viable campylobacters from abattoir and retail beef (Bolton et al. 1982b, Bolton et al. 1985, Fricker and Park 1989, Fukushima et al. 1987, Garcia et al. 1985, Korsak et al. 1998, Kwiatek et al. 1990, Lammerding et al. 1988, Lee et al. 2004, Mayrhofer et al. 2005, Osano and Arimi 1999a, Pezzotti et al. 2003, Whyte et al. 2004, Zhao et al. 2001). Further, viable campylobacters were also not recovered in a recent Alberta ground beef survey which used lower preincubation temperatures (Bohaychuk et al. 2006).

From the direct PCR results, *Campylobacter* DNA (*C. jejuni*, *C. coli*, and *C. hyointestinalis*) were identified in the meat. However, the DNA may have been from dead or damaged organisms, or from campylobacters sufficiently stressed to enter into a viable but non culturable state. Feedlot cattle may carry a variety of *Campylobacter* spp. including *C. jejuni* and *C. coli* in their intestinal tracts, and *C. jejuni* is the most frequently isolated species (Inglis et al. 2006), while *C. coli* is the most common *Campylobacter* species found in swine (Moore et al. 2005). The finding of 27% (38/142) of samples PCR positive for *C. coli* and only 15% (21/142) of samples PCR

positive for *C. jejuni* was interesting. Stores were asked about the cutting and packaging of raw poultry, but not raw pork, and this may be a consideration for future research.

4.6 Conclusions

The prevalence of *Campylobacter* spp. by PCR in this study was moderate to high (46%). PCR results do not necessarily indicate the presence of viable organisms, making it difficult to estimate from these molecular results the true public health risk from retail ground beef. However, continued research into potential interventions in the slaughter-processing-retail continuum may be of use to further lower DNA levels in the meat. The use of HACCP protocols within the beef slaughter industry and minimizing poultry cutting and packaging within store meat departments likely contributed to the low prevalence (0%) of viable *Campylobacter* in retail ground beef, supporting food safety practices in the province of Alberta.

4.6 References

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CHAPTER 5
GENOMICS-BASED MOLECULAR EPIDEMIOLOGY OF *CAMPYLOBACTER JEJUNI* ISOLATES FROM FEEDLOT CATTLE AND PEOPLE IN ALBERTA, CANADA

5.1 Introduction

The Alberta beef industry is economically important to the province as the largest source of farm cash receipts from a single agricultural commodity (2005 data) (Statistics Canada 2006), and Alberta had 2,370,800 cattle on feed; 67% of the national total (CanFax 2007b) in 2005. In that same year, campylobacteriosis was the most common (notifiable) bacterial enteric disease with a provincial rate of 36.1 cases for every 100,000 people (PHAC 2007, Statistics Canada 2007). Because of the relatively high number of human cases and the large numbers of cattle on feed in Alberta, research into the role of feedlot cattle as *Campylobacter* reservoirs has been ongoing (Inglis et al. 2003, Inglis et al. 2004, Inglis et al. 2006). *Campylobacter jejuni (C. jejuni)* is of public health significance as the most common *Campylobacter* spp. isolated from human cases (approximately 85%) (Moore et al. 2005). Recent studies have documented the shedding of *C. jejuni* from Alberta feedlot cattle to be high (32-62 % of animals positive) (Besser et al. 2005, Inglis et al. 2003, Inglis et al. 2004, Inglis et al. 2005, Inglis et al. 2006), reinforcing the need for continued research into the potential importance of cattle as reservoirs for these human pathogens.

Many thermophilic campylobacters are commensals in a wide range of warm blooded hosts and insects, and can persist and maintain viability in water sources,

biofilm and during environmental stress (Adhikari et al. 2004, Murphy et al. 2006). While poultry and poultry products are usually considered the main source for human *Campylobacter* infections (Humphrey et al. 2007), molecular typing studies suggest that cattle play a role in the epidemiology of campylobacteriosis (Colles et al. 2003, Manning et al. 2003, Nielsen et al. 2005), it is possible that other transmission routes exist. Poultry sources have not accounted for 100% of human infections and typing surveys have found human *Campylobacter* strains that do not exhibit similarity (do not cluster) with poultry strains (Dingle et al. 2001a, Manning et al. 2003, Michaud et al. 2005, Nielsen et al. 1997, Nielsen et al. 2005). Cattle and human isolates have been found to be similar using a variety of typing methods (Colles et al. 2003, Johnsen et al. 2006, Nielsen et al. 2005). In a study by Nielsen et al., human and cattle *C. jejuni* isolates were identical based on six molecular typing methods (Nielsen et al. 2000). Further, cattle strains have been able to infect poultry (Ziprin et al. 2003), suggesting that cattle may be a potential reservoir for poultry as well as people.

In 2000, the genetic sequencing of *Campylobacter jejuni* (*C. jejuni* NCTC 11168) by Parkhill et al. (Parkhill et al. 2000) led to the development of whole-genome DNA microarrays that could be used to study the comparative genomics of *C. jejuni* (Dorrell et al. 2001). DNA microarrays have been used in comparative genomic hybridization (CGH) surveys to analyze *C. jejuni* genomic variability (Champion et al. 2005, On et al. 2006, Parker et al. 2006, Taboada et al. 2004) and to explore the possibility of using CGH as a tool for epidemiological investigation (Leonard II et al. 2003).

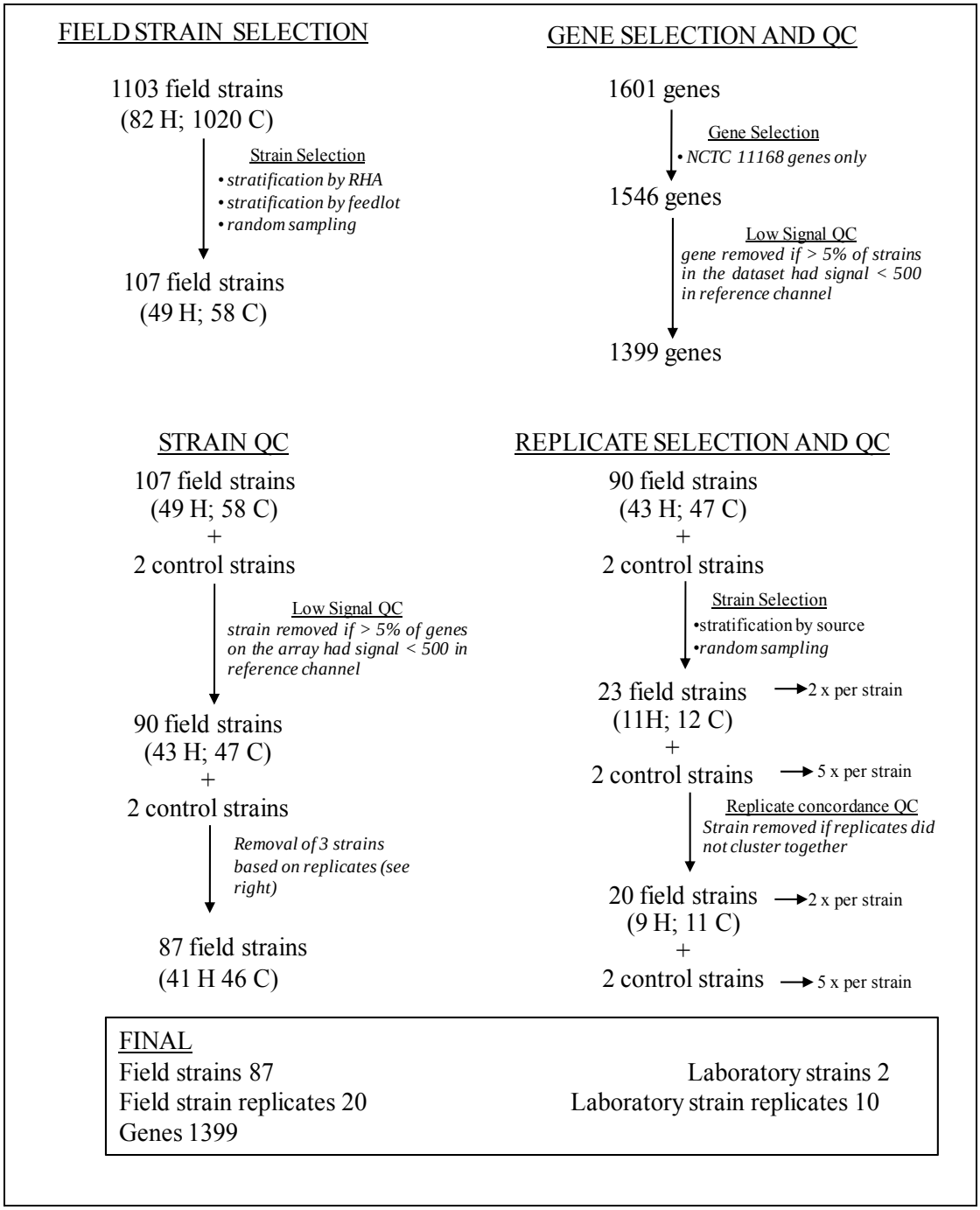
The purpose of this study was to perform comparative high-resolution genotyping (e.g. CGH analysis) on feedlot cattle and human clinical *C. jejuni* isolates obtained from the same geographical regions and during the same time frame in order to identify isolates with high levels of genomic similarity. This was a cross sectional study and it is not known if the persons represented by the human samples had any contact with cattle. Our goal was to use CGH to generate indirect evidence (preliminary assessment) as to the potential for cattle to be a source of *C. jejuni* infection for people based. Human and feedlot cattle isolates for this study were purposefully collected from regional health authorities in southern Alberta in both the winter and summer of 2005, and chosen for DNA microarray testing using stratified random selection.

5.2 Materials and methods

5.2.1 Analytical design

Figure 5.1 describes the pathway of inclusion and exclusion of field isolates, arrays, replicate arrays and genes throughout the analysis process.

Figure 5.1 Flow diagrams of field strains, genes and technical replicates through selection and data analysis



C: cattle, H: human, QC: quality control, RHA: regional health authority.

5.2.2 *C. jejuni* isolation from feedlot cattle

Cattle isolates were collected as part of a prevalence survey of seven large commercial feedlots from four regional health authorities (RHA 1, 2, 3, and 5) in Alberta, Chapter 3. Preliminary identification of *C. jejuni* was made in 1,020 samples based on positive culture (direct) and positive hippurate hydrolysis testing (Morris et al. 1985). Fifty-eight isolates were randomly selected (using Microsoft Office Excel 2007, Microsoft Corporation) after stratification by feedlot and season. Only one isolate was allowed per pen. These were confirmed as *C. jejuni* (based on the *hypO* gene) using multiplex PCR as previously described (Wang et al. 2002), and then subjected to high-resolution genotyping using DNA microarrays.

5.2.3 Human isolates

Eighty-two viable human isolates, identified as *C. jejuni* by diagnostic laboratories in Alberta regional health authorities (RHA 1, 2, and 3), were sent to the Alberta Provincial Laboratory of Public Health (APLPH). Isolates were screened to ensure that patients had not travelled outside Alberta within 30 days of sample submission, and that only one isolate per patient and per household was sent for microarray testing. In Alberta, the campylobacteriosis case definition is based on laboratory confirmation from an appropriate clinical specimen, with or without symptoms in the patient (Gov of AB 2005). Isolates were couriered to the Vaccine and Infectious Disease Organization (VIDO) on ice along with non-identifying information (date of birth, gender of patient, date of specimen submission and regional health authority). Upon receipt, isolates were plated onto Mueller–Hinton (MH) agar for 48

hours at 43°C to ensure pure culture, and then streaked onto three MH plates and incubated for 16-18 hours at 37°C (10%CO_2, 5% O_2, 85% N_2). Growth from three plates per strain was then suspended in a 50% brain-heart infusion-25% glycerol mixture and frozen to -70°C for genotyping at a later date. Human isolates were stratified by RHA and by season, and then randomly sampled. Data from 49 human *C. jejuni* arrays were initially entered into analysis.

5.2.4 Open reading frame *C. jejuni* NCTC 11168 DNA microarray

A *C. jejuni* oligonucleotide microarray from the *Campylobacter jejuni* Genome Oligo Set Version 1.0 was purchased from Operon Biotechnologies, Inc. (Huntsville, AL35805). This product contained 1,601 probes 70 base pairs in length and represented 1,546 open reading frames (ORFs) from *C. jejuni* subsp. *jejuni* NCTC 11168 (GenBank sequence AL111168); 51 ORFs from *C. jejuni* 81-176 virulence plasmid pVir (GenBank sequence AF226280); and four ORFs from *C. jejuni* plasmid pCJ01 (GenBank sequence AF301164). All 1,601 probes were designed within predicted ORFs by Operon using their proprietary software. The probes were normalized to a melting temperature of 71ºC (±5ºC). Triplicate spots were included for each ORF on the chip. The microarray slides were produced from this set of oligonucleotides by The Biomedical Genomics Center, University of Minnesota (Minneapolis, MN, USA). Conditions required for optimal hybridization of these arrays using Cyanine dye detection systems are outlined below. For this study, only genes from *C. jejuni* NCTC 11168 (n=1,546) were analyzed; plasmid data available on the arrays were not included in this analysis.

