DRUG RESISTANCE AND TOXIGENIC PROPERTIES OF *Campylobacter jejuni* AND *Campylobacter coli* ISOLATED FROM THE INTESTINAL TRACT OF CHICKENS ON PRINCE EDWARD ISLAND

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  - Ancient 0289
  - Linguistics 0290
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- Literature
  - General 0401
  - Classical 0294
  - Comparative 0295
  - Medieval 0297
  - Modern 0298
  - African 0316
  - American 0591
  - Asian 0305
  - Canadian (English) 0352
  - Canadian (French) 0355
  - English 0593
  - Germanic 0311
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- Religion
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- American Studies 0323
- Anthropology
  - Archaeology 0324
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- Business Administration
  - General 0310
  - Accounting 0272
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  - Management 0454
  - Marketing 0338
- Canadian Studies 0385
- Economics
  - General 0501
  - Agricultural 0503
  - Commerce-Business 0505
  - Finance 0508
  - History 0509
  - Labor 0510
  - Theory 0511
- Folklore 0358
- Geography 0366
- Gerontology 0351
- History
  - General 0578
  - Ancient 0579
  - Medieval 0581
  - Modern 0582
  - Black 0328
  - African 0331
  - Asia, Australia and Oceania 0332
  - Canadian 0334
  - European 0335
  - Latin American 0336
  - Middle Eastern 0333
  - United States 0337
- History of Science 0585
- Law 0398
- Political Science
  - General 0615
  - International Law and Relations 0616
  - Public Administration 0617
- Recreation 0814
- Social Work 0452
- Sociology
  - General 0626
  - Criminology and Penology 0627
  - Demography 0938
  - Ethnic and Racial Studies 0631
  - Individual and Family Studies 0628
  - Industrial and Labor Relations 0629
  - Public and Social Welfare 0630
  - Social Structure and Development 0700
  - Theory and Methods 0344
- Transportation 0709
- Urban and Regional Planning 0999
- Women's Studies 0453

THE SCIENCES AND ENGINEERING

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- Agriculture
  - General 0473
  - Agronomy 0285
  - Animal Culture and Nutrition 0475
  - Animal Pathology 0476
  - Food Science and Technology 0359
  - Forestry and Wildlife 0478
  - Plant Culture 0479
  - Plant Pathology 0480
  - Plant Physiology 0817
  - Range Management 0777
  - Wood Technology 0746
- Biology
  - General 0306
  - Anatomy 0287
  - Biostatistics 0308
  - Botany 0309
  - Cell 0379
  - Ecology 0329
  - Entomology 0353
  - Genetics 0369
  - Limnology 0793
  - Microbiology 0410
  - Molecular 0307
  - Neuroscience 0317
  - Oceanography 0416
  - Physiology 0433
  - Radiation 0821
  - Veterinary Science 0778
  - Zoology 0472
- Biophysics
  - General 0786
  - Medical 0760

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- Biogeochemistry 0425
- Geochemistry 0996
- Geodesy 0370
- Geology 0372
- Geophysics 0373
- Hydrology 0388
- Mineralogy 0411
- Paleobotany 0345
- Paleoecology 0426
- Paleontology 0418
- Paleozoology 0985
- Palynology 0427
- Physical Geography 0368
- Physical Oceanography 0415

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- Environmental Sciences 0768
- Health Sciences
  - General 0566
  - Audiology 0300
  - Chemotherapy 0992
  - Dentistry 0567
  - Education 0350
  - Hospital Management 0769
  - Human Development 0758
  - Immunology 0982
  - Medicine and Surgery 0564
  - Mental Health 0347
  - Nursing 0569
  - Nutrition 0570
  - Obstetrics and Gynecology 0380
  - Occupational Health and Therapy 0354
  - Ophthalmology 0381
  - Pathology 0571
  - Pharmacology 0419
  - Pharmacy 0572
  - Physical Therapy 0382
  - Public Health 0573
  - Radiology 0574
  - Recreation 0575
  - Speech Pathology 0460
  - Toxicology 0383
- Home Economics 0386

PHYSICAL SCIENCES
Pure Sciences
- Chemistry
  - General 0485
  - Agricultural 0749
  - Analytical 0486
  - Biochemistry 0487
  - Inorganic 0488
  - Nuclear 0738
  - Organic 0490
  - Pharmaceutical 0491
  - Physical 0494
  - Polymer 0495
  - Radiation 0754
- Mathematics 0405
- Physics
  - General 0605
  - Acoustics 0986
  - Astronomy and Astrophysics 0606
  - Atmospheric Science 0608
  - Atomic 0748
  - Electronics and Electricity 0607
  - Elementary Particles and High Energy 0798
  - Fluid and Plasma 0759
  - Molecular 0609
  - Nuclear 0610
  - Optics 0752
  - Radiation 0756
  - Solid State 0611
- Statistics 0463

Applied Sciences
- Applied Mechanics 0346
- Computer Science 0984
- Engineering
  - General 0537
  - Aerospace 0538
  - Agricultural 0539
  - Automotive 0540
  - Biomedical 0541
  - Chemical 0542
  - Civil 0543
  - Electronics and Electrical 0544
  - Heat and Thermodynamics 0348
  - Hydraulic 0545
  - Industrial 0546
  - Marine 0547
  - Materials Science 0794
  - Mechanical 0548
  - Metallurgy 0743
  - Mining 0551
  - Nuclear 0552
  - Packaging 0549
  - Petroleum 0765
  - Sanitary and Municipal 0554
  - System Science 0790
- Geotechnology 0428
- Operations Research 0796
- Plastics Technology 0795
- Textile Technology 0994

PSYCHOLOGY
- General 0621
- Behavioral 0384
- Clinical 0622
- Developmental 0620
- Experimental 0623
- Industrial 0624
- Personality 0625
- Physiological 0989
- Psychobiology 0349
- Psychometrics 0632
- Social 0451

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iii-iv

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ABSTRACT

Campylobacter jejuni and *C. coli* were isolated from 119 (51.5%) of 231 samples of chicken cecal contents. Of these 101 (85%) were identified as *C. jejuni* and 18 (15%) as *C. coli* based on the Hippurate hydrolysis test. Antimicrobial resistance was determined using the disk diffusion method. Resistance to the eight antimicrobials used was as follows: ampicillin 53%; erythromycin 7%; tetracycline 2% and kanamycin 4%. All the isolates were susceptible to enrofloxacin, chloramphenicol and nalidixic acid. None of the isolates was found to be susceptible to cephalothin.

Plasmid DNA was isolated from two tetracycline resistant (TET^r) *C. jejuni* strains RO 28 and RO 33 from broiler chickens using three different protocols: rapid TELT mini-prep, rapid mini-prep with heat, and Promega™ mini-prep. Of these three only Promega™ mini-prep gave a consistent yield of plasmid DNA. The two TET^r isolates RO 28 and RO 33 each had a plasmid DNA in the Agarose gel electrophoresis corresponding to the 45 Kbp TET^r plasmid and a smaller band corresponding to the 12 Kbp of the marker DNA. A TET^r strain of *C. jejuni* ATCC 43502 with a 45 Kbp a TET^r plasmid was used as a control.

The production of enterotoxin by *C. jejuni* and *C. coli* was evaluated by adding cell-free filtrates of 48 hour cultures of the bacteria to CHO-K1 cells grown in Ham's F12 media in 96 well plates. Two human *C. jejuni* strains 4483 and 6704 were included in each test as positive and negative controls respectively. Of the 119 isolates of *C. jejuni* and *C. coli* that were tested, 56 (47%) produced toxins that caused elongation of the CHO-K1 cells within 48-72 hours following incubation at 37°C in a 5% CO_2 incubator. Strong toxin effect was observed at dilutions of 1:2, 1:4 and 1:8. The toxin titers of the isolates tested varied considerably. The toxin effects were best demonstrated when the F12 media used to dilute the bacterial toxin contained 3% Fetal Bovine Serum (FBS) compared to F12 with 10% FBS used for maintaining the CHO-K1 cells. This might be due to nonspecific binding reaction between the bacterial toxin and the serum proteins.

In conclusion, understanding the rate of carriage of drug resistant and toxigenic *C. jejuni* by chicken is crucial to the development of control measures to reduce the contamination of the pathogen on poultry meat and thus lower the risk of infection to consumers.

To my Parents:

Nur Ahmed Castelli and Faduma Godane
(May God Bless their Souls)

To my Canadian family:

Dwight and Barbara Fulford

To my brothers and sisters

To all those who helped me during hard times

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1. REVIEW OF LITERATURE

1.1 General Introduction

GENUS *CAMPYLOBACTER*: HISTORIC BACKGROUND AND DESCRIPTION

The genus name *Campylobacter* was derived from the Greek word for curved rod. Sebald and Vèron (1963), proposed to include in this genus microaerophilic bacteria that were unrelated to *Vibrio cholerae* and other vibrios on the basis of their nucleotide base composition (mol% G+C), as well as their inability to use sugars either oxidatively or fermentatively (Alexander, 1957; Lecce, 1958; Owen and Leaper, 1981; Sebald and Vèron, 1963).