5.2.5 Genomic DNA extraction and labeling

Genomic DNA isolation was performed using a modification of the hexadecyltrimethyl ammonium bromide (CTAB) procedure (Wilson et al. 1987). Briefly, cells were suspended in 567 µL of TE (10mM Tris HCl and 1 mM EDTA pH 8.0). Proteinase K and sodium dodecyl sulfate were added to a final concentration of 100 µg/mL and 0.5% respectively. After incubation for one hour at 37˚C 100 µL of 5 M NaCl was added and the suspension was mixed thoroughly. 80 µL of 10% CTAB in 0.7 M NaCl were added and the mixture was incubated at 65˚C for 10 minutes. An equal volume of chloroform/isoamyl alcohol (24:1) was added and after thorough mixing the CTAB-protein/polysaccharide complex was removed by centrifugation. The aqueous supernatant was transferred to a fresh tube and the remaining protein was extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant was precipitated with 0.6 volumes of isopropanol and the precipitate was dissolved in 250 µL of water.

Three microlitres of random primers (Invitrogen Corporation, Carlsbad, CA, USA, 3µg/µl) were added to approximately 6 µg of genomic *C. jejuni* DNA (Cy3 for reference NCTC 11168, Cy5 for test strain) in 1.5 ml amber tubes (Diamed Lab Supplies Inc., Mississauga, ON, Canada). Distilled water was then added so that each tube contained a total of 40.5 µl. Contents were then denatured at 95-97˚C for six minutes, kept on ice for two minutes and then left at room temperature for five minutes. Five microlitres of 10 x Klenow reaction buffer (USB Corporation, Cleveland, OH, USA), 1.5 µl Cy labeled dCTP (Amersham Biosciences Inc., Sunnyvale, CA, USA), 1 µl dNTP (Amersham Biosciences Inc., Sunnyvale, CA, USA), and 20 units exonuclease-free Klenow (USB Corporation, Cleveland, OH, USA) were added and

tubes incubated at 37˚C for two hours. Then, 2.5µl of 0.5M EDTA was added to each tube and left for one minute at room temperature. Tubes were then heated at 95-97˚C for two minutes, kept on ice for five minutes and left to sit at room temperature for five minutes. Cleanup of probes were carried out using the Qiagen MinElute Reaction Cleanup Kit as per manufacturer's specifications (Cat #28206, Qiagen Inc., Mississauga, ON, Canada) with a final elution volume of 13 µl distilled H_2O. Labeled DNA was quantified using a spectrophotometer (Ultrospec® 3000, Pharmacia Biotech) to calculate the number of pmol/µl in each tube. In a fresh 1.5 ml amber tube, 40 pmol of both the reference strain (Cy3) and test strain (Cy5) were combined, and distilled H_2O was added to bring the total volume to 20 µl.

5.2.6 Microarray hybridization

Each array was submerged in warm prehybridization solution (Genicon Sciences Corporation, now part of Invitrogen, Carlsbad, CA, USA) and incubated for 30 minutes at 42˚C. Each array was then washed in fresh distilled H_2O 10 times and in 100% isopropanol 10 times. Arrays were then dried with filtered air. Lifter slips (#25x601-2-4789,Erie Scientific Co., Portsmouth, NH, USA) were washed in distilled H_2O followed by 100% ethanol and left to dry. Damp paper towels were placed onto a metal leveling block in a large plastic container. Arrays were then labeled and placed, with the probes facing upward, on the paper towel and lifter slips added. SlideHyb Buffer #2 (55µl, Ambion, Austin, TX, USA) was then added to the 20 µl tube from the quantification step, mixed gently and the tube placed in the heat block for five minutes (65-67˚C). In a darkened room the entire contents of the tube was then pipetted along one edge of the lifter slip to wick up the slide. The lid was then placed on the container and it was

incubated at 42˚C in the humidified chamber for 18-24 hours. After incubation, the lifter slips were removed and the arrays immersed in fresh wash 1 solution (1x sodium chloride-sodium citrate (SSC)/0.1% sodium dodecyl sulfate, 42˚C for five minutes). This step was then repeated twice. Arrays were then immersed in wash solution 2 (1xSSC) at 42˚C for five minutes (repeated twice), and then in wash solution 3 (0.1xSSC) at 42˚C for five minutes (repeated twice). Slides were then rinsed in warm distilled H_2O (42˚C), dried with filtered air, placed into clean slide mailers and protected from direct light until scanned.

5.2.7 Scanning, data acquisition, and preliminary data analysis

Arrays were scanned using GenePix Pro version 4.1 (GenePix 4000B scanner, MDS Analytical Technologies, Mississauga, ON, Canada) or Jaguar, 2.0 (ArrayScanner 428, Affymetrix Inc, Santa Clara, CA,USA). Cy3 and Cy5 were scanned at wavelengths of approximately 532 nm and 635 nm respectively, both with 100% power. Primary image analysis (ArrayVision, version 8.0, rev. 3.0; Imaging Research Inc.) and global loess normalization (ArrayPipe) (Hokamp et al. 2004) were performed. The background was then subtracted from the raw spot intensity for both reference and test strain, giving a net intensity for each spot. Prior to all subsequent analyses, anomalous spots resulting from printing errors were removed from the dataset. The average net intensity data in reference and test channels for each ORF on the array were then obtained by averaging the net intensity of remaining replicate spots.

5.2.8 Quality control and gene absence/divergence analysis

Taboada et al previously showed that low intensity CGH data may behave less reliably than high intensity data upon subsequent analysis of gene divergence/absence (Taboada et al. 2005). Preliminary analysis of our data set revealed that low intensity data reduced the concordance of data from replicate arrays (see description in "validation of clustering results" section below). A custom script was written in Visual Basics for Applications (Microsoft Office Excel, Microsoft Corporation, 2007) to test pixel intensity cut-offs from 200 to 1,200 pixel units (in 20 unit increments) while monitoring concordance of replicate data. This analysis allowed us to determine intensity and log ratio cut-offs which would maximize the amount of reliable data retained for subsequent analysis and minimize the adverse effects of low signal data on replicate concordance. ORFs and arrays in which greater than 5% of the data yielded less than 500 pixel units in the reference channel were excluded from subsequent analysis. A "log ratio" or $\log_2$ (net test signal/net reference signal) threshold of -1.1 was chosen to differentiate divergent/absent from present genes. Spots for which the log ratio was < -1.1 were categorized as "divergent/absent", or categorized as "conserved" if the log ratio was ≥ -1.1). A log ratio was calculated for each ORF in each array that passed QC (1,399 ORFs; 119 arrays). Raw and processed log ratio data for this dataset are available at NCBI's Gene Expression Omnibus website (www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE13228. Log ratio data were visualized and analyzed in TIGRs MultiExperiment Viewer (TMEV, version 3.1) (Saeed et al. 2003).

5.2.9 Global cluster analysis and validation of clustering results

Average linkage hierarchical clustering (Eisen et al. 1998) was used to cluster samples based on similarity of binarized gene conservation profiles, and was performed in TMEV (Saeed et al. 2003) using Euclidean distance as a distance metric. Support tree bootstrapping within TMEV (500 bootstrap re-samplings) (Saeed et al. 2003) was then used to test the reliability of the clustering patterns. Tree data were coded into Newick format prior to visualization using Treeview version 1.6.6 (Page 1996).

Arrays from 90 study *C. jejuni* isolates and replicate arrays from 23 randomly selected isolates stratified by source (human or bovine) were included in the preliminary cluster analysis. Six self-self arrays (laboratory strain NCTC 11168) and six arrays comparing the reference NCTC 11168 to the laboratory test strain RM 1221 (hereafter referred to as RM 1221) were also included in the dataset. These technical replicate arrays were used to validate our data based on the expectation that replicates should group together on the dendrogram. Three isolates for which replicate arrays showed low concordance were identified and were removed from subsequent analyses. The remaining 87 isolates were included in the analysis of gene association.

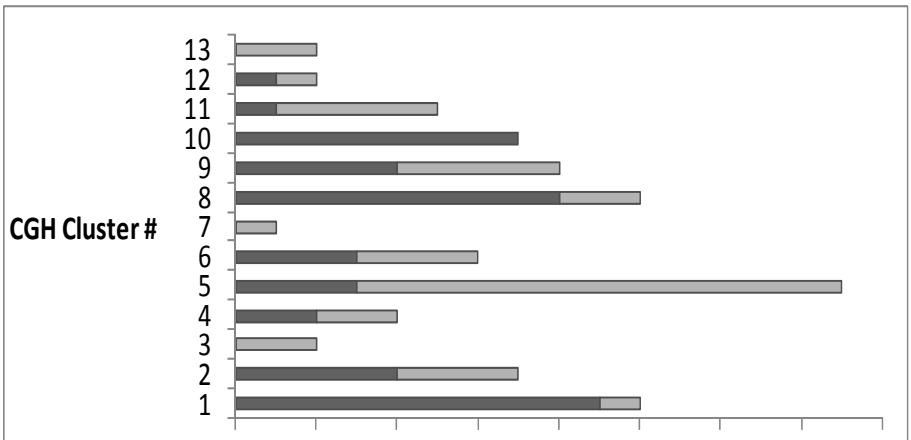
5.2.10 Statistical analyses of gene association

A multi-step process was used to investigate genes which might be differentially distributed between human and cattle sources. Genome-wide gene association analyses were conducted using an in-house Microsoft Excel script developed to compare differential conservation rates for each array gene (n=1,399), comparing groups of strains using the two tailed Fisher's exact test (Taboada et al. 2007b). The 87 isolate dataset was separated by source, and the number of absent and conserved genes between

the cattle and human isolates compared on a gene-by-gene basis. Because of obvious clonality present in the data, it was then decided to analyze CGH clusters that might have the potential for niche adaptation. Comparative genomic hybridization clusters were combined based on their apparent affinity for either human or cattle hosts. The first group was comprised of CGH clusters composed mainly of cattle isolates (CGH1, CGH8, CGH10; 24 cattle, 3 human) and designated as "cattle enriched" (CGH CE). The second group was comprised of CGH clusters composed mainly of human isolates (CGH3, CGH5, CGH11, CGH13; 4 cattle, 20 human) and designated "human enriched" (CGH HE). A third group, not used for analytical purposes, was comprised of CGH clusters composed of similar number of cattle and human isolates (CGH2, CGH4, CGH6, CGH7, CGH10; 14 cattle, 13 human) and designated at "intermediate" (CGH I), Figure 5.2. On a gene-by-gene basis (n=1,399), we tested the null hypothesis that each gene present in isolates from the CGH HE group would be present in the CGH CE group, and the alternate hypothesis that the gene would have an unequal distribution between the two groups using the Fisher's exact test ($p \leq 0.05$).

The Fisher's exact test p values from both of the above analyses (cattle vs human and CGH CE vs CGH HE) were adjusted for multiple comparisons using the Westfall and Young correction (WY, $p \leq 0.05$) (Westfall and Young 1993) based on 20,000 bootstrap resamplings (SAS version 9.2 SAS Institute Inc., Cary, NC, USA).

Figure 5.2 Distribution of human and cattle *C. jejuni* isolates within all comparative genomic hybridization clusters and designation into human enriched, or cattle enriched groups for gene association testing



	1	2	3	4	5	6	7	8	9	10	11	12	13
# Cattle Isolates	9	4	0	2	3	3	0	8	4	7	1	1	0
# Human Isolates	1	3	2	2	12	3	1	2	4	0	4	1	2
Designation	CE	I	HE	I	HE	I	I	CE	I	CE	HE	I	HE

CE: cattle enriched, CGH: comparative genomic hybridization, HE: human enriched, I: intermediate. n=79 isolates (isolates that did not cluster not shown)

5.3 Results

5.3.1 Assessment of clustering results using technical replicates

Technical replicates were included in the study, Figure 5.1. The inclusion of these arrays was a means of validating our data as replicate arrays should group together. Initially, 25 replicate sets were included in analysis, representing 11 human and 12 cattle field isolates and two laboratory strains (NCTC 11168 and RM 1221). The field isolates each had two replicates and the two laboratory strains (NCTC 11168 and RM 1221) each had six replicates.