Campylobacters are gram-negative bacteria, 0.5 to 8μm long and 0.2 to 0.5μm wide with characteristically curved, or S-shaped cells (Penner, 1988). The motility of the bacteria is characteristically rapid and darting in corkscrew fashion, a feature by which their presence among other bacteria can be detected by phase-contrast microscopy (Karmali and Fleming, 1979). Their guanine-plus-cytosine (G+C) content is low, ranging from 28 to 38 mol% (Smibert, 1984; Vèron and Chatelaine, 1973).

There are few biochemical reactions by which *Campylobacter* species can be differentiated, due to their inability to ferment or oxidize the usual carbohydrate substrates available in the diagnostic laboratory (Penner, 1988). They have a respiratory type of metabolism and use amino acids and intermediates of the tricarboxylic acid cycle.

Smith and Orcutt (1927) described another group of "Vibrio-like" bacteria from the feces of cattle with diarrhea. Later, a casual relationship was shown between these thermotolerant vibrios and bovine dysentery and the organisms were subsequently named as *Vibrio jejuni* (Laanbroek et al., 1977; La Riviere and Kuenen, 1989). Similar organisms were also found in aborted sheep fetuses (Bryans et al., 1960; Smibert, 1978) and in the blood of humans with diarrhea (King, 1957; 1962). In 1944, Doyle isolated vibrios from the feces of pigs with diarrhea (Doyle, 1944) and they were named *Vibrio coli* (Doyle, 1948).

Butzler and co-workers, in Belgium and Skirrow in the United Kingdom emphasised the importance of these organisms as human enteropathogens by introducing a filtration technique and selective growth media in isolation procedures (Dekeyser et al., 1972; Butzler et al., 1973; Skirrow, 1977). At present, it is known that these organisms occur, mostly as commensals, in cattle, pigs, sheep, poultry, various domestic animals, birds, primates, rodent and even insects (Blaser et al., 1979; Garcia et al., 1983; Rosef and Kapperud, 1983). They are the bacterial enteropathogens most frequently isolated from children (Blaser et al., 1979). Campylobacter enteritis was first reported by Escherich in 1886, where he described finding in the feces of children with diarrhea, spiral bacteria that could not be cultured on solid media (Kist, 1985). Other authors made similar observations over the following decade but interest died down and these early contributions went unrecognized for many years until the organisms were rediscovered by Skirrow from humans with diarrhea (Skirrow, 1977).

The campylobacters of medical significance, *C. jejuni*, *C. coli* and *C. laridis*, show optimal growth at 42-43°C. This thermophilic characteristic can be used to differentiate these species from *C. fetus*, which grows at 25°C and rarely at 37°C. Among the thermophilic campylobacters, *C. jejuni* causes the majority (98%) of the cases of gastroenteritis. The closely related species *C. coli* is often attributed to about 2 to 5% of all cases (Karmali et al., 1983).

C. jejuni and *C. coli* each have two colony forms on primary isolation as described by Smibert (1974; 1978) as follows: a flat, greyish, translucent type which tends to coalesce, or colonies which are round, raised and have a distinct margin. These two forms can occur simultaneously.

The incubation period associated with detection of the organisms in primary cultures of *C. jejuni* is dependent on the size of the inoculum and their number in the specimen (Butzler and Skirrow, 1979). Colony growth is generally recognizable within 24-48 hours, but an incubation time of 48-72 hours is required when only a few organisms are present in the primary isolation.

Isolation of *C. jejuni* is dependent on the application of specialized laboratory techniques. Appropriate methods of transportation and storage of specimens are important in maintaining viability of the organisms prior to laboratory culture (Shane and Montrose, 1985). A semi-solid brucella medium with 10% sheep blood has been recommended for transport of cultures of *C. jejuni* and storage for up to three weeks at 25°C (Wang et al., 1980). Leuchtefeld et al. (1981) compared the survival of *C. jejuni* in cecal specimens derived from infected turkeys, utilizing six transport media

at various temperatures. Media included Cary Blair media with decreased agar, buffered glycerol saline, alkaline peptone water, thiogylocollate medium, Campy-thio^{R} (Pasco laboratories) and Culturettes^{R} (Marion Scientific) with modified Stuart's medium. Of all these, Cary Blair medium with decreased agar supported the best viability and was recommended for samples stored longer than three days.

The realization that *Campylobacter spp.* are a common causes of enteritis in humans was due to the development of highly selective media able to suppress all the other fecal microbes thus supporting the growth of campylobacters often present in the intestinal milieu as a minor population. Skirrow's (1977) medium is fairly good for isolating campylobacters from feces and is still widely used today. Many different formulations for selective media have been suggested. The performance of each of the most commonly used preparations was reviewed by Bolton et al. (1986), Goossens et al. (1986), and Merino et al. (1986). Preston blood-free medium is excellent for the routine isolation of *C. jejuni*, *C. coli* and *C. laridis* (Bolton and Coates, 1983; Fricker et al., 1983) from feces. It contains cefoperazone as the selective agent which inhibits most of the unwanted fecal flora, and it is more inhibitory to competing flora than most other recommended media and this probably explains its greater sensitivity (Fricker, 1987).

The identification of *Campylobacter spp.* can be difficult because of their fastidious growth requirements and their inability to ferment sugars. There are limited biochemical tests that give adequate discrimination. For example, separation of *C. coli* from *C. jejuni* relies almost exclusively on hippurate hydrolysis. However, hippurate

negative strains of *C. jejuni* and hippurate positive *C. coli* have been reported (Goossens and Butzler, 1992). Resistance to various agents, temperature tolerance and growth requirements are among the phenotypic tests used in characterizing campylobacters. The following tests have been recommended for these organisms: oxidase; catalase; H_2S production in Triple Sugar Iron (TSI) agar; hippurate hydrolysis, growth at 15°, 25° and 42°C; and tests on susceptibility to cephalothin and nalidixic acid. *Campylobacter jejuni* grows at 37°C or 42°C, is catalase and hippurate positive. It is resistant to cephalothin and sensitive to nalidixic acid. Growth of *C. jejuni* and *C. coli* at 37°C or 42°C, hippurate hydrolysis and susceptibility to cephalothin and nalidixic acid provides relatively good discrimination among enteropathogenic campylobacters such as *C. jejuni, C. coli, C. laridis* and other animal pathogens.

1.2 Pathogenic properties of *Campylobacter jejuni*

The report by King (1957) on the isolation of *C. jejuni* which was formerly classified among the "related vibrios" from the blood of children with diarrhea, represented the first account of the pathogenicity of the organism.

Infection with *C. jejuni* was subsequently described in chickens as avian vibrionic hepatitis (Peckham, 1958) and avian infectious hepatitis (Hofstad et al., 1958). The exact mechanism by which campylobacters induce disease is not clear. However, based on the clinical symptoms of the disease, three mechanisms have been postulated by which *C. jejuni* is believed to cause illness. Levine et al. (1983) categorized enteric pathogens into five groups on the basis of their pathogenesis. Thermophilic

campylobacters have clinical characteristics that resemble the pathogens in three of these groups, namely (i) adherence and production of enterotoxins, inducing secretory diarrhea; (ii) invasion and proliferation within the intestinal epithelium, causing damage and an inflammatory response clinically manifested as dysentery and diarrhea with fecal leucocytes; and (iii) translocation in which the organisms penetrate the intestinal mucosa, causing minimal damage and proliferate in the lamina propria and mesenteric lymph nodes, with further extraintestinal infections such as meningitis, cholecystitis, urinary tract infections and mesenteric lymphadenitis. Although not conclusive, there is clinical and laboratory evidence that supports the existence of these three mechanisms.

Campylobacter jejuni has been recognized as a significant cause of enteritis in humans and a wide range of domestic animals (Butzler and Skirrow, 1979; Fox, 1982; Prescott and Munroe, 1982). It is quite clear that C. jejuni can also infect domestic animals.

In a series of experiments conducted by Butzler and Skirrow (1979), chicken embryo cells and 8-day-old chicks were infected with strains of *C. jejuni* that had been isolated from the feces of diarrheic children. Five isolates were seen to be pathogenic for chick embryo tissue culture cells producing degenerative changes within 36 hours after inoculation. Multiplication of *C. jejuni* in the cecal mucosa of infected chicks was observed together with bacteremia in 50% of the infected chicks (Shane and Montrose, 1985). The organisms were re-isolated from the liver and intestines.

The pathogenesis of campylobacters is often associated with direct invasion of mucosal cells. Ruiz-Palacios et al. (1981) demonstrated the penetration and invasion of intestinal mucosa of chicken by *Campylobacter jejuni* using electron microscopy and immunofluoroscence. These authors demonstrated diarrhea and enteritis in 3-day-old chickens infected orally with a strain of *C. jejuni* recovered from a diarrheic human patient. Diarrhea was observed in 88% (22/25) of the experimentally infected chicks and was accompanied by weight loss and mortality of 32% (8/25).

In another study conducted by Manninen et al. (1982) inoculation of *C. jejuni* failed to induce diarrhea and weight loss in 3-day-old chicks and two-week-old gnotobiotic chicken after experimental infection. The lesions found in all dead birds were located in the jejunum and ileum and consisted of mononuclear infiltration, which was the principal histological change. The study found that the appearance of gastrointestinal signs is influenced by the age of the host. Infection of chicks 12 hours after hatching resulted in onset of diarrhea in experimental subjects but infection at three days of age with 10^9 organisms failed to produce any detectable clinical change (Welkos, 1984). The toxigenic and invasive properties of *C. jejuni* strains may influence the severity of clinical changes.

Pathogenic isolates derived from humans with enterocolitis, induced diarrhea of short duration in newly hatched chicks (Ruiz-Palacios et al., 1981; Sanyal et al., 1984; Welkos, 1984). Gross lesions in neonatal chicks include distention of the jejunum with accumulation of mucus and fluid (Sanyal et al., 1983) or disseminated areas of hemorrhage (Welkos, 1984).

Microscopic lesions attributed to *C. jejuni* infection include edema of the mucosa of the ileum and cecum, with *C. jejuni* present in the brush border of enterocytes (Sanyal et al., 1983). In more severe cases, mononuclear infiltration of the submucosa and villous atrophy occur, resulting in intra-luminal accumulation of mucus, erythrocytes, and mononuclear and polymorphonuclear cells (Welkos, 1984).

1.3 Epidemiology of campylobacters

Campylobacters are ubiquitous organisms. Epidemiology these organisms is complex and it includes many animal species and locations. Campylobacters have been isolated from the intestines of cattle (Morris and Park, 1973; Waterman et al., 1984), pigs (Morris and Park, 1973; Fernie et al., 1975), rats (Fernie and Parks, 1977; Moreno et al., 1990), bank voles (Fernie and Healing, 1976) and dogs and cats (Moreno et al., 1990). Campylobacters are widespread in the gut of gulls and several other wild birds (Fricker et al., 1983). Occurrence of *C. jejuni* in the intestines of sheep is also common. The organism is considered an important cause of epizootic infectious abortion in sheep (King, 1957). However, importance is given to the occurrence of these organisms in the intestines of chicken, because during the process of slaughtering, *C. jejuni* spreads from the intestinal contents to the carcasses. In those flocks in which intestinal carriage was not detected, carcasses were not contaminated (Criuckshank et al., 1982). Chlorine washing does not remove all campylobacters from the giblets and carcasses (Leuchtefeld, & Wang, 1981; Cruickshank et al., 1982). Freezing and storage may diminish the magnitude of contamination (Simmons and Gibbs, 1979; Mehle et al.,

1982). In the United States, the majority of chickens sold at retail markets are contaminated with *C. jejuni* (Eiden and Dalton, 1980; Grant et al., 1980; Park et al., 1981). Processing and packaging of chicken carcasses provide suitable conditions for survival of campylobacters, and surveys at point of sale often show over 50% of samples to be contaminated (Blaser et al., 1984; Fricker and Park, 1989). Feces from infected poultry may also contaminate the surface of eggs.

The significance of *C. jejuni* as a foodborne pathogen is confirmed by the prevalence studies conducted in the United States and England. A national *C. jejuni* isolation rate of 40/100,000 in humans was determined in the USA in 1984 (Tauxe et al., 1992). The Centers for Disease Control, which is responsible for Campylobacter surveillance in the USA recorded a marked seasonal distribution of cases during the summer months of July through September with a second peak extending from mid-November through mid-December. Jones and Telford (1991) also reported a similar seasonal pattern of distribution. Large numbers of campylobacters were found in the water systems during the winter months, but small numbers during the summer months of May, June and July. Campylobacters are sensitive to direct sunlight and high temperatures, this perhaps explains why infections are at their lowest during the summer months. Both these studies indicate the seasonal pattern of the pathogens that could also aid in predicting possible outbreaks during those months.

In England, a human campylobacteriosis prevalence rate of 80/100,000 was calculated for 1989, representing a four-fold increase over a decade (Skirrow, 1990).

Based on USA data, it is estimated that 60,000 to 170,000 clinical cases occurred in that country during the year 1982 (Shane, 1992).

Although non-chlorinated ground water and raw milk are implicated in outbreaks of *C. jejuni* in rural populations (Skirrow, 1991), undercooked chicken is a significant source of infection in urban communities (Finch and Blake, 1985). A survey conducted in Seattle, USA, during 1983-84 revealed 48% of cases of *C. jejuni* enteritis were associated with consumption of chicken which represented the predominant risk factor (Harris et al., 1986).

Bruce et al. (1977) showed the similarity of *Campylobacter spp.* derived from human patients and poultry, suggesting a common source of infection. A study conducted in Australia (Shanker et al., 1982) showed that 98% of 46 isolates of *C. jejuni* from humans with enterocolitis and 82% of 19 isolates from chickens conformed to biotype I. Based on circumstantial evidence, five cases of campylobacter gastroenteritis described by Hayek and Cruickshank (1977) were attributed to either cross-contamination or consumption of infected chicken. All infected persons showed agglutinating antibodies to *C. jejuni* and the organism was isolated from the feces of one individual.

An extensive epidemiological study in Southeastern England during 1985 and 1986 demonstrated the chain of *C. jejuni* infection extending from the broiler farm through a processing plant, wholesaler, catering college and retail outlet to consumers (Pearson et al., 1987). Approximately 31% of the *C. jejuni* isolates derived from patients were of the same serotype as 91% of the chicken isolates on the affected farm.

A case control study in Colorado, USA, demonstrated a significant correlation between handling or preparing of raw chicken and the occurrence of confirmed *C. jejuni* enterocolitis (Hopkins and Scott, 1983). In a similar study, Istre et al. (1984) documented an association between campylobacteriosis and barbecued and presumably undercooked chicken. This work confirmed the earlier observations of Nokrans and Svedhem (1982) in which chicken was implicated as the most probable source of infection in nine patients known to have acquired campylobacteriosis in an urban community in Sweden during 1978-1980.

Free-ranging chickens maintained in close contact with humans represent a significant potential source of *C. jejuni* infections (Marquis et al., 1990). In a study conducted in Peru, it was seen that children in contact with infected chickens had a 12-fold relative risk of campylobacteriosis compared to households without chickens. Employment in a poultry processing plant may also predispose workers to campylobacteriosis (Grados et al., 1983). A serological survey showed that 27% to 68% of personnel in poultry and red meat plants showed complement-fixing antibodies to *Campylobacter spp.*, compared to 3% of rural field workers (Jones and Robinson, 1981). Outbreaks of the disease involving abattoir workers were also reported in Sweden by Christenson et al. (1983).

Serotyping based on heat-stable (Penner and Hennessy, 1980) and heat-labile (Lior et al., 1982) antigens comprise standard techniques to characterize strains of *C. jejuni* isolated in epidemiological investigations. Salama et al. (1990) reported on phagetyping as a method of differentiating among specific Penner serotypes. A rigorous

evaluation of 10 techniques to differentiate *C. jejuni* strains was conducted by the Centers for Disease Control (Patton et al., 1991). Heat-labile and heat-stable serotyping, multilocus enzyme electrophoresis, DNA restriction endonuclease analysis, Southern blot hybridization and ribotyping were all effective in identifying strains associated with water-borne and milk-borne outbreaks of campylobacteriosis. Although these advanced methods can be performed in appropriately equipped laboratories, serotyping was suggested as a practical and inexpensive technique for field and epidemiologic studies (Shane, 1992).