5.3.2 Cluster analysis of human and cattle isolates

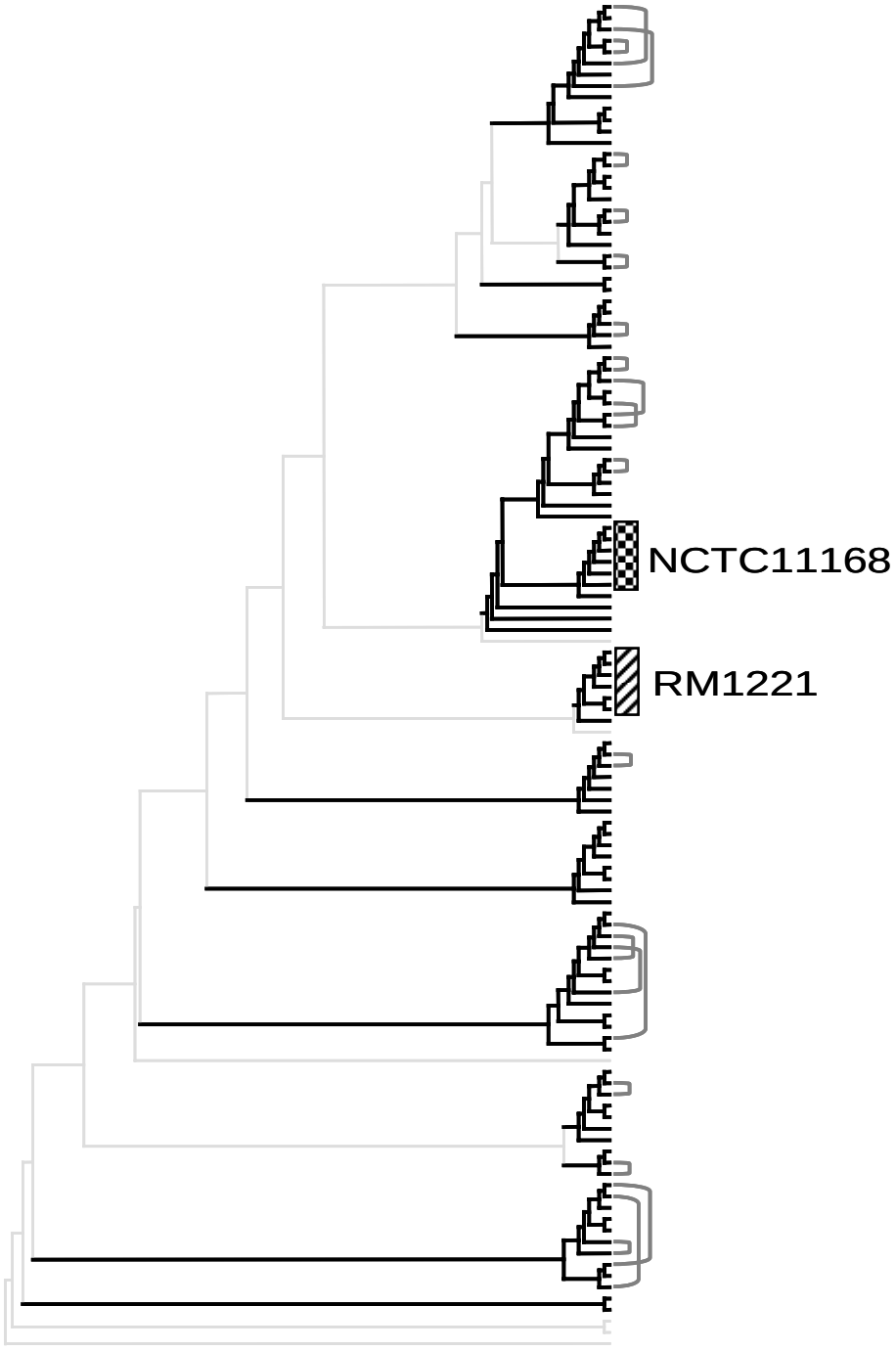
As expected, most replicate sets grouped within the same CGH cluster (22/25 sets). Three replicate pairs from three field isolates, however, did not group within the same CGH cluster and these isolates were removed from all subsequent analyses. A dendrogram representing 119 arrays (87 field isolates: 67 with single arrays, 20 with duplicate arrays; two laboratory strains each with six replicate arrays) is shown in Figure 5.3.

Forty-one arrays from human *C. jejuni* isolates (24 male, 17 female; ages 1-81 years old) and 46 arrays from cattle *C. jejuni* isolates arrays were included in the final study dataset, Table 5.1. Of the 87 field isolates, eight isolates did not cluster with others (designated NC in tables and figures), and isolate #4121 (CGH 7) clustered only with the RM 1221 laboratory strain. Of the 13 CGH clusters identified, nine contained human and cattle isolates, three contained only human isolates, and one contained only cattle isolates, Figures 5.2 and 5.4.

5.3.3 Molecular epidemiological analysis of temporal distribution

Nine of 13 CGH clusters contained isolates from both summer and winter seasons, and of these three clusters contained both cattle and human isolates from both winter and summer, Figure 5.5. Further, five clusters (CGH 2, 5, 6, 9, and 11) contained both human and cattle *C. jejuni* isolates submitted/collected within two week time frames, Table 5.1.

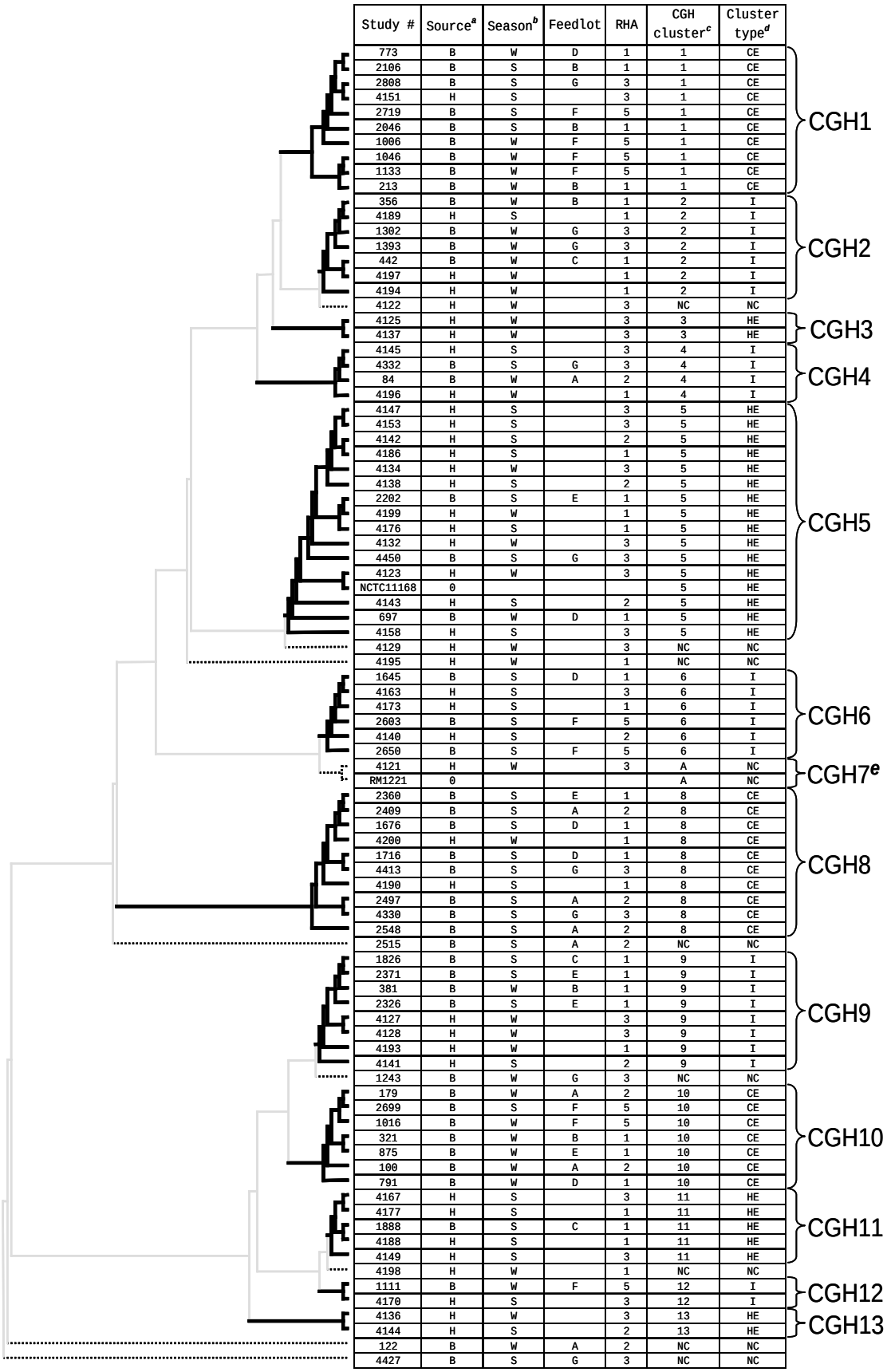
Figure 5.3 Dendrogram of validated *C. jejuni* technical replicates, laboratory strains and field isolates



Heavy black branch lines indicate greater than 75% bootstrap support. Grey brackets join the 20 sets of field isolate replicates on the dendrogram. Boxes denote the two sets of laboratory strain replicates. n=119 arrays.

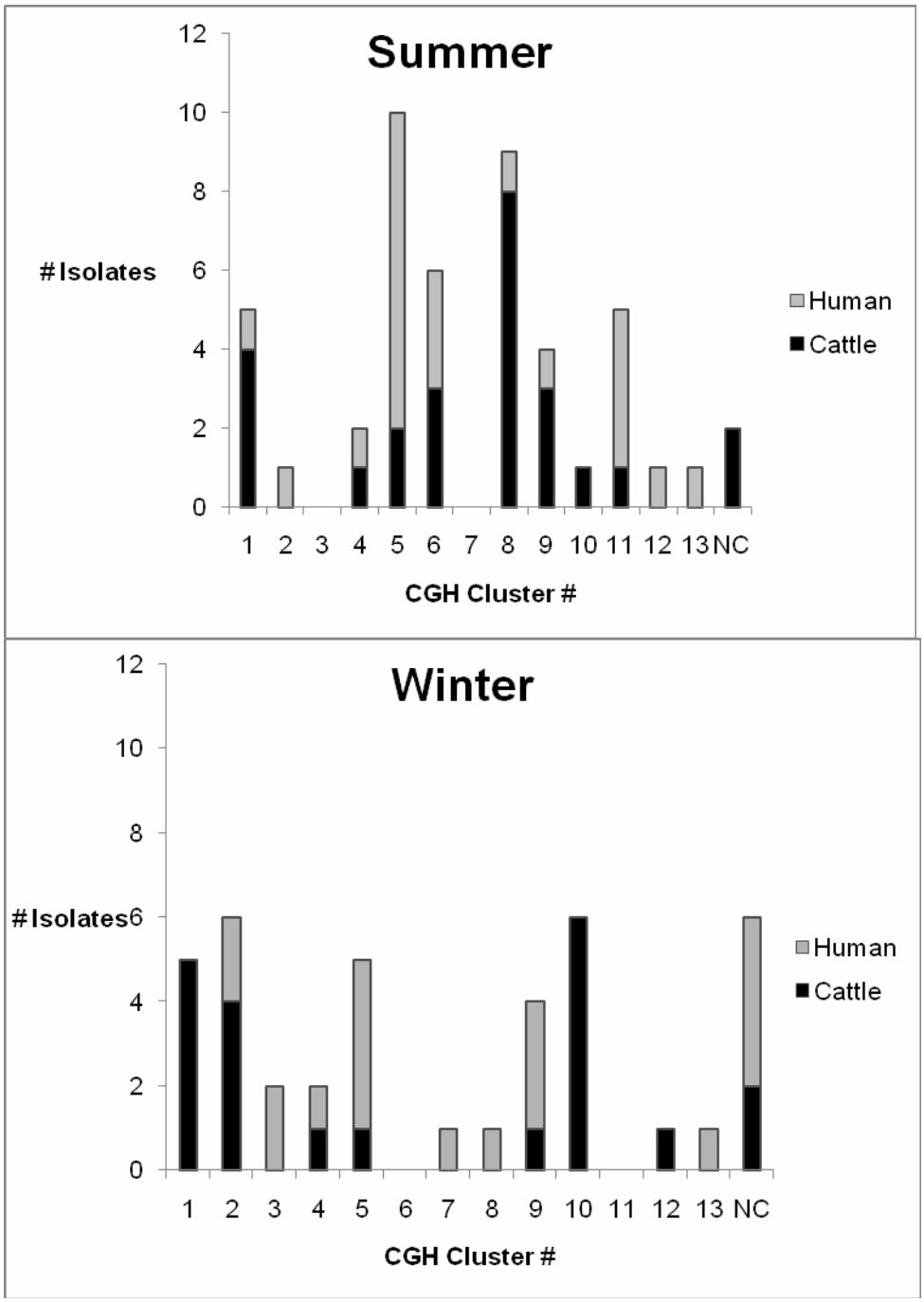
Figure 5.4 Global clustering dendrogram of cattle and human *C. jejuni* isolates

[a] B: bovine, H: human; [b] S: summer, W: winter; [c] NC: not clustered;
[d] CE: cattle-enriched, HE: human-enriched, I: intermediate, NC: not clustered; [e:] CGH7 contains a single study isolate and laboratory strain RM 1221. Heavy black branch lines indicate greater than 75% bootstrap support; dashed lines represent isolates that did not cluster with other study isolates. Figure includes 87 field isolate arrays and two laboratory strain arrays.