1.3.1 Animal species other than poultry

C. jejuni is an intestinal commensal of cattle. Detection rates in the herds peak in summer months and decline in the winter. Individual cows may excrete the same serotype for at least several months, sometimes for life (Robinson, 1982). Several serotypes may be found in a herd at a given time (Taylor et al., 1982). Transmission to calves occurs but has not been shown to occur among adult cows. However, there is substantial evidence that *C. jejuni* may be the cause of intestinal infection and abortion in cattle. Firehammer and Myers (1981) demonstrated *C. jejuni* infection in 40% of diarrheic calves derived from 14 herds in contrast to a low prevalence in clinically unaffected animals. However, Prescott and Brun-Mosch (1981) were not able to demonstrate a significant difference between healthy and diarrheic cattle. Reported isolation frequencies have varied from herd to herd, but this may be due to the use of different methods for isolation of the organisms. As with poultry, methods that use

enrichment techniques have shown a significantly higher isolation rate. Carcasses may become contaminated with intestinal contents; however, this is infrequent, and when it occurs the level of contamination is often low (<1 organism/cm^2) (Turnbull and Rose, 1982). Unpasteurized cow's milk has also been implicated, as a vehicle, in several outbreaks of Campylobacter enteritis (Robinson and Jones, 1981a). Either contamination with fecal contents or mastitis (Lander and Gill, 1980) are likely situations leading to introduction of *C. jejuni* into the milk. In an experimental model of Campylobacter mastitis, milk became contaminated with large numbers of organisms (Lander and Gill, 1980), but whether this phenomena occurs in nature is unknown. The presence of *C. jejuni* in a herd however does not necessarily lead to contamination of milk.

Swine usually carry *C. coli* and occasionally *C. jejuni* as intestinal commensals (Oosterom, 1980; Svedhem and Kaijser, 1981; Luechtefeld and Wang, 1982; Sticht-Groh, 1982). Studies in the United States, the Netherlands and Germany have shown that more than half of commercially raised pigs excrete these organism. Rosef (1981) recovered *C. coli* from 58% of pig gallbladders at slaughter. A study done in Germany showed a campylobacter isolation rate of 70% of campylobacters from the feces of healthy slaughter pigs (Sticht-Groh, 1982). Isolates of campylobacter from pigs were found to belong to a distinct biotype that are uncommon among human diarrhea-associated isolates (Butzler and Skirrow, 1979). Contamination of swine carcasses is more common than that of sheep and cattle carcasses and possibly occurs as a result of intestinal spillage during slaughtering (Blaser et al., 1983). In many flocks it exists as a long-term intestinal commensal without causing any apparent disease to the animals.

Surveys done on the intestinal contents of sheep have shown that isolation of *C. jejuni* is common, but infection of carcasses occurs less often (Blaser et al., 1983).

In dogs and cats, *C. jejuni* and *C. coli* are present in the stools of both healthy animals and those with diarrhea. Isolation is higher in puppies than in mature dogs. Isolation rates are also higher in kennel populations than among household dogs (Blaser et al., 1980; Bruce et al., 1980). Surveys of dog shelters in England (Hastings, 1978; Bruce et al., 1980; Fox et al., 1988) showed prevalence rates of 38-39% even though only a small percentage of dogs had diarrhea. It is important to recognize that asymptomatic animals colonized with *C. jejuni* and subjected to physical stress may cause the sudden onset of diarrhea and thus excretion of the organisms into the environment. This is particularly relevant in dogs placed on experimental regimens, which may include environmental, physiologic or surgical stress. Of potential zoonotic significance is the finding that 20/36 (56%) of the *C. jejuni* isolates from dogs over a 5 year survey belonged to 20 of the most common serotypes of *C. jejuni* frequently found in humans with diarrhea (Penner and Hennessy, 1980). Zoonotic spread of *C. jejuni* is well documented in diarrheic humans who had been exposed to pet dogs and cats feces infected with *C. jejuni* (Lindquist et al., 1978; Svedham and Norkrans, 1980; Blaser et al., 1983). In one study, 45% of 56 clinically normal cats examined yielded campylobacters while another study (Blaser et al., 1979) reported only 5% of 75 cats positive. Usually kittens are more frequently culture-positive for *C. jejuni* than adult cats (Blaser et al., 1980), and higher isolation rates are found in kennel populations (Bruce et al., 1980). Of 14 stray cats captured in the vicinity of a poultry processing

plant, nine (64%) were positive for campylobacters, and because poultry carcasses are known to harbour campylobacters (Bruce et al., 1977; Ribiero, 1978; Simmons and Gibbs, 1979), these cats may have scavenged contaminated poultry meat.

There are a number of suggestions that animal species other than those described above suffer from naturally occurring Campylobacter enteritis. These suggestions have been generally made on the basis of clinical and bacteriological findings. Atherton and Ricketts (1980) isolated *C. jejuni* from the feces of foals in a sporadic outbreak of diarrhea. Luechtefeld et al. (1981) recovered *C. jejuni* from the feces of 14 of 44 diarrheic and 32 of 575 non-diarrheic animals at a zoo. Several studies on laboratory-reared primates have shown that Campylobacter infection is common (Lauwers et al., 1981), although infection rates among animals in the wilderness and at a zoo are lower (Luechtefeld at al., 1981). Monkeys apparently suffer naturally occurring *C. jejuni* enteritis; Tribe et al. (1979) recovered the organism from the feces of 60% of diarrheic and 18% of normal, newly imported monkeys. Experimental reproduction of *C. jejuni* infection in these animals resulted in mild disease (Fitzgeorge et al., 1981). Other zoo animals that have low *C. jejuni* isolation rates include felines (2%), and ungulates (6%), whereas pigeons and other birds at a zoo had higher carriage rates of 17% and 10%, respectively (Luechtefeld et al., 1981).

1.3.2 Prevalence of *C. jejuni* in poultry

Most commercially raised poultry and free-living birds are natural reservoirs of thermophilic Campylobacters (Shane, 1991a). Prevalence rates are a function of

intensity of surveillance and the strictness of laboratory isolation procedures. Recent studies that use enrichment methods suggest that contamination may be universal (C[illegible]erom, and Beckers, 1982) and may start early in life, although some flocks [illegible]rently escape infection completely (Cruickshank et al., 1982). *Campylobacter jejuni* was recovered from 10% of 445 birds representing 13 groups in a study in Louisiana (Yogasandrum et al., 1989). In wild birds, Galliformes showed the highest prevalence (25%) followed by Anseriformes and Columbiformes which had rates of 13% and 8%, respectively. Feral pigeons are frequently carriers of *C. jejuni* (Luechtefeld et al., 1981; Kinjo et al., 1983), and may infect commercial avian and mammalian species. Among the free-living Galliformes, pheasants (Volkheimer and Wuthe, 1986) and quail (Minakshi and Ayyagari, 1988) can be infected with *C. jejuni*. Free-living marine species including gulls (Kaneuchi et al., 1987), puffins (Kapperud et al., 1983) and shore birds such as waders and gulls (Fricker and Metcalfe, 1984) are frequently infected with *C. jejuni*. A wide range of migratory Anseriformes serve as asymptomatic carriers of *C.jejuni* (Pacha et al., 1988) and are believed to be responsible for contamination of waterways, ponds and reservoirs.

All commercial poultry have been shown to be infected in areas where surveys have been carried out on both household and commercial flocks. A study in Canada showed that 85% of broilers in 28 groups yielded *C. jejuni* from cloacal swabs obtained at slaughter (Prescott and Gellner, 1984). In another study done in Israel on commercial chickens and turkeys comprising 349 birds from 124 farms (Pokamunsky et al., 1984), approximately half of the broiler birds sampled were infected, with an overall

prevalence rate of 31% in birds over four weeks of age. Similar values were obtained in growing turkeys sampled at 6 weeks of age and older, in a survey of 12 broiler flocks in Ireland (Neill et al., 1984). The number of infected farms reached 90% by 70 days of age, and the prevalence rate in individual houses ranged from 50 to 75% based on examination of cloacal swabs. Longitudinal surveys conducted in California (Smitherman et al., 1984) and in Sweden (Engvall et al., 1986) had similar findings. Initial recovery of *C.jejuni* was possible only during the second and third weeks after placement. Subsequently, prevalence rates increased until a high proportion of birds in an infected flock excreted *C. jejuni* consistent with cecal colonization (Genigeorgis et al., 1986). A similar situation occurred in turkeys in which fecal shedding begins at two weeks of age (Acuff et al., 1982).

Cage-housed hens maintained for table-egg production are frequently infected with *C. jejuni*. Flock prevalence rates ranging from 13 to 62% have been observed, based on examination of cloacal swabs (Figueroa et al., 1983; Shane et al., 1986). Breeding flocks of turkeys (Acuff et al., 1982) and broilers (Shanker et al., 1986) may be colonized with *C.jejuni*. Despite maternal infection, it is considered unlikely that vertical transmission occurs under commercial conditions. Eggs from cage-housed table-egg flocks known to carry *C. jejuni* were uninfected in a field study (Shane et al., 1986). Shell penetration by *C. jejuni* occurs at an extremely low rate, as indicated in laboratory studies, and the shell membranes serve as an effective barrier to infection of albumen (Doyle, 1984). The organism can be introduced into eggs by immersion in *C. jejuni* (Neill et al., 1985) or by contamination of the shell followed by application of

pressure and temperature differential treatments (Doyle, 1984). These experimental procedures do not, however, reflect the commercial conditions. Shanker et al. (1986) failed to show vertical transmission from infected breeders to progeny. Monitoring 14 groups of broiler chicks during growth, confirmed the absence of *C. jejuni* during a 6-week period, despite a 74% carriage rate in parents. It is believed that separation of soiled eggs from clean ones and rigorous decontamination of shells reduces bacterial contamination.

Environmental contamination is regarded as the principal source of infection for newly introduced broilers and turkeys. Litter has been shown to be capable of transmitting *C. jejuni* to specific-pathogen-free chicks under controlled conditions (Montrose et al., 1985). Infected chicks can excrete *C.jejuni* for up to 63 days when housed under conditions which do not permit coprophagy. Fecal contamination of litter is regarded as the most probable source of infection of young flocks hatched in units which have been improperly cleaned or contain recycled litter (Cruickshank et al., 1982; Genigeorgis et al., 1986; Pokamunski et al., 1986). Persistence of *C.jejuni* in litter depends on adequate temperature, moisture (Smitherman et al., 1984) and pH (Doyle and Roman, 1982), but the organism can survive for at least 10 days at 20°C (Luechtefeld et al., 1981). Kazwala et al. (1990) have investigated the factors responsible for the introduction and spread of the bacteria in commercial poultry production. They concluded that a likely source of *C. jejuni* for young chicks was the environment in the proximity of the rearing houses, and that infection could be readily introduced by the footwear and clothing of farm staff. Thermophilic Campylobacters

were found in the air, litter and drinking water containers in the rearing and finishing houses. Water contaminated with *C. jejuni* introduces infection into poultry houses (Engvall et al., 1986) and lead to subsequent dissemination within flocks (Smitherman et al., 1984). Similar observations have been made by Shanker et al. (1990) who demonstrated rapid horizontal spread of *C. jejuni* in three days of infection with water containing 10^5 CFU/ml. The potential role of viable but not culturable *C. jejuni* in transmission of infection by water requires evaluation in commercial conditions (Rollins et al., 1987). This possible source can be significant in cases where flocks are supplied with water from an open, non-chlorinated source.

Rats in the vicinity of broiler houses have been found to be infected with *C. jejuni* in a study conducted in Yugoslavia (Annan-Prah and Janc, 1988). High prevalence rates in ducklings were reflected in recovery from 87% of rodents in a survey carried out in California (Kasrazadeh and Genigeorgis, 1987). Wild birds, which may shed *C.jejuni*, may infect poultry flocks if the access is allowed to the interior of houses (Acuff et al., 1982; Annan-Prah and Junc, 1988). House flies (*Musca domestica*) captured in the vicinity of poultry houses have shown *C. jejuni* infection rates of up to 50% in a survey conducted in Norway (Rosef and Kapperud, 1983) and Yugoslavia (Annan-Prah and Junc, 1988). This conflicts with a 2% level of infection in flies recorded in suburban gardens in England (Wright, 1983).

The movement of personnel and equipment among farms is a feature of integrated poultry production. The risk of infection of *C. jejuni* is inversely proportional

to the appropriate measures required to restrict transfer of moist, fecal-contaminated material from infected units to susceptible young flocks (Kazwala et al.,1990).

1.3.3 Waterborne infections

Waterborne infections by campylobacter organisms occur following consumption of unboiled water or surface water, contamination of ground water with surface water, inadequate disinfection, and contamination of water by wild bird feces (Tauxe, 1992). The presence of campylobacter organisms in surface water appears to be seasonally affected by both temperature and sunlight, and is lowest in the summer when the number of waterborne outbreaks decline in this season (Bolton et al., 1987). Drinking water is known as an additional transmission route of campylobacter infection as documented by several outbreaks in humans in the past decade (Mentzing, 1981). Numbers have ranged from a few people to three thousand. Most of the cases of waterborne infections are sporadic and isolated cases. Only in a few cases it is possible to isolate the organisms from infected people and the implicated water samples. Therefore in waterborne outbreaks, it is quite difficult to establish a link between the occurrence of infection and its source. This is due mainly to the existence of viable but not culturable campylobacters in water, the short life of the bacteria in aqueous environments as a result of stressful conditions such as direct sunlight and higher oxygen tension as well as the existence of multiple source of infection which make it difficult to determine the exact source of infection.

The content of campylobacters in surface water appears to depend chiefly on rain fall, water temperature and the presence of water fowl in the vicinity of water sources (Bolton et al., 1987; Stelzer et al., 1989). Isolation of *C. jejuni* from waters is often low; < 10 organisms/ml of water were detected (Stelzer et al., 1989). In river systems, the highest frequency of isolation and the highest counts of 10-230 campylobacters/100 ml water were associated with sites close to or downstream of sewage (Bolton et al., 1987). Sewage treatment plant effluents are considered to be one of the major sources of campylobacters occurring in rivers (Arimi et al., 1988; Bolton et al., 1987). There are only limited data available on campylobacter contamination in sewage sludge. Sewage can be heavily contaminated with *C. jejuni*, but waste water treatment can reduce bacterial counts considerably.

Isolation of *C. jejuni* from water seems to depend upon the water temperature. Different concentrations of the bacteria were found during a half year study of surface water in Germany, the highest contamination was found at water temperatures between 5° C - 10° C. Drinking of untreated water represents a risk of campylobacter infections. Aho et al. (1989) reported an outbreak associated with the consumption of untreated water in Finland where 85% of 88 people had gastrointestinal symptoms after consuming untreated surface water. *Campylobacter jejuni* heat-labile serotype 3/43/59 was isolated from people and from the source of surface water used by them. It is therefore important to chlorinate and disinfect all surface water used for human consumption, including the clear and cold stream water. Generally, *C. jejuni*, similar to other pathogens, survives better at low temperatures which suggests that cold water may

be a more effective vehicle of transmission if there is a source of contamination. Gondrosen (1986) compared the survival of *C. jejuni* in drinking water and river water kept at different temperatures. At 20° C no viable cells could be isolated after more than 48 hours. But at lower temperature, survival was detected for several weeks.

Campylobacter jejuni cannot adapt to a free-living existence in water. In plating experiments with water samples, the organisms survived up to four weeks when kept at 4° C. However, survival was reduced at higher temperatures (Blaser et al., 1980). Sunlight also has a lethal effect on these bacteria (Jones and Telford, 1991).

Although *C. jejuni* cannot multiply in the environment, continued survival and existence of these organisms in environmental conditions that are very different from those in the gut of their animal hosts is likely to induce metabolic modifications besides morphological changes, such as the development of coccoid forms of the bacteria (Jones et al., 1993). The metabolic changes are believed to enable the organisms to survive in normal atmospheric conditions by adapting to aerobic conditions. Survival of air-adapted or aerotolerant strains of *C. jejuni* can play an important role in the epidemiology of waterborne outbreaks of campylobacter infections.

1.3.4 Milkborne infections

Unpasteurized milk is the most frequently recognized vehicle of transmission in human outbreaks of *C. jejuni* infections. Some of the largest outbreaks followed the consumption of unpasteurized milk (Robinson and Jones, 1981; Skirrow, 1982). Large common-source outbreaks associated with consumption of unpasteurized milk are

frequently reported in England, a country where consumption of raw milk is more common. Epidemiological studies have been successful in demonstrating an association between campylobacter enteritis and the consumption of unpasteurized milk. Although attempts to isolate the pathogens from milk have not generally been successful, campylobacters that are of the same serotype have been isolated from cattle and milk filters at implicated farms (Blaser et al., 1984). Birkhead et al. (1988) reported the first milkborne outbreak in which the same two serotypes of *C. jejuni* were isolated both from patients with diarrhea and the dairy cows. Contamination of raw milk is believed to result from fecal contamination during milking, perhaps due to chronic carriage in the cattle (Warner et al., 1986). Also, it can also be the result of secretion of the organisms into the milk by cows with asymptomatic mastitis caused by *C. jejuni.* Mastitis has been reported to be the source of two cases of human infections (Hudson et al., 1984; Hutchinson et al., 1985). Humphrey and Beckett (1987) found that 83% of 12 herds examined had *C. jejuni* in their feces. All positive herds in this study drank from rivers or streams when grazing. *C. jejuni* is frequently found in natural water courses, therefore drinking water may be the main source of campylobacter in infected cows. Other cows represent a less likely source of infection except for calves.