Study #	Source[a]	Season[b]	Feedlot	RHA	CGH cluster[c]	Cluster type[d]	
773	B	W	D	1	1	CE	CGH1
2106	B	S	B	1	1	CE	
2808	B	S	G	3	1	CE	
4151	H	S		3	1	CE	
2719	B	S	F	5	1	CE	
2046	B	S	B	1	1	CE	
1006	B	W	F	5	1	CE	
1046	B	W	F	5	1	CE	
1133	B	W	F	5	1	CE	
213	B	W	B	1	1	CE	
356	B	W	B	1	2	I	CGH2
4189	H	S		1	2	I	
1302	B	W	G	3	2	I	
1393	B	W	G	3	2	I	
442	B	W	C	1	2	I	
4197	H	W		1	2	I	
4194	H	W		1	2	I	
4122	H	W		3	NC	NC	
4125	H	W		3	3	HE	CGH3
4137	H	W		3	3	HE	
4145	H	S		3	4	I	CGH4
4332	B	S	G	3	4	I	
84	B	W	A	2	4	I	
4196	H	W		1	4	I	
4147	H	S		3	5	HE	CGH5
4153	H	S		3	5	HE	
4142	H	S		2	5	HE	
4186	H	S		1	5	HE	
4134	H	W		3	5	HE	
4138	H	S		2	5	HE	
2202	B	S	E	1	5	HE	
4199	H	W		1	5	HE	
4176	H	S		1	5	HE	
4132	H	W		3	5	HE	
4450	B	S	G	3	5	HE	
4123	H	W		3	5	HE	
NCTC11168	0				5	HE	
4143	H	S		2	5	HE	
697	B	W	D	1	5	HE	
4158	H	S		3	5	HE	
4129	H	W		3	NC	NC	
4195	H	W		1	NC	NC	
1645	B	S	D	1	6	I	CGH6
4163	H	S		3	6	I	
4173	H	S		1	6	I	
2603	B	S	F	5	6	I	
4140	H	S		2	6	I	
2650	B	S	F	5	6	I	
4121	H	W		3	A	NC	CGH7[e]
RM1221	0				A	NC	
2360	B	S	E	1	8	CE	CGH8
2409	B	S	A	2	8	CE	
1676	B	S	D	1	8	CE	
4200	H	W		1	8	CE	
1716	B	S	D	1	8	CE	
4413	B	S	G	3	8	CE	
4190	H	S		1	8	CE	
2497	B	S	A	2	8	CE	
4330	B	S	G	3	8	CE	
2548	B	S	A	2	8	CE	
2515	B	S	A	2	NC	NC	
1826	B	S	C	1	9	I	CGH9
2371	B	S	E	1	9	I	
381	B	W	B	1	9	I	
2326	B	S	E	1	9	I	
4127	H	W		3	9	I	
4128	H	W		3	9	I	
4193	H	W		1	9	I	
4141	H	S		2	9	I	
1243	B	W	G	3	NC	NC	
179	B	W	A	2	10	CE	CGH10
2699	B	S	F	5	10	CE	
1016	B	W	F	5	10	CE	
321	B	W	B	1	10	CE	
875	B	W	E	1	10	CE	
100	B	W	A	2	10	CE	
791	B	W	D	1	10	CE	
4167	H	S		3	11	HE	CGH11
4177	H	S		1	11	HE	
1888	B	S	C	1	11	HE	
4188	H	S		1	11	HE	
4149	H	S		3	11	HE	
4198	H	W		1	NC	NC	
1111	B	W	F	5	12	I	CGH12
4170	H	S		3	12	I	
4136	H	W		3	13	HE	CGH13
4144	H	S		2	13	HE	
122	B	W	A	2	NC	NC	
4427	B	S	G	3	NC	NC	

Figure 5.5 Distribution of summer and winter feedlot cattle and human clinical *C. jejuni* isolates within all comparative genomic hybridization clusters



CGH: comparative genomic hybridization; NC: isolates that did not cluster with any other field or laboratory strains. Cattle n=46, human n=41, summer n=47, winter n=40.

Table 5.1 Human and feedlot cattle *C. jejuni* isolate information (order same as dendrogram Figure 5.4)

CGH #	Study #	# Reps	Pop'n	Sson	Feedlot	RHA	Date[a]	Gender	Age
1	773	1	B	W	D	1	24-Jan-05	-	-
	2106	1	B	S	B	1	25-Aug-05	-	-
	2808	1	B	S	G	3	13-Sep-05	-	-
	4151	0	H	S	-	3	5-Aug-05	M	34
	2719	0	B	S	F	5	12-Sep-05	-	-
	2046	0	B	S	B	1	25-Aug-05	-	-
	1006	0	B	W	F	5	31-Jan-05	-	-
	1046	0	B	W	F	5	31-Jan-05	-	-
	1133	0	B	W	F	5	31-Jan-05	-	-
	213	0	B	W	B	1	18-Jan-05	-	-
2	356	0	B	W	B	1	18-Jan-05	-	-
	4189	0	H	S	-	1	3-Jul-05	Fe	43
	1302	1	B	W	G	3	1-Feb-05	-	-
	1393	0	B	W	G	3	1-Feb-05	-	-
	442	0	B	W	C	1	19-Jan-05	-	-
	4197	1	H	W	-	1	7-Jan-05	Fe	41
	4194	0	H	W	-	1	17-Dec-04	Fe	30
NC	4122	1	H	W	-	3	8-Nov-04	M	57
3	4125	0	H	W	-	3	15-Nov-04	Fe	74
	4137	0	H	W	-	3	11-Jan-05	Fe	1
4	4145	0	H	S	-	3	1-Aug-05	M	64
	4332	0	B	S	G	3	13-Sep-05	-	-
	84	1	B	W	A	2	17-Jan-05	-	-
	4196	0	H	W	-	1	20-Dec-04	M	23
5	4147	0	H	S	-	3	2-Aug-05	Fe	63
	4153	1	H	S	-	3	7-Aug-05	M	2
	4142	1	H	S	-	2	28-Jun-05	M	50
	4186	1	H	S	-	1	29-Jun-05	M	28
	4134	0	H	W	-	3	13-Dec-04	M	81
	4138	0	H	S	-	2	5-Jul-05	Fe	28
	2202	1	B	S	E	1	6-Sep-05	-	-
	4199	0	H	W	-	1	12-Jan-05	Fe	1
	4176	0	H	S	-	1	8-Jun-05	M	19
	4132	0	H	W	-	3	6-Dec-04	M	7
	4450	0	B	S	G	3	13-Sep-05	-	-
	4123	0	H	W	-	3	14-Nov-04	Fe	3
	NCTC 11168	5				Laboratory strain			
	4143	0	H	S	-	2	1-Jun-05	M	23
	697	0	B	W	D	1	24-Jan-05	-	-
	4158	0	H	S	-	3	11-Aug-05	M	40
NC	4129	0	H	W	-	3	3-Dec-04	M	23
NC	4195	0	H	W	-	1	19-Dec-04	M	49
6	1645	0	B	S	D	1	22-Aug-05	-	-
	4163	1	H	S	-	3	15-Aug-05	Fe	77
	4173	0	H	S	-	1	7-Jun-05	Fe	34
	2603	0	B	S	F	5	12-Sep-05	-	-
	4140	0	H	S	-	2	13-Sep-05	M	43
	2650	0	B	S	F	5	12-Sep-05	-	-
7	4121	0	H	W	-	3	9-Dec-04	Fe	64
	RM 1221	5				Laboratory strain			

CGH #	Study #	# Reps	Pop'n	Sson	Feedlot	RHA	Date[a]	Gender	Age
8	2360	0	B	S	E	1	6-Sep-05	-	-
	2409	1	B	S	A	2	8-Sep-05	-	-
	1676	1	B	S	D	1	22-Aug-05	-	-
	4200	1	H	W	-	1	27-Jan-05	M	3
	1716	0	B	S	D	1	22-Aug-05	-	-
	4413	0	B	S	G	3	13-Sep-05	-	-
	4190	0	H	S	-	1	5-Jul-05	M	32
	2497	0	B	S	A	2	8-Sep-05	-	-
	4330	0	B	S	G	3	13-Sep-05	-	-
	2548	0	B	S	A	2	8-Sep-05	-	-
NC	2515	0	B	S	A	2	8-Sep-05	-	-
9	1826	0	B	S	C	1	23-Aug-05	-	-
	2371	0	B	S	E	1	6-Sep-05	-	-
	381	0	B	W	B	1	18-Jan-05	-	-
	2326	0	B	S	E	1	6-Sep-05	-	-
	4127	0	H	W	-	3	21-Nov-04	M	47
	4128	0	H	W	-	3	22-Nov-04	M	19
	4193	0	H	W	-	1	17-Dec-04	M	57
	4141	0	H	S	-	2	11-Sep-05	Fe	28
NC	1243	0	B	W	G	3	1-Feb-05	-	-
10	179	1	B	W	A	2	17-Jan-05	-	-
	2699	1	B	S	F	5	12-Sep-05	-	-
	1016	0	B	W	F	5	31-Jan-05	-	-
	321	0	B	W	B	1	18-Jan-05	-	-
	875	0	B	W	E	1	25-Jan-05	-	-
	100	1	B	W	A	2	17-Jan-05	-	-
	791	0	B	W	D	1	24-Jan-05	-	-
11	4167	0	H	S	-	3	16-Aug-05	Fe	25
	4177	0	H	S	-	1	9-Jun-05	M	18
	1888	0	B	S	C	1	23-Aug-05	-	-
	4188	1	H	S	-	1	30-Jun-05	M	45
	4149	0	H	S	-	3	3-Aug-05	Fe	40
NC	4198	0	H	W	-	1	9-Jan-05	Fe	24
12	1111	0	B	W	F	5	31-Jan-05	-	-
	4170	1	H	S	-	3	26-Aug-05	Fe	48
13	4136	0	H	W	-	3	3-Jan-05	M	33
	4144	0	H	S	-	2	8-Aug-05	M	47
NC	122	0	B	W	A	2	17-Jan-05	-	-
NC	4427	0	B	S	G	3	13-Sep-05	-	-

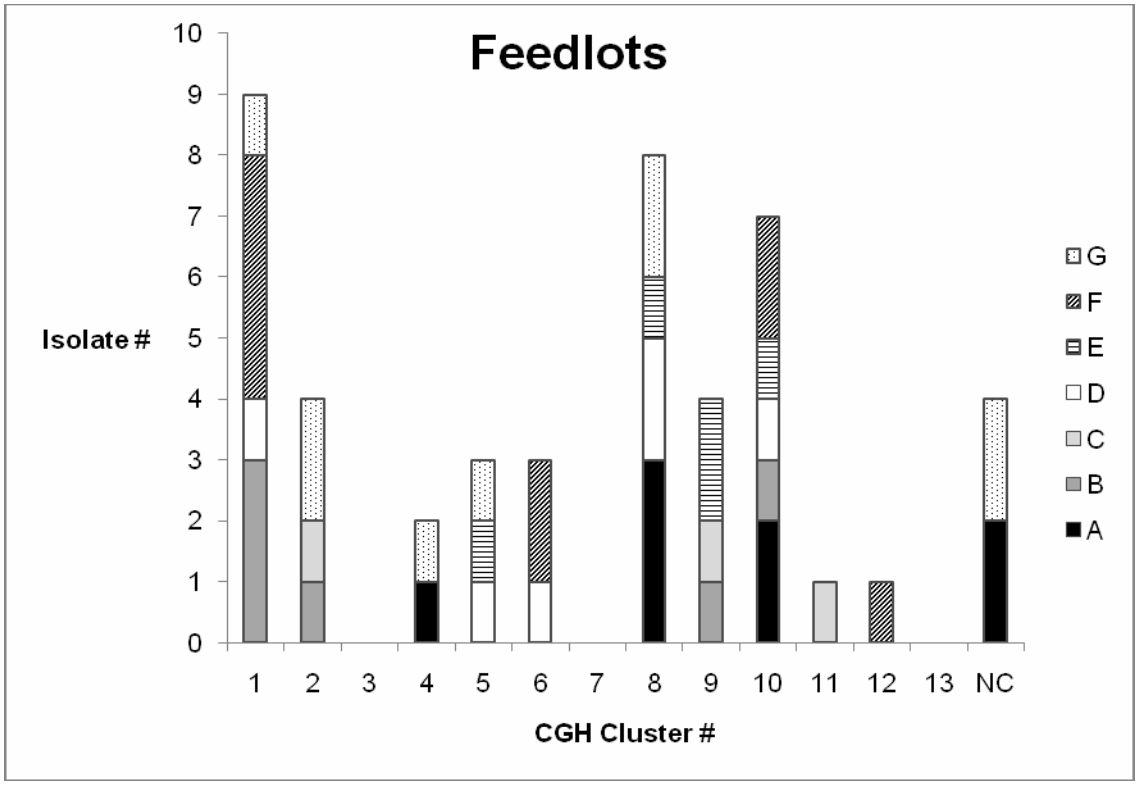
[a]: Submission or sampling date.

A-F: feedlot designations, CGH: comparative genomic hybridization, Dash (-): not applicable, Fe: female, M: male, NC: isolates that did not cluster with any other field or laboratory strains, Pop'n: population, RHA: regional health authority, Reps: replicates, Sson: season, S: summer, W: winter. n=89 *C. jejuni* isolates, 87 field, 2 laboratory strains.

5.3.4 Molecular epidemiological analysis of geographical distribution

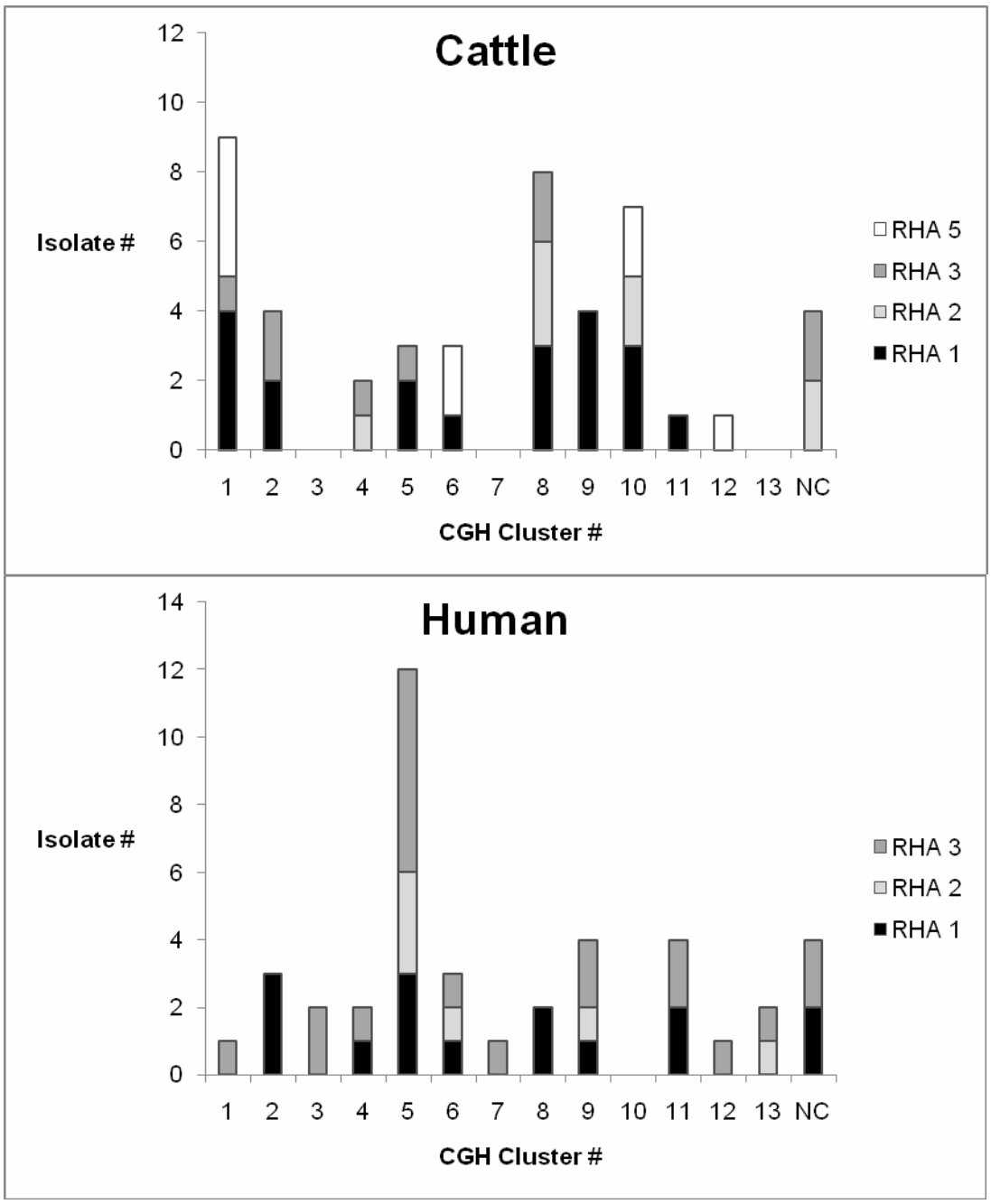
Two or more feedlots were represented in eight of the 13 CGH clusters, and three of the 13 CGH clusters contained cattle isolates from four or more feedlots, Figure 5.6. Only one CGH cluster was composed of isolates from a single RHA (CGH3, n=2). Another cluster, CGH7, contained a single field isolate and the laboratory strain RM 1221 (one field and one laboratory strain). The other 11 CGH clusters contained isolates from two or more RHAs, and one cluster (CGH6) contained isolates from all four RHAs, Figure 5.7.

Figure 5.6 Distribution of feedlots within all *C. jejuni* comparative genomic hybridization clusters



Legend at the right gives the feedlot designation, figure includes feedlot cattle *C. jejuni* isolates only n=46. CGH: comparative genomic hybridization, NC: isolates that did not cluster with any other field or laboratory strains.

Figure 5.7 Distribution of Alberta regional health authorities within all *C. jejuni* comparative genomic hybridization clusters



CGH: comparative genomic hybridization. NC: isolates that did not cluster with any other field or laboratory strains. Cattle n=46, human n=41.

5.3.5 Gene association testing to compare cattle and human isolates

The comparison of all human (n=41) and cattle (n=46) *C. jejuni* isolates originally identified 25 of 1,399 genes in our dataset as having an unequal distribution between the two groups (gene absent more in one group than the other, $p \leq 0.05$). The WY correction was used to account for testing of multiple comparisons, and adjusted the Fisher's exact *p* values to minimize the possibility of false positive results. In gene-by-gene comparison of the cattle and human clinical *C. jejuni* isolates (with WY correction), only three genes were identified with statistically significant differences in presence/absence between the two groups: Cj 0617, a hypothetical protein; Cj0628, a putative lipoprotein; Cj1668, a putative periplasmic protein. It was also observed that seven CGH clusters were dominated by either cattle or human isolates, Figure 5.2. We hypothesized that there could be potentially meaningful genetic differences between the human-dominated or cattle-dominated clusters and decided to investigate these source-biased groupings further. CGH clusters predominantly composed of human isolates (CGH HE) and those predominantly composed of cattle isolates (CGH CE) were compared using gene association testing. This focused cluster comparison identified 37 of 1,399 genes unequally distributed between these two groups using both the Fisher's exact test and the WY correction ($p \leq 0.05$), Table 5.2.

Table 5.2 Results of *C. jejuni* gene association testing for comparison of CGH "cattle enriched" and "human enriched" groups

All genes are statistically significant ($p \leq 0.05$) based on the unadjusted Fisher's exact test p value and the Westfall and Young (WY) corrected p value, 1,399 genes tested. CE: cattle enriched, CGH: comparative genomic hybridization, HE: human enriched, P: protein, put.: putative. Gene product information is from the following reference: (Gundogdu et al. 2007).

Gene	Absent CGH CE (n=27)	Absent CGH HE (n=24)	CGH CE vs CGH HE		Gene Product
			Unadjusted *p* value	WY *p* value	
Cj0202	27	14	0.00015	0.00210	hypothetical P
Cj0300	19	24	0.00464	0.05000	put. molybdenum transport ATP-binding P
Cj0302	16	24	0.00033	0.00400	put. molybdenum-pterin binding P
Cj0303	11	24	<0.00001	<0.00001	put. molybdate-binding lipoprotein
Cj0304	15	24	0.00013	0.00135	put. biotin synthesis P
Cj0399	17	24	0.00081	0.01145	colicin V production P homolog
Cj0485	13	21	0.00352	0.03890	put. oxidoreductase
Cj0617	27	6	<0.00001	<0.00001	hypothetical P
Cj0628	0	8	0.00116	0.01525	put. lipoprotein
Cj1051	2	12	0.00118	0.01555	restriction modification enzyme
Cj1136	3	16	0.00005	0.00055	put. glycosyltransferase
Cj1137	4	17	0.00006	0.00085	put. glycosyltransferase
Cj1138	5	17	0.00023	0.00325	put. glycosyltransferase
Cj1139	10	22	0.00010	0.00115	beta-1,3 galactosyltransferase
Cj1140	9	19	0.00172	0.01970	alpha-2,3 sialyltransferase
Cj1141	3	17	0.00001	0.00005	sialic acid synthase (N-acetyl neuraminic acid synthetase)
Cj1142	6	18	0.00023	0.00360	put. UDP-N-acetylglucosamine 2-epimerase
Cj1143	2	16	0.00002	0.00010	two-domain bifunctional P (beta-1,4-N-acetylgalactosaminyltransferase /CMP-Neu5Ac synthase)
Cj1144	6	16	0.00194	0.02315	hypothetical P
Cj1145	5	16	0.00066	0.00885	coding sequence merged with Cj1144
Cj1146	12	22	0.00038	0.00455	put. glucosyltransferase
Cj1150	19	24	0.00464	0.05000	D-beta-D-heptose 7-phosphate kinase/D-beta-D-heptose 1-phosphate adenylyltransferase
Cj1297	10	19	0.00424	0.04385	hypothetical P
Cj1389	1	16	<0.00001	<0.00001	pseudogene (put. C4-dicarboxylate anaerobic carrier
Cj1421	6	18	0.00023	0.00360	put. sugar transferase
Cj1422	7	20	0.00006	0.00080	put. sugar transferase
Cj1428	10	20	0.00145	0.01710	GDP-L-fucose synthetase
Cj1429	6	19	0.00007	0.00090	hypothetical P
Cj1430	8	18	0.00191	0.02225	put. dTDP-4-dehydrorhamnose 3,5-epimerase
Cj1432	3	16	0.00005	0.00055	put. sugar transferase
Cj1433	2	17	<0.00001	<0.00001	hypothetical P
Cj1434	5	19	0.00002	0.00025	put. sugar transferase
Cj1435	8	19	0.00105	0.01410	put. phosphatase
Cj1439	0	15	<0.00001	<0.00001	UDP-galactopyranose mutase
Cj1440	6	16	0.00194	0.02315	put. sugar transferase
Cj1520	9	20	0.00054	0.00665	removed from coding sequences
Cj1729	1	13	0.00007	0.00150	flagellar hook subunit P

5.4 Discussion

This study describes the use of DNA microarray as a high-resolution genotyping tool for the molecular epidemiological investigation of *C. jejuni*. This dataset represents the largest published comparisons of human and feedlot cattle *C. jejuni* isolates using DNA microarrays, and focused on feedlot cattle because of their potential as *Campylobacter* reservoirs. The isolates tested by DNA microarray in this study were purposefully collected within a defined geographical and temporal framework in order to generate data on the presence and persistence of strains in feedlot cattle and people in Alberta.

Although the microarray-based CGH approach described is not used in routine molecular epidemiology due to high cost and low throughput compared to conventional genotyping methods, it has the potential to provide an unprecedented level of discriminatory power (Dorrell et al. 2005b, Leonard II et al. 2004, Taboada et al. 2007a). Further, analysis by DNA microarray-based CGH has recently been shown to correlate with clonal complexes identified by multi-locus sequence typing (MLST), the "gold standard" in molecular typing, in *Streptococcus pneumoniae* (Dagerhamn et al. 2008) and in *C. jejuni* (Taboada et al. 2008). The data obtained from genomic DNA microarray studies can not only create high-resolution genetic profiles for global clustering but can also be directly applied in gene association studies to study potential genotype-phenotype links. These advantages are reflected in a recent investigation of *C. jejuni* strains implicated in Guillain-Barré and Miller Fisher syndromes (Taboada et al. 2007b). In the past, finding associations using conventional molecular typing methods between neuropathogenic *C. jejuni* typing markers and clinical phenotype has

been difficult (Dingle et al. 2001b). With the use of data obtained from microarray-based CGH, it has been possible to extend applications beyond lineage diversity to successfully identify factors commonly shared by neuropathogenic strains using a gene association approach.

In this study, global clustering of *C. jejuni* isolates based on whole-genome profiles showed that there was a high degree of similarity between cattle and human isolates. The distribution of isolates from both sources within most of the 13 CGH clusters suggests that both people and cattle may have access to the same transmission routes. Nine out of 13 CGH clusters contained both bovine and human isolates, and within five clusters genetic clones (isolates with high genomic similarity and belonging to the same CGH cluster) were identified from both cattle and people within very confined temporal periods (two weeks). Often the isolates within a cluster represented multiple geographical regions and feedlots from both seasons and from both cattle and human sources. It is not known if human campylobacteriosis patients in our study had contact with feedlot cattle or were from urban or rural backgrounds. Specifically designed epidemiological studies would be required to link cattle contact to human cases. However, our findings, although indirect, suggest that transmission of *C. jejuni* strains may be occurring between people and feedlot cattle, and that the distribution of *C. jejuni* strains able to cause human disease is widespread in southern Alberta.

Clonality was a prominent feature observed in our dataset. One known campylobacteriosis outbreak occurred in RHA 3 during the course of our study. However, isolates from this outbreak were not included in order to maximize genetic variability in our dataset. CGH cluster 5, in which 12 of 15 field isolates were of human

origin, was an important strain in the dataset as isolates with this genetic profile were persisting, widespread, and clearly pathogenic. Patients infected with this *C. jejuni* strain sought clinical care over a 44 week period (seven different months) in three different RHAs. Identifying CGH clusters dominated by either human or cattle sources is consistent with MLST studies which have suggested that niche adaptation may play a role in the over-representation of sources within clonal groups (Dingle et al. 2001a, Dingle and Maiden 2005). It is possible that sampling issues could have played a role in our findings. The culture process used could have selected for colonies with particular genetic make-up, therefore not representative of the full spectrum of *C. jejuni* genetic variability. In addition, only one colony per plate was selected for molecular testing, which may have resulted selection bias. However, it is also possible that the genotypic clusters identified in our study may represent phenotypic separations within the dataset. It is plausible that *C. jejuni*, considered a commensal in cattle but usually pathogenic in people, could have differential infection and colonization rates between hosts species based on differences in strain attributes or exposure patterns.

The ability to mine microarray data using gene association testing, in addition to global clustering, is one of the main advantages of the DNA microarray platform. Global clustering gives an overview of similarity between isolates but does not specify which parts of the genomes are similar or different. Our comparison of human and bovine isolates using gene association testing identified only three of 1,399 genes with statistically significant differences in conservation rates between sources. Because it is possible that the small number of genes identified resulted from confounding factors or lack of power, in addition to similarity between human and cattle isolates, it was decided

to explore source-biased clusters more thoroughly. Our comparison of "cattle enriched" and "human enriched" clonal groupings identified 37 of 1,399 genes absent in one group but not in the other. This represents a very small number of differences and supports the overall similarity between human and cattle isolates. The concept of feedlot niche adaptation may be plausible based on our findings of clonal groupings dominated by particular host sources and is interesting, from an epidemiological perspective, as the genetic composition of different clonal strains may have potential clinical relevance.