1.3.5 Campylobacter infections in humans

Campylobacteriosis now is considered an important disease throughout the world. Its prevalence can only be estimated at this time, because the reported infections represent a minor portion of the total segment. Infection often occurs sporadically and

is not always associated with food as the source. The signs and symptoms of *C. jejuni* infections lack distinctive clinical features and cannot be differentiated from illnesses caused by other enteric pathogens (Franco, 1988). Following an average incubation period of 3 to 5 days there is a febrile period accompanied by malaise, headache, dizziness, myalgia and severe abdominal pain. This is followed by diarrhea characterized by watery, bile-stained, malodorous stools which may contain blood (Shane and Montrose, 1985). The diarrheic phase persists for three days but symptoms may recur for periods of up to two weeks. The diarrhea may be slight, but in some cases it is severe and watery and the patient may experience dehydration. A characteristic symptom of the disease is abdominal pain. This may be quite severe and may persist for several days. Complications may follow infection with *C. jejuni*. These include meningitis, cholecystitis and urinary tract infection. The main sites of human infection are the jejunum and ileum (Ruiz-Palacios et al., 1981), but there is evidence that the colon may be involved (Karmali and Fleming, 1979). *Campylobacter jejuni* can be encountered as an opportunistic pathogen in a wide range of infections, especially in debilitated patients or immunocompromised persons (Shane and Montrose, 1985). Fatalities due to campylobacter infections are extremely rare (Mandal et al., 1984; Walker et al., 1986). The exact mechanism by which *C. jejuni* causes disease is not fully understood. However, various forms of the disease recognized and claimed by some workers are believed to be due to production of toxins.

1.4 Virulence mechanisms of *C. jejuni* and *C. coli*

Campylobacter jejuni and its relative, *C. coli*, have recently emerged as major causes of bacterial enterocolitis in humans. Majority of these cases (~98%) are caused by *C. jejuni*. It is increasingly apparent that these species are pathogenic to humans. *Campylobacter jejuni* causes abortion in sheep, and enteritis in piglets, calves, and lambs. Poultry are major carriers of the organism (Blaser et al., 1983). *Campylobacter coli* similarly causes enteritis in several domestic and wild animals but to a lesser extent. Pigs are the major reservoirs of *C. coli* (Sticht-Groh, 1982). Infections with *C. jejuni* and *C. coli* occur by ingestion of contaminated food of animal origin and water (Blaser et al., 1979; Gill and Harris, 1982); the number of organisms required to cause infection in humans can be relatively small (Robinson, 1981). The colonisation of mucosal surfaces by *C. jejuni* and *C. coli* depends on the ability of the bacteria to maintain close proximity to and attachment to the mucosa to avoid being swept away by the movements of the intestinal contents (Griffith and Park, 1990). A number of factors play important roles in the establishment of the infection in vertebrate hosts.

1.4.1 Association with intestinal mucosa

The mechanism of association of thermophilic Campylobacters with the intestinal mucosa seems to be facilitated by various factors like the morphology of the bacterium, motility and chemotaxis (Walker et al., 1986); the spiral shape of the organism and its characteristic cork screw-like motility enable it to penetrate and pass through the thick

intestinal mucus and thus establish a close contact with the intestinal mucosa (Griffith and Park, 1990).

Campylobacter jejuni exhibits chemotaxis towards L-fucose and L-serine which are major components of mucus glycoproteins. Hugdahl et al. (1988) determined that *C. jejuni* is chemo-attracted to mucin and to L-fucose, which is likely the principal chemoattractant in mucin. Furthermore, these authors observed that mucin can serve as a sole substrate for growth of *C. jejuni*. The viscous glycoprotein gel covering the epithelial cells of the intestinal tract is likely to be the initial point of contact between host and *C. jejuni* (Field et al., 1986). The effectiveness of colonisation of various sites within the intestine is probably due to the interaction between its motility and adhesion.

1.4.2 Adhesion

Adhesion of bacteria to mucosal surfaces is necessary for colonisation and subsequent pathogenesis (Beery et al., 1988). Although *C. jejuni* and *C. coli* lack fimbria (Walker et al., 1986), they possess other systems of adherence including flagella. Lipopolysaccharides (LPS) (Perez-Perez and Blaser, 1985) and outer membrane proteins (OMPs) (Trust and Logan, 1984) have also been suggested to act as adhesins. Immunoblotting techniques have revealed considerable antigenic relatedness between the major outer membrane proteins of campylobacters belonging to diverse serotypes (Blaser et al., 1984). Although these molecules are candidates for a vaccine, information on the antigenic nature of shared domains of the molecule is not yet available (Walker et al., 1986).

The flagella of *C. jejuni* is involved in its adhesion to intestinal surface (Walker et al., 1986; Griffith and Park, 1990) and is vital for colonisation of the mucus layer. It may contain adhesins for epithelial surfaces since aflagellate strains were unable to colonise the intestine (Newell et al., 1983; 1984; Mills and Bradbury, 1984).

Campylobacter jejuni and *C. coli* are able to adhere to different cell lines *in vitro*, particularly HeLa, INT 407 cells (Walker et al., 1986) and porcine brush border preparations (Naess and Hofstad, 1984). An aflagellate variant of *C. jejuni* was reported to adhere poorly to INT 407 cells (McBride and Newell, 1983), which suggests the presence of adhesins on flagella similar to those seen in *V. cholerae* (Attridge and Rowley, 1983). There are, however, multiple adhesins involved in adhesion. Although flagellated organisms attach better to cell monolayers, aflagellated organisms were shown to attach better than parental flagellated type to target cells in suspension (McBride and Newell, 1983). The explanation to this phenomena might be that there are other means by which the bacteria adhere to the monolayer cells.

Numerous substances and treatments can interfere with *in vitro* adherence of *C. jejuni* to epithelial cells. L-fucose and asparagus pea lectin which recognize L-fucose determinants on cells inhibit binding to INT 407 cells (Cinco et al., 1984). Partial inhibition of adhesion to INT 407 cells was also seen with glucose, galactose and mannose (Newell et al., 1983). Reduced adherence of *C. jejuni* to INT 407 cells by at least 50% was achieved with D-mannose, D-fucose and proteolytic enzymes suggesting the presence of a variety of receptors for the organisms (Walker et al., 1986).

1.4.3 Lipopolysaccharides (LPS)

The LPS are important outer membrane constituents of gram-negative organisms. Biological and chemical (Naess and Hofstad, 1984) studies have indicated that LPS are present in *C. jejuni*. Electrophoretic and chemical analyses indicate that *C. jejuni* LPS are predominantly of the low-molecular type (Logan and Trust, 1984) found in pathogens such as *Neisseria*, *Haemophilus* and *Bordetella spp*. However, in contrast to the LPS from these pathogens, the LPS of *C. jejuni* appears to be antigenically diverse, conferring a large number of serotypes. Furthermore, *C. jejuni*, unlike other enteric pathogens lacks extensive O side chains (Taylor et al., 1983). Fecal *E. coli* strains, deficient in O side chains of LPS were better able to colonize mice than fecal strains that had the smooth type of LPS molecules (Goldman and Leive, 1980). The presence of rough LPS may be a significant factor in the ability of *C. jejuni* to colonize the intestinal epithelium. Epithelial cell surfaces appear to be the preferred binding site of both flagella and LPS (Naess and Hofstad, 1984). Outer membrane proteins of *C. jejuni* were also suggested to function as adhesins (Walker et al., 1986).

1.4.4 Invasiveness

Campylobacter jejuni is clearly an invasive organism. Evidence for this is given in the nature of the clinical symptoms in humans, detection of the organism in peripheral blood in acute phase of the disease and the rapid development of high antibody titer after infection (Blaser et al., 1979). Invasiveness of Campylobacters is considered by some authors (Butzler and Skirrow, 1979) to be a prerequisite virulence

factor for establishing enteritis. This concept is supported by the appearance of bloody diarrhea, bacteremia, cholecystitis and ulceration of the gut in infected human patients (Field et al., 1981).

Campylobacter jejuni like entero-invasive *Shigella spp.* is able to penetrate and proliferate within the intestinal epithelium of different experimentally infected animals such as 3-11 days old chicken, hamster and mice. Penetration seems to be facilitated by a cytotoxin produced by the organism which appears to cause damage and induce death of the epithelial cells thus allowing its penetration.

Like *Salmonella* and *Yersinia spp.*, *C. jejuni* possesses the ability to migrate [translocate] across the intestinal epithelium and reach the lamina propria and mesenteric lymph nodes where it proliferates and causes serious lymphadenitis and systemic infection. An additional property that the organism shares with *Salmonella typhimurium* is its remarkable ability to survive within monocytes and macrophages. Studies done on human monocytes and murine and chicken macrophages (Myszewski and Stern, 1991) showed that *C. jejuni* is internalized more rapidly and vigorously by human monocytes and murine macrophages and that the organism survived better in these cells whereas almost all the bacteria phagocytized by the chicken macrophages were killed within six hours.