The feedlot environment seems a dynamic and important niche in the epidemiology of campylobacters. Our results supported our expectation that *C. jejuni* strains collected within a feedlot would be similar and would group within the same CGH cluster. Seven CGH clusters were found to contain two or more isolates from the same feedlot. However, it was surprising that individual feedlots contained so many genetically diverse strains. Three or more strains (CGH clusters) were identified within each of the seven feedlots. Multiple strains within each feedlot niche are mostly likely the result of a combination of influences including human, wild bird, insects, other reservoir hosts, fomite and water exposures. Further, clones from both winter and summer collections within individual feedlots (B and F) were identified. Our findings suggest that some genotypic clones may persist over the course of the year in the feedlot environment, and that *C. jejuni* genomic profiles may be relatively stable and not undergoing major recombination events. They also suggest that cattle may be exposed to multiple strains of *C. jejuni* over time.

Human samples were acquired from diagnostic laboratories across southern Alberta. As protocols for *C. jejuni* isolation are not standardized across the province, it

is possible that a variety of isolation methods were used for the human isolations and that these were different from protocols for isolation of bovine strains. This may have resulted in selection pressure for certain strains based on the isolation conditions used, resulting in an underrepresentation of isolate genetic variability.

Both cattle and human isolates were collected in winter and summer 2005 from three geographical areas (RHA 1, 2, 3) in southern Alberta. In order to include more bovine isolates, cattle in a fourth area (RHA 5) were sampled over the same time frame. Clustering of *C. jejuni* strains did not seem to be influenced by season, as nine of 13 CGH clusters contained isolates from both summer and winter. RHA was also not a segregating factor as 11 of 13 CGH clusters contained *C. jejuni* isolates from two or more RHAs. These findings suggest that movement of strains between the different geographical regions is occurring and may be common in southern Alberta.

Alberta patients are provided with medical treatment, including laboratory services, under Canada's universal, publicly insured health care plan. Community and hospital physician services are accessed within regional health authorities and while patients have the option to access these services outside of RHAs, the majority of primary care and laboratory services are accessed within the RHA of residence. While it was assumed that the RHA submitting the human isolate was the same region in which the patient lived, it is possible that regional misclassification may have occurred if the patient saw a physician outside of their area of residence or if the samples were sent to diagnostic laboratories in a different RHA.

5.5 Conclusions

This study used DNA microarray as a platform to investigate *C. jejuni* isolates from feedlot cattle and people in Alberta. Using this study design, the time, labour and cost of conducting whole-genome CGH studies with DNA microarrays does not make the platform conducive for time sensitive outbreak investigation or detection surveys. However, the advantages of DNA microarray technology in generating high-resolution data useful for both global clustering and gene association studies represents significant value-added compared to other molecular typing techniques (Taboada et al. 2007b, Taboada et al. 2008). As a result, DNA microarray may be advantageous for use in molecular epidemiological contexts that require comprehensive genetic data but not immediate reporting. This molecular study has enabled us to describe the genetic variability of human and cattle isolates, both globally and gene-by-gene. Study isolates clustered regardless of seasonal or geographical frameworks, suggesting that *C. jejuni* strains may be stable and endemic in Alberta. Further, the common distribution of human clinical and bovine *C. jejuni* isolates within the same genetically-based clusters suggests that dynamic and important transmission routes between cattle and human populations may exist.

5.6 References

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CHAPTER 6
CONCLUSIONS

6.1 Summary

Campylobacter species, in particular *Campylobacter jejuni (C. jejuni)*, are important human pathogens in both the developed and developing world (Friedman et al. 2000, Oberhelman and Taylor 2000). In people, campylobacters commonly cause enteric disease which may include costly and serious sequelae such as Guillain-Barré syndrome, Miller Fisher syndrome and reactive arthritis (Nachamkin 2002).

As carriers of campylobacters, feedlot cattle are potential reservoirs for human infection, for transmission to other reservoir hosts including wild birds and insects, and for contamination of soil and water sources. The effects of the Walkerton waterborne outbreak emphasized the importance of cattle as human enteric bacteria reservoirs and the need for continued research into transmission routes for these bacteria to people (O'Connor 2002). *Campylobacter* research in Alberta has included a targeted prevalence study in international travelers, knowledge assessment of prevention techniques for traveler's diarrhea, and human risk factor identification (Johnson et al. 2006, Johnson et al. 2008, Russell et al. 1993); pathogen detection in water (Walters et al. 2007); antimicrobial resistance studies in people including feedlot workers, and in cattle (Inglis et al. 2005, Inglis et al. 2006, Johnson et al. 2008, Lefebvre et al. 2006, Read et al 2004); and molecular typing of campylobacters from cattle (Besser et al. 2005).

The purpose of this thesis was to investigate the hypothesis that feedlot cattle in Alberta could be plausible and potential sources of *Campylobacter* spp. and *C. jejuni* to Albertans. A prevalence study of *Campylobacter* spp. and *C. jejuni* in feedlot cattle feces, and a preliminary risk factor study of associations between *Campylobacter* spp. isolation rates and pen and feedlot variables were conducted. During the same time frame, the potential public health importance of retail ground beef as a source of *Campylobacter* spp. was investigated. Through enrichment culture and polymerase chain reaction (PCR) techniques, retail ground beef was tested for *Campylobacter* spp. and an initial risk factor study of associations related to the presence of *Campylobacter* spp. DNA in the meat was conducted. DNA microarray technology was then used to describe and compare *C. jejuni* genomic profiles in feedlot cattle and human clinical *C. jejuni* isolates from southern Alberta.

6.1.1 Feedlot cattle fecal prevalence, seasonality and risk factor analysis

Overall and seasonal (summer and winter) prevalence estimates for *Campylobacter* spp. and *C. jejuni* isolation rates in feedlot cattle near slaughter weight from large commercial feedlots in Alberta were identified. While this study sampled only seven feedlots, this was one of the largest samplings of feedlot cattle feces for *Campylobacter* spp. in Alberta, and utilized an efficient and cost effective swab sampling methodology to obtain the *Campylobacter* isolates. From the collection of 2,800 feedlot cattle fresh fecal samples within a geographical and temporal framework, 1,486 *C. jejuni* isolates were obtained for use in this and future studies.

Fecal prevalences of *Campylobacter* spp. (87%) and *C. jejuni* (61% summer estimate) were found to be very high in commercial feedlot cattle in Alberta. It was expected that winter and summer *Campylobacter* spp. estimates would be different, in keeping with human and poultry trends (Kovats et al. 2005, Meldrum et al. 2004). However this was not supported by study data as the summer (88%) and winter (86%) fecal prevalence estimates were not statistically significantly different in univariable analysis accounting for pen and feedlot clustering.

In multivariable analysis accounting for pen and feedlot clustering, the longer cattle remained in the feedlot, the lower the odds of shedding campylobacters. While this finding was similar to that from a previous Alberta study looking at growth implants in feedlot cattle (Lefebvre et al. 2006), it was contradictory to longitudinal studies which have found an increasing prevalence with time in the feedlot ((Besser et al. 2005, Inglis et al. 2004, Inglis et al. 2006). Further, feedlot size was associated with *Campylobacter* spp. isolation rates in this study. In particular, reduced rates were seen in mid-sized feedlots compared to small, likely reflecting differences in management between feedlots.

After accounting for pen level clustering, feedlot was associated with the *Campylobacter* spp. isolation rates, illustrating that differences exist between feedlots. Feedlot *Campylobacter* spp. prevalences varied from 76 to 95% and *C. jejuni* identification among *Campylobacter* positive isolates varied from 59 to 74% across the seven feedlots.

6.1.2 Retail ground beef prevalence and risk factor analysis

Twelve hundred regular and lean ground beef packages were collected from 60 stores in three regional health authorities (RHAs 1, 2 and 3) in southern Alberta. The ground beef samples were intended to represent chains and stores with the largest sales volume of ground beef to consumers in those RHAs. The assumption was not that the feedlot cattle sampled in the fecal survey would be sources of this meat, as most ground beef would come from central processors and be distributed over large areas of the province. Specific study designs including farm-to-fork tracking would be required to make such an assumption. The meat was sampled, however, under the assumption that people would most likely purchase meat within their area of residence, and therefore that ground beef and human isolates from the same temporal and geographical samplings would be similar. No ground beef *Campylobacter* spp. isolates were obtained using culture in this ground beef survey.

Poultry cutting was initially thought to be a potential source of contamination for retail ground beef. However 2/3 of stores did not cut poultry on-site and brought in pre-packaged poultry cuts for consumers. No association was found between poultry cutting and the presence of *Campylobacter* DNA in retail ground beef in the risk factor study.

An interesting finding in this study was the high level of *Campylobacter coli* DNA found in ground beef samples. *C. coli* can be carried by feedlot cattle, although usually in much lower prevalences than *C. jejuni* (Inglis et al. 2003, Inglis et al. 2006), and is more commonly associated with swine and pork. As a result, it was surprising to find more *C. coli* (26.8%) than *C. jejuni* (14.8%) in the ground beef samples tested with PCR.

While *Campylobacter* DNA was present in the ground beef sampled in this study, the levels of culturable or viable campylobacters for retail consumption were extremely low. The prevalence of *Campylobacter* DNA using PCR detection, however, was moderate to high (46%), thus continued research into potential interventions in the slaughter to retail continuum could be of use. While culture results indicate viable organisms, the PCR results could indicate the presence of viable, viable but non culturable, or dead organisms, so it is difficult to evaluate public health risk based on the PCR findings. In addition, the high levels of *Campylobacter* DNA in the beef suggest that breaks in food safety protocols within slaughter plants, processors or grocery stores could have potentially important public health repercussions.

6.1.3 Molecular epidemiology using DNA microarray

Feedlot cattle and human *C. jejuni* isolates from the same geographical and temporal framework were compared using DNA microarray, with the inclusion of technical replicates and control strains as validation of the technique. The results indicated that human and feedlot cattle *C. jejuni* isolates in southern Alberta are very similar. Human and feedlot cattle *C. jejuni* isolates were both identified in nine of the 13 CGH clusters (three CGH clusters contained only human isolates and one CGH cluster contained only feedlot isolates). Because clusters were often found to contain both human and cattle *C. jejuni* isolates, to contain isolates from both winter and summer, and to contain isolates and from two or more RHAs, this suggests that some strains of *C. jejuni* may be endemic and stable over time, and that geographical movement is taking place. These may be important contributions to our knowledge of *C. jejuni* epidemiology in Alberta.

In addition, gene association testing comparing human clinical and feedlot cattle *C. jejuni* isolates or comparing targeted host-based genetic groupings found only a small number of the 1,399 genes to be absent more in one group than the other. Based on these findings, it seems that human and cattle *C. jejuni* isolates from Alberta are genetically very similar, and that transmission between the two populations may be occurring.

While feedlot cattle may be a source of human infection, it is always possible that the opposite is true; that people may be a source of campylobacters to cattle. It is possible that fomite transmission and human sewage issues could lead to contamination of feedlot cattle water supplies. In a recent study of river waters in southern Alberta, ruminant bacterial indicators were linked to *Escherichia. coli* O157:H7 and *Salmonella* water contamination while campylobacters were linked to human bacterial markers (Walters et al. 2007). However, due to the large quantities of cattle manure produced each year in Alberta to be treated and used in agricultural endeavours, the human-to-cattle hypothesis seems less likely. More plausible may be that people and cattle may be exposed to campylobacters through similar sources and transmission routes such as water. In addition, it is possible that cattle may have an important but indirect role in the 'web of transmission' of campylobacters to people. For example, bird or insect access to cattle manure within feedlots prior to manure treatment or composting may be a means of transmitting bovine *Campylobacter* strains to other reservoir species such as poultry, followed by transmission to people.

6.2 Strengths of the research

The fecal samples collected as part of this research represented 2,800 individual cattle and is one of the largest *Campylobacter* studies published based on feedlot cattle

feces sampling in Alberta. The purposeful sampling strategy (20 pens per feedlot per sampling, 10 samples per pen, collected in winter and summer from seven large commercial Alberta feedlots) resulted in the collection of *Campylobacter* spp. and *C. jejuni* isolates which may be used for future research.

One of the strengths of this study was the sampling of feedlot cattle near slaughter weight. This timing was targeted so that estimates would have as much relevance as possible to public health risk, short of slaughterhouse/processing sampling. In addition, swabbing fresh, pen-floor fecal pats was an efficient, economically feasible and non-invasive *Campylobacter* sampling technique for feedlot cattle. In Alberta in 2005, 58% of cattle production came from 35 operators feeding 10,000 head or more (CanFax 2006). Even though feedlots were not randomly selected, this study focused on seven of these 35 sites in an attempt to estimate *Campylobacter* prevalences on sites responsible for larger proportions of the provincial industry production compared to smaller head capacity feedlots.

The preliminary risk factor study evaluated variables which were potential predictors of *Campylobacter* spp. isolation rates in feedlot cattle. It was interesting that feedlot size and the number of days on feed predicted *Campylobacter* isolation rates in this study. The feedlot and pen-level variance components identified from this model could be used in future studies for clustered sample size calculations.

This study is one of the largest surveys of *Campylobacter* spp. in retail ground beef in Canada to date. The sampling frame contained stores from four major chains in the province, and after stratification by city and by chain, stores were randomly selected for inclusion in the study. In addition, ground beef packages were randomly selected

from retail meat counters and the number of packages selected per store per sampling time was limited to five to avoid oversampling the same ground beef batches. These measures were incorporated to attempt to minimize selection bias in results.

DNA microarray was chosen for this research because it allowed both global clustering of isolates and also a gene-by-gene comparison between human clinical and feedlot cattle *C. jejuni* isolates. The ability to accomplish both goals with the same dataset was an efficient use time and resources. This research is the largest comparison of feedlot cattle and human clinical *C. jejuni* isolates using DNA microarray technology, and used randomly sampled cattle and human clinical *C. jejuni* isolates collected from similar geographical regions during the same time frame. DNA microarray is an extremely high resolution technique which allows researchers' flexibility in data analysis as investigations can be as broad or as focused as required.

6.3 Limitations to this research

Enrolling only seven feedlots in this study reduced the ability to generalize results to a greater extent, and likely limited our ability to find statistically significant differences for feedlot level variables in risk factor analyses. In addition, the seven feedlots were enrolled based on willingness to participate, which may have resulted in volunteer bias. These feedlots may have been different from other Alberta feedlots based on size, management protocols, use of veterinarians or other factors.

Fecal samples were collected using swabs instead of grams of feces. Collecting more fecal matter may have improved *Campylobacter* recovery with culture, resulting in higher prevalence levels. However, the swab transport media used in this study seemed

to work well in protecting campylobacters from temperature and atmospheric damage, and very high prevalence estimates were identified using our methodology.

Seasonal effects were assessed in this study using winter and summer point estimates, and as such generalization of these estimates to seasonal trends should be cautious. Specifically designed longitudinal studies would be required for this end.

It is possible that the plating media used for fecal sample culture could have potentially selected for faster growing campylobacters, or for strains with genetic compositions that favoured growth in our protocol conditions (temperature, atmosphere). In addition, only one colony was selected from each culture plate as a means of identifying *Campylobacter* spp./*C. jejuni* in samples. This may have resulted in reduced prevalences from selection bias, or in less genetic variability of *C. jejuni* isolates, particularly pertinent to the microarray study that looked at similarities and differences between isolates.

Campylobacter spp. were not isolated in the ground beef survey. It is possible that some component of the culture protocol, whether in enrichment, plating media, length of incubation, temperature or atmospheric conditions, limited the sensitivity of the technique. As the prevalence of campylobacters in ground beef was known to be low (0.5-3%) based on previous research (Zhao et al. 2001, Whyte et al. 2004) very few positive packages were expected. It is also possible that a lengthier incubation period, perhaps with time at 37°C, may have improved recovery. However it is difficult to speculate on this when the potential number of naturally contaminated samples is so small. In addition, our spiked meat trials were able to isolate very low levels ($1x10^1$ cfu/g) of *C. jejuni* in ground beef using the study protocol. Only a small portion of

ground beef from the centre of the meat package was tested. It is possible that campylobacters may not have been distributed uniformly throughout the ground beef and that sampling larger amounts of meat from several areas of the package may have resulted in positive cultures.

The selection of the 142 ground beef samples for testing with PCR was not random. Initially every 10^{th} ground beef sample was selected and frozen for later testing, but this systematic approach did not continue for the entire study. However, 52 of the 60 stores were represented, 60 samples from winter and 82 from summer, and samples were tested from all chains and most stores in all three cities. In addition, power was likely limited in the risk factor investigation because only 142 samples were tested.

Because DNA microarray is relatively new technology, protocols for DNA labeling, scanning and analysis are not standardized, including cut points for identifying genes as present or absent. In this study, laboratory strain arrays and technical replicates were used to validate clustering results. In addition, DNA is quite expensive and time-consuming, and technical expertise is required for labeling, scanning and data analysis. As a result, the technique used here would be less appropriate for time sensitive analyses such as outbreak investigation or organism detection surveys.

6.4 Future research

The purpose of this research was to identify the prevalence of campylobacters, in particular *C. jejuni*, in commercial feedlot cattle feces and retail ground beef. The isolates identified were to be used for further molecular characterization using DNA microarray and to create a collection of *Campylobacter* isolates for use in future research.

The number of feedlots enrolled in the study was small, particularly related to risk factor analyses. In the future a larger number of feedlots should be enrolled to increase the power and external validity of study findings. From our results, reasons for differences between feedlots should be a topic of future research as it may be possible to reduce *Campylobacter* shedding through interventions and feedlot management. The identification and inclusion of a larger number of management variables, both at feedlot and pen levels, should be incorporated in analyses.

Little research has been published on seasonality in feedlot cattle *Campylobacter* shedding. The point estimates identified here were interesting as differences between seasons were not found. Using this research as a start point, seasonal *Campylobacter* shedding trends in commercial feedlot cattle should be evaluated using longitudinal surveys which sample animals at regular intervals over the course of the year. The study should span several years so that periodicity in shedding can be documented. This type of research should include antimicrobial use data (both feed and individual treatment) to aid in interpretations of results.

From the ground beef survey, the prevalence of culturable thermophilic campylobacters seems extremely low. However, *Campylobacter* DNA was detected in 48% of 142 ground beef samples using PCR, suggesting that improvements in ground beef handling at the slaughterhouse and grocery store levels may be possible. These findings also reinforce awareness that breaches in current ground beef handling may have the potential to result in human infection. In the future, it may also be worthwhile to include meat department pork cutting practices in risk factor studies as our PCR results identified *C. coli* in 26.8% of the 142 packages tested.

This research assessed the prevalence of campylobacters in feedlot cattle near slaughter weight and in ground beef as a preliminary assessment of the importance of campylobacters in the Alberta beef industry. To more directly assess the role of these enteric bacteria, cattle/beef should be sampled at feedlot, slaughter plant, processor and retail levels over several years. Feedlot cattle are the source of approximately 70% of the contents of ground beef (Young et al. 1997). Often beef from leaner cattle (cull cows, bulls and dairy) may be added to ground beef from fed cattle to meet consumer preferences for reduced fat levels in the product. As a result, farm to fork assessment of *Campylobacter* levels should also incorporate data from these non-feedlot cattle. This type of complex farm to fork study would need to be large and to have the support of a number of stakeholders, including feedlots, non-feedlot cattle suppliers, slaughter plants, processors and retail chains to obtain required data.

6.5 Overall conclusions

DNA microarray was evaluated as an epidemiologic tool. Due to the time, labour, cost and expertise required, this technology does not, at present, seem appropriate for time sensitive analyses such as outbreak investigation although automation and the use of robotics are dealing with these issues. However, the high resolution data acquired and the value-added ability to accomplish both genomic profiling and gene association investigations with the same dataset makes this technology of worth as a molecular epidemiologic tool.

Based on the high levels of *Campylobacter* isolation rates in feedlot cattle near slaughter weight and the high degree of similarity found between human and feedlot cattle *C. jejuni* isolates within the same time frame and geographical regions, it seems

plausible that feedlot cattle could be important reservoirs of campylobacters related to human health and that further exploration of the transmission routes between cattle and people may be warranted

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APPENDIX A
LITERATURE REVIEW KEYWORDS

Appendix Table A.1 Literature review keywords

Main search July 3-16, 2007 [a]

Keywords

((feedlot OR cattle OR bovine OR bovid* OR steer* OR heifer* OR cow* OR bull* OR calf OR calv*) AND (feces OR fecal OR pat*) AND (campylobact* OR jejuni) AND (prevalence OR survey)) [b]

((beef OR "ground beef" OR "red meat") AND (retail OR grocer*) AND (campylobact* OR jejuni))

((beef OR "ground beef" OR "red meat") AND (campylobact* OR jejuni)) [b]

(("DNA microarray" OR microarray OR array OR chip OR "comparative genom* hybridization" OR CGH OR "whole genome") AND (campylobact* OR jejuni)) [b]

((feedlot OR cattle OR bovine OR bovid* OR steer* OR heifer* OR cow* OR bull* OR calf or calv*) AND (season*) AND (campylobact* OR jejuni))

((campylobact* OR jejuni) AND (outbreak OR case) AND (human OR people) AND (Canad*))

MeSH Terms:

Campylobacter AND cattle AND feces [b]

Cattle AND Meat AND Campylobacter [b]

Oligonucleotide array sequence analysis AND Campylobacter [b]

Microarray Analysis AND Campylobacter [b]

Seasons AND Cattle AND Campylobacter

Campylobacter AND human AND outbreak AND Canada

Campylobacter AND Canada [b]

[a] Some modifications to keyword formatting were required based on the specific requirements of some databases.
[b] Terms set up in PubMed (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov) as weekly notifications for newly published articles.

APPENDIX B
ETHICS ANALYSIS

The following ethics analysis was written prior to initiation of this project as a means of assessing the principles of respect for persons, respect for animals, justice, non-maleficence and beneficence related to the research.

Respect for persons

The principle of respect for persons will be met as the investigators in this study will not have access to any identifying information from human samples. Study procedures are also compliant with the requirements of the Alberta Health Information Act. Alberta diagnostic laboratories will supply *Campylobacter jejuni* (*C. jejuni*) positive human isolates to the Alberta Provincial Laboratory of Public Health (APLPH, in accordance with Section 24 of the Alberta Public Health Act). The APLPH acts as a "custodian" as per section 1(1) (f)(iii) of the Alberta Health Information Act. The APLPH will then submit to the investigator the first positive isolate per person, the health region of residence of the person, the age and gender of the patient. In addition, samples will be excluded for any person who has traveled outside of Canada or Alberta within 30 days. The human samples will then be genotyped using DNA microarray techniques. The human subjects that submitted the fecal samples will not be contacted at any time during this study. The research team for this project has expertise in all areas necessary, including public health, molecular and diagnostic laboratory techniques,

veterinary, epidemiological, and cattle industry experience. Diagnostic work will be completed at the APLPH and the Vaccine and Infectious Disease Organization, both which have appropriate and regulated facilities fit for this study.

Respect for Animals

Respect for animals is also a requirement of this study. Humane animal handling and sample collection techniques will be used throughout this study, in accordance with Section 446 of the Canadian Criminal Code, the Recommended Code of Practice for the Care and Handling of Farm Animals—Beef Cattle (Agriculture Canada 1870/E), the Canadian Council on Animal Care, and the University of Saskatchewan Animal Care Committee.

Justice

The laboratory samples were taken prior to the onset of this work, and were directly for the health benefit of the people, not for the benefit of this research. Fees may be paid to the APLPH to help reimburse staff and transport costs for sample collection and shipping.

Non-maleficence

The human subjects supplying the fecal samples have minimal risk of harm as their samples have been collected previously for purposes not associated with this study, and the subjects will not be contacted. Further, the subjects cannot incur the harm of breach of privacy as the researchers will not be able to identify them. The APLPH will not include any identifying information with the samples, so as to protect the privacy of the individuals.

In the short term, if meat samples are found to be *Campylobacter* positive, the published results could negatively influence consumer confidence, and possibly reduce meat purchases in Alberta. This could be detrimental to the Alberta Beef Industry and to the Alberta retail supermarkets. However in the long term, if campylobacters are found in the meat, it is in the best interest of the beef industry, the retail meat industry and the public health sectors to have this information so that timely and effective preventive measures may be introduced, and risk to the public reduced (i.e., there is social benefit to this study). In this study, Alberta supermarkets will not be informed that their meat will be sampled, as it is important to eliminate possible bias that could be introduced if the supermarket managers have knowledge of the project. As a means of protecting stores, steps will be taken to avoid identification of supermarket store or chain in publication and communication of results.

The prevalence of *C. jejuni* in ground beef in the USA and Italy have been estimated to be very low, 0.5% and 1.3% respectively (Pezzotti et al. 2003, Zhao et al. 2001). We believe the type of risk to Alberta consumers from ground beef is similar to the risk presently incurred by purchasing other raw supermarket meats, or to the risk from ground beef due to other organisms like *E. coli* and *Salmonella.* At present in Canada, public education programs have been implemented to educate consumers on the handling of raw meats (including specific recommendations for preventing disease from *E. coli* http://www.hc-sc.gc.ca/english/iyh/food/hamburger.html and *C. jejuni* http://www.inspection.gc.ca/english/corpaffr/foodfacts/ campye.shtml). Thus even if campylobacters were found in this survey, public health programs to address handling and cooking of meat in the home are already in place. It is expected that the time

between the collection and the culture of samples will be sufficiently long that recall of meat found positive for *Campylobacter* spp. would not be necessary, and based on a personal communication with Dr J Kamanzi (Director, Food Microbiology and Chemical Evaluation, Food Safety, Canadian Food Inspection Agency), there is no legal obligation to report findings of *C. jejuni* in ground beef to the Canadian Food Inspection Agency.

This research is important to the continued improvement of food safety in Alberta and Canada. All attempts will be made to interpret the results appropriately, and to publish the results in a way that minimizes impact on consumer confidence, supermarkets, and the Alberta beef industry. However, in order to best address Alberta food safety, it is imperative that findings be published. Whether the result is protection of consumer confidence with low prevalence, or the need to commit research and funds toward identifying prevention and control interventions, public safety is best served by the continuation of this research.