1.4.5 Toxin production

One important mechanism in which *Campylobacter jejuni* and *C. coli* induce diarrhea is thought to be the production of potent toxins (Daikoku et al., 1990; Johnson

and Lior, 1986). Bacterial toxins are classified as either membrane damaging, such as hemolysins and phospholipases, or enterotoxins, such as the toxins elaborated by *C. diphteriae*, *Vibrio cholerae* and *Shigella dysenteriae* (Middlebrook and Dorland, 1984). The intracellular-acting toxins are probably directly associated with the mechanisms for inducing diarrhea. These toxins are proenzymes that share several modes of action among them (Walker et al., 1986). They bind to specific receptors on the plasma membrane, similar to the GM1 ganglioside for cholera toxin (CT), enter the cell and interact with targets such as intracellular adenylate cyclase (CT and LT of *E. coli*) or the 60S subunit ribosome (Shigella toxin). In the case of Cholera toxin, this interaction causes fluid secretion, but the Shiga toxin causes the shutdown of protein synthesis and cell lysis (Middlebrook and Dorland, 1984).

Campylobacter jejuni is known to produce at least two types of exotoxins: a heat-labile enterotoxin and a cytotoxin (Johnson and Lior, 1986). The *Campylobacter jejuni* enterotoxin is similar in structure, receptor, mechanism of action and antigenic characteristics to cholera toxin and the heat-labile toxin (LT) of *E. coli* (Ruiz-Palacios et al., 1983; Klipstein and Engert, 1984a; 1985; Goossens et al., 1985; Johnson and Lior, 1986).

The enterotoxin produced by *C. jejuni* acts in a manner identical to that of the cholera toxin (Fishman, 1990) and *E. coli* LT (Gyles, 1992); they stimulate membrane bound adenylate cyclase in intestinal epithelial cells. These enterotoxins activate adenylate cyclase by catalyzing the transfer of ADP-ribose from NAD to an amino acid in the stimulatory guanine nucleotide binding regulatory protein (Gs), leading to

activation of the cyclase catalytic unit and increased cyclic AMP levels. This stimulation results in persistent elevation of cyclic AMP, which leads to hypersecretion of electrolytes and water usually manifested as watery diarrhea (Walker et al., 1986; Gyles, 1992). There is no detectable intestinal damage, only an 'overstimulation' of normal mechanisms. Heat-labile enterotoxins are therefore said to be cytotonic toxins, in contrast to cytotoxic toxins. They cause impaired cell functions by inducing loss of fluids without any evidence of structural damage (Gyles, 1992).

Although the *C. jejuni* enterotoxin has not yet been fully characterized, it is known to be a large protein with a molecular mass of about 60 to 70 kDa (McCardell et al., 1984; Daikoku et al., 1990); it is heat-labile and can be completely inactivated at 56 °C for one hour or at 96 °C for 10 minutes (Walker et al., 1986). The toxin is partially inactivated at pH 4 and is completely destroyed at pH 2 and pH 8. The toxic activity of the crude toxin is progressively lost after storage for one month at 4°C or for 1 week at - 20 °C or -70 °C (Klipstein and Engert, 1984b; Ruiz-Palacios et al., 1983). Because *C. jejuni* toxin (CJT) shares functional and immunological properties with cholera toxin and *E. coli* LT, the same assays have been applied to detect it. Tissue culture assays with either Chinese Hamster Ovary (CHO) cells (Ruiz-palacios et al., 1983) or Y-1 mouse adrenal cells (Gubina et al., 1981; McCardell et al., 1984; Ruiz-palacios et al., 1983) were used as convenient systems to test for toxin production. Johnson and Lior (1986) studied the toxin production of *C. jejuni* and *C. coli* using various cell lines such as Vero (African green monkey kidney cells), HeLa (Human cervical carcinoma cells), CHO (Chinese hamster ovary cells), and Y-1 mouse

adrenal cells. The most sensitive cells were found to be Vero cells for cytotoxic factors and CHO cells for the enterotoxin. HeLa cells were less responsive than Vero to cytotoxin, and Y-1 cells were less sensitive than CHO to enterotoxin. The enterotoxin of *C. jejuni* causes cytotonic changes in either confluent monolayers or cells in suspension, inducing elongation of CHO cells and rounding of Y-1 mouse adrenal cells (Gubina et al., 1981; Ruiz-Palacios et al., 1983; McCardell et al., 1984). The *C. jejuni* toxin was also seen to induce accumulation of fluid and electrolytes in both rat (Fernandez et al., 1983; Ruiz-Palacios et al., 1983; Saha et al., 1988) and rabbit (McCardell et al., 1984) ileal loops, but not in the infant mouse assay (Ruiz-Palacios et al., 1983); it causes increased permeability in the rabbit skin test (McCardell et al., 1984).

1.5 Importance of Campylobacters in the meat industry

For more than 10 years the thermophilic Campylobacters *C. jejuni*, *C. coli* and *C. laridis* have gained recognition as human pathogens transmissible through foods. Campylobacteriosis is considered a foodborne infection and the foods that are most frequently incriminated are the ones of animal origin, particularly poultry (Grant et al., 1980). *Campylobacter jejuni* now rivals *Salmonella spp.* as a major cause of gastroenteritis in humans. However, the principal food-borne pathogen has been and remains *Salmonella* species. Both these pathogens have been isolated from animal and poultry carcasses after slaughter (Oosterom, 1980; Oosterom et al., 1983a; Stern et al., 1985) and from meat products at retail outlets (Park et al., 1981; Gill and Harris 1982;

Stern et al., 1985). A survey study done in Canada by Lammerding and co-workers (1988) to assess the prevalence of thermophilic Campylobacters and *Salmonella* in foods revealed that *C. jejuni* and *C. coli* are quite commonly present in food animals in slaughterhouse. Thermotolerant campylobacters were frequently recovered from broiler chicken and turkey carcasses. This is not surprising since poultry slaughtering and cleaning process leads to contamination of carcasses with intestinal bacteria (Oosterom et al., 1983b; Yusufu et al., 1983). Contamination rate of individual turkey carcasses with *C. jejuni* is often greater than that of other birds (Yusufu et al., 1983; Lammerding et al., 1988). In Norway, Rosef et al. (1984) also noted that turkeys were more frequently contaminated with *C. jejuni* than either hens or broiler chickens. In contradiction to this, some comparative studies have concluded that the levels of *C. jejuni* are lower in meat products derived from turkeys than those from commercial broilers (Harris et al., 1986; Baker et al., 1987; Kwiatek et al., 1990). This difference of contamination levels may be attributed to efficiency of washing, plant layout, operation of evisceration, size of turkey carcass and degree of farm contamination (Shane, 1992). Despite differences in intestinal carriage rates among poultry and turkeys delivered to processing plants, immersion scalding, defeathering, evisceration and continuous waterbath chilling serve to disseminate infection to all carcasses processed. In the study conducted by Wempe et al. (1983a), the level of contamination of ready-to-cook poultry following processing ranged from 67 to 100%. High rates of *C. jejuni* were recovered from scalding water, feather and offal channels and from equipment at various points on the processing line.

The presence of significant levels of *C. jejuni* on poultry carcasses and portioned products represents a potential for food-borne campylobacteriosis. Handling and storage following processing does not reduce levels of contamination. Hänninen (1981) determined a 0.5-2.0 log reduction in surface *C. jejuni* over a 12-week period when whole carcasses were stored at -20°C. Campylobacters survived for 21 days on chicken drumsticks stored at 4°C in carbon dioxide-enriched atmosphere. The survival of *C. jejuni* in packaged turkey rolls stored under microaerophilic conditions was evaluated by Phebus et al. (1991). The bacteria were detected after holding the products for 18 days at 4°C. These studies demonstrate that *C. jejuni* can persist in poultry products under commercial conditions of handling and storage.

The recovery rate of *C. jejuni* on livestock carcasses are often higher than those reported for *Salmonella.* It should be noted, however, that published rates of contamination of meats with *Salmonella* vary widely. Widespread occurrence in the intestines of cattle, sheep and pigs suggests that campylobacters would be common on eviscerated carcasses. This is true at the slaughterhouse but the number of organisms falls rapidly, perhaps due to the sensitivity of the bacteria to drying. However, they can be isolated from meats from retail outlets using enrichment of 25 g samples (Fricker and Park, 1987). Garcia et al. (1983) reported that many of the serotypes of *C. jejuni* isolated from cattle are similar to those found in human disease. Therefore, a proportion of human campylobacteriosis could be traced back to beef products. The organism was frequently found in gallbladders, large and small intestines, and livers. Most of the serogroups and biotypes of the organisms are similar for humans and cattle. Cattle are

not always strongly implicated in transmission of campylobacters through meat products. In a three-year survey done in Canada, Lammerding et al. (1988) found that of the thermophilic *Campylobacter* biotypes isolated, *C. jejuni* biotypes I and II were isolated more frequently than all other biotypes, representing 63% of the total number of Campylobacter isolates.