Beneficence

The diagnostic techniques used in this study, enrichment culture and hippurate hydrolysis testing, have both been used previously to detect campylobacters in both meat and fecal samples. DNA microarray has been used previously for characterizing *C. jejuni* and is an exciting new molecular tool. As a result, the use of these tools will further the body of knowledge in diagnosis and description of *Campylobacter* spp., significant contributions to science. This study will try to link *Campylobacter* genotypes from human, feedlot cattle, and beef sources. These results have important public health ramifications as they will allow further understanding of beef as a potential source of

infection for people. In addition, knowledge of *Campylobacter* prevalence in cattle on their sites may be of benefit to feedlot operators in terms of understanding the zoonotic potential to themselves and their staff, and increasing awareness related to food safety.

Conclusions

It is expected that the results of this study will have positive social benefit to the people of Alberta and Canada by improving the current state of knowledge. At present, *C. jejuni* is a significant cause of enteric disease in people. However, little is known about the prevalence of campylobacters in red meat, in particular ground beef in Canada. It is critical to identify if beef is a potential or significant source of *Campylobacter* spp. If campylobacters are not found in any of the meat, this will support consumer confidence in the food supply. If *Campylobacter* spp. are found in the meat, education programs on the importance of proper handling and cooking procedures in the home are already in place. In addition, the Medical Officers of Health in the three Regional Health Authorities supplying human isolates will be provided with copies of the published results of this study. If positive culture results are found, this preliminary study will encourage further scientific research into appropriate interventions to reduce the prevalence of *Campylobacter* in feedlot cattle and ground beef.

References

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Zhao C, Ge B, De Villena J, Sudler R, Yeh E, Zhao S, White DG, Wagner D, Meng J. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork and beef from the Greater Washington, D.C., area. Appl Environ Microbiol 2001;67:5431-5436.

APPENDIX C
GENE ASSOCIATION TESTING RESULTS

In Chapter 5 detailed methodology for *Campylobacter jejuni* gene association testing for was described. The following tables give full results for that testing. Two analyses are presented: 1) cattle isolates compared to human isolates, and 2) "cattle enriched" comparative genomic hybridization (CGH) clusters compared to "human enriched" CGH clusters. See Figure 5.2 for a description of groupings.

In brief, gene conservation rates were compared between groups using the Fisher's exact test (unadjusted p value). In addition, two adjustments for multiple comparisons were made. The Westfall and Young correction (Westfall and Young 1993) is a moderately conservative adjustment, while the Holm step-down method (Westfall and Young 1993) is an extremely conservative adjustment. Both are presented in the tables below. Genes that were unequally distributed between the groups with statistical significance $p \leq 0.05$ in both the unadjusted Fisher's exact p value and the Westfall and Young p value are marked (^{a}). In addition, the gene products from Gundogdu et al. 2007 are also given.

Appendix Table C.1 Results of *C. jejuni* gene association testing for comparison of cattle and human isolate groups

Gene	Absent Cattle (n=46)	Absent Human (n=41)	Cattle vs Human			Gene Product
			Unadjusted *p*-value	WY *p*-value	HSD *p* value	
Cj0208	37	40	0.01662	0.19040	1.00000	put. DNA modification methylase (adenine-specific methyltransferase)
Cj0261	46	37	0.04550	0.41720	1.00000	put. SAM-dependent methyltransferase
Cj0303	32	38	0.00733	0.09755	1.00000	put. molybdate-binding lipoprotein
Cj0422	39	41	0.01301	0.16195	1.00000	put. H-T-H containing P
Cj0617	37	20	0.00306	0.04945[a]	1.00000	hypothetical P
Cj0628	2	12	0.00248	0.04255[a]	1.00000	put. lipoprotein
Cj0938	40	41	0.02745	0.28505	1.00000	put. 2-acylglycerophospho-ethanolamine acyltransferase/ acyl-acyl carrier P synthetase
Cj1141	12	20	0.04438	0.39515	1.00000	sialic acid synthase (N-acetyl-neuraminic acid synthetase)
Cj1143	11	19	0.04155	0.37910	1.00000	two-domain bifunctional protein (beta-1,4-N-acetylgalactosaminyl-transferase/CMP-Neu5Ac synthase)
Cj1146	28	36	0.00675	0.09140	1.00000	put. glucosyltransferase
Cj1293	40	41	0.02745	0.28505	1.00000	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase
Cj1323	27	33	0.03718	0.35705	1.00000	hypothetical P
Cj1421	14	24	0.01009	0.13215	1.00000	put. sugar transferase
Cj1422	16	25	0.01856	0.21270	1.00000	put. sugar transferase
Cj1428	18	27	0.01802	0.20515	1.00000	GDP-L-fucose synthetase
Cj1429	13	21	0.04688	0.42180	1.00000	hypothetical P
Cj1433	8	16	0.03135	0.31575	1.00000	hypothetical P
Cj1434	12	21	0.02621	0.27320	1.00000	put. sugar transferase
Cj1435	13	21	0.04688	0.42180	1.00000	put. phosphatase
Cj1438	13	21	0.04688	0.42180	1.00000	put. sugar transferase
Cj1439	4	14	0.00685	0.09380	1.00000	UDP-galactopyranose mutase
Cj1520	19	30	0.00461	0.06805	1.00000	removed from CS
Cj1562	46	36	0.02028	0.23885	1.00000	hypothetical P
Cj1668	44	29	0.00248	0.04255[a]	1.00000	put. periplasmic P
Cj1729	4	14	0.00685	0.09380	1.00000	flagellar hook subunit P

[a] $p \leq 0.05$ for both the Fisher's exact unadjusted *p* value and the Westfall and Young adjusted p value. CS: coding sequences; HSD: Holm step-down; P: protein; Put: putative; WY: Westfall and Young.

Appendix Table C.2 Results of *C. jejuni* gene association testing for comparison of CGH "cattle enriched" and "human enriched" groups

CS: coding sequences, P: protein, put.: putative, mod.: modification.

Gene	Absent CGH CE (n=27)	Absent CGH HE (n=24)	CGH CE vs CGH HE			Gene Product
			Unadjusted *p* value	WY *p* value	HSD *p* value	
Cj0030	24	13	0.01054	0.09840	1.00000	hypothetical P
Cj0057	27	19	0.01809	0.15630	1.00000	put. periplasmic P
Cj0138	25	16	0.03258	0.24630	1.00000	conserved hypothetical P
Cj0201	26	16	0.00850	0.08520	1.00000	put. integral membrane P
Cj0202	27	14	0.00015	0.00210[a]	0.21228	hypothetical P
Cj0300	19	24	0.00464	0.05000[a]	1.00000	put. molybdenum transport ATP-binding P
Cj0302	16	24	0.00033	0.00400[a]	0.44978	put. molybdenum-pterin binding P
Cj0303	11	24	<0.00001	<0.00001[a]	0.00268[b]	put. molybdate-binding lipoprotein
Cj0304	15	24	0.00013	0.00135[a]	0.17513	put. biotin synthesis P
Cj0305	20	24	0.01066	0.10355	1.00000	conserved hypothetical P
Cj0380	27	19	0.01809	0.15630	1.00000	hypothetical P
Cj0399	17	24	0.00081	0.01145[a]	1.00000	colicin V production P homolog
Cj0417	18	22	0.04249	0.28860	1.00000	hypothetical P
Cj0422	21	24	0.02391	0.20490	1.00000	put. H-T-H containing P
Cj0481	10	17	0.02456	0.20910	1.00000	put. dihydrodipicolinate synthase
Cj0482	11	17	0.04849	0.33655	1.00000	put. altronate hydrolase N-terminus
Cj0483	13	19	0.04104	0.26400	1.00000	put. altronate hydrolase C-terminus
Cj0484	13	20	0.01764	0.14095	1.00000	put. MFS (Major Facilitator Superfamily) transport P
Cj0485	13	21	0.00352	0.0389[a]	1.00000	put. oxidoreductase
Cj0486	14	20	0.02091	0.17240	1.00000	put. sugar transporter
Cj0488	12	19	0.02058	0.16775	1.00000	conserved hypothetical P
Cj0489	10	17	0.02456	0.20910	1.00000	put. aldehyde dehydrogenase N-terminus
Cj0490	11	17	0.04849	0.33655	1.00000	put. aldehyde dehydrogenase C-terminus
Cj0522	15	21	0.01570	0.13895	1.00000	put. Na+/Pi cotransporter P
Cj0617	27	6	<0.00001	<0.00001	0.00001[b]	hypothetical P
Cj0619	27	19	0.01809	0.15630	1.00000	put. MATE family transport P
Cj0628	0	8	0.00116	0.01525[a]	1.00000	put. lipoprotein
Cj0691	22	11	0.01000	0.09225	1.00000	put. membrane P
Cj0755	18	6	0.00473	0.05325	1.00000	ferric enterobactin uptake receptor
Cj0968	17	22	0.02153	0.17895	1.00000	CS merged with Cj0969 pseudogene (put. periplasmic P)
Cj0970	11	19	0.00967	0.08700	1.00000	hypothetical P
Cj0973	18	22	0.04249	0.28860	1.00000	hypothetical P
Cj1051	2	12	0.00118	0.01555[a]	1.00000	restriction mod. enzyme
Cj1135	11	18	0.02292	0.18655	1.00000	put. two-domain glucosyltransferase
Cj1136	3	16	0.00005	0.00055[a]	0.06864	put. glycosyltransferase

Gene	Absent CGH CE (n=27)	Absent CGH HE (n=24)	CGH CE vs CGH HE			Gene Product
			Unadjusted *p* value	WY *p* value	HSD *p* value	
Cj1136	3	16	0.00005	0.00055[a]	0.06864	put. glycosyltransferase
Cj1137	4	17	0.00006	0.00085[a]	0.08631	put. glycosyltransferase
Cj1138	5	17	0.00023	0.00325[a]	0.31148	put. glycosyltransferase
Cj1139	10	22	0.00010	0.00115[a]	0.13489	beta-1,3 galactosyl-transferase
Cj1140	9	19	0.00172	0.01970[a]	1.00000	alpha-2,3 sialyltransferase
Cj1141	3	17	0.00001	0.00005[a]	0.02005[b]	sialic acid synthase (N-acetyl neuraminic acid synthetase)
Cj1142	6	18	0.00023	0.0036[a]	0.32195	put. UDP-N-acetyl-glucosamine 2-epimerase
Cj1143	2	16	0.00002	0.0001[a]	0.02370[b]	two-domain bifunctional P (beta-1,4-N-acetylgalactos-aminyltransferase/CMP-Neu5Ac synthase)
Cj1144	6	16	0.00194	0.02315[a]	1.00000	hypothetical P
Cj1145	5	16	0.00066	0.00885[a]	0.91341	CS merged with Cj1144
Cj1146	12	22	0.00038	0.00455[a]	0.52228	put. glucosyltransferase
Cj1150	19	24	0.00464	0.05[a]	1.00000	D-beta-D-heptose 7-phosphate kinase/D-beta-D-heptose 1-phosphate adenylyltransferase
Cj1297	10	19	0.00424	0.04385[a]	1.00000	hypothetical P
Cj1331	19	23	0.02601	0.22700	1.00000	acylneuraminate cytidylyl-transferase (flagellin mod.)
Cj1389	1	16	<0.00001	<0.00001[a]	0.00271[b]	pseudogene (put. C4-dicarb-oxylate anaerobic carrier
Cj1394	20	24	0.01066	0.10355	1.00000	put. fumarate lyase
Cj1415	27	20	0.04252	0.30860	1.00000	put. adenylylsulfate kinase
Cj1421	6	18	0.00023	0.0036[a]	0.32195	possible sugar transferase
Cj1422	7	20	0.00006	0.0008[a]	0.08277	possible sugar transferase
Cj1428	10	20	0.00145	0.0171[a]	1.00000	GDP-L-fucose synthetase
Cj1429	6	19	0.00007	0.0009[a]	0.09391	hypothetical P
Cj1430	8	18	0.00191	0.02225[a]	1.00000	put. dTDP-4-dehydro rhamnose 3,5-epimerase
Cj1432	3	16	0.00005	0.00055[a]	0.06864	put. sugar transferase
Cj1433	2	17	<0.00001	<0.00001[a]	0.00690[b]	hypothetical P
Cj1434	5	19	0.00002	0.00025[a]	0.02574[b]	put. sugar transferase
Cj1435	8	19	0.00105	0.0141[a]	1.00000	put. phosphatase
Cj1438	7	16	0.00504	0.06095	1.00000	put. sugar transferase
Cj1439	0	15	<0.00001	<0.00001[a]	0.00057[b]	UDP-galactopyranose mutase
Cj1440	6	16	0.00194	0.02315[a]	1.00000	put. sugar transferase
Cj1441	9	16	0.02545	0.21815	1.00000	UDP-glucose 6-dehydrogenase
Cj1520	9	20	0.00054	0.00665[a]	0.74973	removed from CS
Cj1668	27	19	0.01809	0.15630	1.00000	put. periplasmic P
Cj1714	27	20	0.04252	0.30860	1.00000	small hydrophobic P
Cj1729	1	13	0.00007	0.0015[a]	0.09587	flagellar hook subunit P

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