Grau (1988) characterized the presence of *Campylobacter* spp. in the intestinal tract and on carcasses of calves and cattle. He found that calves carried the organism more frequently than did adult cattle and that feedlot cattle were more likely to harbour the pathogen in their intestines and on their carcasses than pasture-fed cattle, and that chilling the carcasses overnight significantly reduced the numbers of *Campylobacter* spp. associated with carcass. Similar findings were also reported by Lammerding et al. (1988) who found that higher levels of campylobacters in veal calves compared to beef which may also reflect differences in intestinal flora due to age or rearing practices. A study by Garcia et al. (1983) also showed higher recovery of thermophilic Campylobacters from young heifers (40%) than from cows (22%).

Several reports described the association of *Campylobacter* spp. and beef products. Earliest reports were from England (Turnbull and Rose, 1982) where campylobacters were isolated from 1.6% of 6,000 meats sampled. Fukishima et al. (1987) reported levels of *Campylobacter* spp. in raw beef, pork and chicken similar to those found in the British study. Rosef (1981) described the presence of *Campylobacter* spp. in gallbladders and bile ducts of slaughtered pigs in Norway; 58% of the pigs sampled yielded the organisms. In Sweden, Svedhem and Kaijser (1981) reported that

95% of the pigs sampled at slaughterhouses carried *Campylobacter* spp. Serological studies on pig isolates of *C. jejuni* from Western Europe and Canada concluded that in isolates from pigs, *C. coli* in particular, belonged to serotypes not commonly found in humans. Therefore, meats and other products derived from pigs do not seem to represent a major source of human campylobacteriosis.

1.6 Research objectives

The work reported in this thesis was undertaken to gain information on intestinal colonization of thermophilic campylobacters, particularly *Campylobacter jejuni* in broiler chickens at poultry meat plants in Prince Edward Island (PEI). The main need for the poultry industry in PEI, as elsewhere, is to reduce the contamination of chickens with *C. jejuni* which represents a health risk to consumers.

The objectives of this thesis are as follows:

1. To gain information on the intestinal-carriage rate of pathogenic campylobacters in broiler chickens at poultry meat plant in PEI.

2. To investigate the degree of antimicrobial resistance of these isolates using eight antibiotics some of which are currently used for the treatment of enteric diseases of bacterial origin;

3. Perform plasmid DNA isolations on those strains of *C. jejuni* that showed resistance to tetracycline; and

4. To study the prevalence of enterotoxin-producing isolates of *C. jejuni* and *C. coli* in chickens available to PEI consumers and to study their enterotoxigenic properties on CHO-K1 cells.

CHAPTER 2. ISOLATION AND IDENTIFICATION OF CAMPYLOBACTERS FROM CHICKEN

2.1 Introduction

Successful isolation of *Campylobacter* species from clinical specimens depends on several factors which are as follows: (i) culture medium, including growth and selective supplements; (ii) temperature; and (iii) atmosphere of incubation. *Campylobacter* spp. are naturally resistant to vancomycin, polymyxin B, bacitracin, cephalothin, trimethoprim, and novobiocin (Taylor and Courvalin, 1988). A number of selective culture media containing various combinations and concentrations of the antibiotics are widely used for the isolation of these pathogens from fecal samples. Most of the contaminating fecal flora are inhibited. *C. jejuni* and *C. coli* grow well at a temperature of 42-43°C as well as at 37°C. Growth requires microaerophilic conditions of 5% O_2, 10% CO_2 and 85% N_2.

The principal sites of localization of *C. jejuni* are the large intestines and cloaca. Samples taken from the ceca provide a higher rate of recovery compared to other parts of the intestine and cloaca (Beery et al., 1988; Luechtefeld et al., 1980). Cecal tissue can be collected at the time the viscera are being removed from the body. *Campylobacter* species may be isolated either directly from feces or from rectal swabs. Ideally the specimens should be inoculated onto selective plate media within two hours following collection, although *C. jejuni* will grow on ordinary blood agar media and, isolation is enhanced by inoculating fecal specimens and rectal swabs directly onto

selective agar media. Detection of campylobacters used in this study was done by direct plating of cecal contents of slaughtered birds onto selective media. The media used for the isolation of *C.jejuni* and *C. coli* from chicken included Skirrow media (Oxoid Canada Inc., Napean, ON. Canada) and Preston blood-free agar (Oxoid, Canada). Skirrow medium is fairly good in supporting growth of the pathogens; it contains three antibiotics: polymyxin B, trimethoprim and vancomycin that inhibit many of the contaminating gram positive and gram negative organisms. Preston blood-free media, on the other hand, is excellent for the isolation of *C. jejuni* and *C. coli*. It is routinely used for the detection of bacteria from fecal specimens. It contains the antibiotic cefoperazone as the selective agent which inhibits most of the unwanted fecal flora. It inhibits more of the competing flora than most other recommended media. Other media used were Preston blood-free formulation containing amphotericin B (2mg/l) to suppress yeast growth and Butzler medium containing 40,000 U of colistin to inhibit gram negative contaminants.

2.2 Materials and methods

2.2.1 Sample collection

Collection of samples was done during May and August 1991. A total of 231 cecal samples from broilers and older birds from a poultry processing plant in Prince Edward Island (PEI) were collected to determine the prevalence of thermophilic campylobacter species in poultry processed in this plant. Birds in this study came from different commercial poultry producers on PEI. Sampling was done in a systematic way.

Every Tuesday, the main slaughter day for the palnt, 15-20 samples of ceca were collected. It was ensured that the sampled chickens came from different farms in PEI, on each day of collection.

Viscera of freshly killed birds were collected immediately on a paper tray during the evisceration process. For each bird, one or both ceca were collected; the ceca were aseptically cut, tied and placed in sterile plastic bags that were kept on ice in an insulated cooler box and transported to the laboratory within two hours of processing.

2.2.2 Culture of samples

The cecal samples were placed in a biological containment hood in sterile plastic petri dishes (Polar plastics Ltd, Montreal, Canada). The surface of the intestines was cauterized by placing a flamed spatula and then was cut open with sterile forceps. For each sample, a loopful of cecal content was streaked onto modified Preston blood free agar (Oxoid, Canada) which contained cefoperazone (32 mg/l) that inhibits most of the gram negative contaminants, and Skirrow selective medium (Oxoid, Canada) which contained blood agar base #2, horse blood and antibiotic supplements, consisting of polymyxin B (2500 U/l), trimethoprim (5 mg/l) and vancomycin (10 mg/l), to which *C. jejuni* and *C. coli* organisms are intrinsically resistant. Inoculated plates were placed in anaerobe jars and incubated at 42°C for 48 hours in a gas generating kit (Oxoid, Canada) with catalyst that gives microaerophilic conditions consisting of 10% CO_2, 5% O_2 and 85% N_2.

One liver specimen obtained from an older bird was also included in the sampling. The organ was slightly enlarged, pale and had numerous isolated white foci on the surface. A sterile swab was streaked on the surface of the liver, and inoculated on the Preston medium. The surface of the liver was cauterized with flamed spatula and then cut with a sterile scalpel. The exposed liver parenchyma was streaked with a sterile cotton-tipped swab that was inoculated on selective media and incubated as described earlier.

2.2.3 Identification of the isolates

The Campylobacter strains were identified on the basis of the typical morphology of the colonies. Isolates showing characteristic small, raised or flat white-greyish colonies with regular edges were gram-stained. Cultures which had gram-negative, slender curved to spiral or comma-shaped rods were subcultured onto blood agar plates and incubated at 42°C for 48 hours in microaerophilic conditions in an anaerobic jars as described previously.

All strains were subjected to a Latex agglutination test using "MERITECH CAMPY jcl" kit (Meridian Diagnostics, Cincinnati, Ohio, USA) to differentiate *C. jejuni, C. coli* and *C. laridis* from other *Campylobacter* spp. The test utilizes the principle of latex agglutination in which rabbit antibodies specific for common antigens of *C. jejuni, C. coli* and *C. laridis* bound to latex particles react with these three *Campylobacter* species giving a visible agglutination. Two to three colonies were picked to create a suspension with an antigen extraction reagent consisting of diluted

hydrochloric acid solution and mixed on a special agglutination plate provided by the manufacturer of the kit. Neutralization reagent containing glycine buffer and a preservative was added to the suspension. Finally, a latex detection reagent which consists of rabbit antiserum to common antigens of selected Campylobacter species bound to latex particles suspended in buffer containing a preservative was added to the bacterial suspension. The suspension was mixed carefully and placed on a shaker platform (Orbit Shaker, Lab-line instruments, Illinois, USA) at 100 rpm for 15-20 minutes at room temperature. The test gives a visible agglutination on the latex-coated slide when reacted with colonies of the three thermophilic campylobacters: *C. jejuni*, *C. coli* or *C. laridis*. Control strains of *C. jejuni*, *C. coli* and *C. laridis*, *C. hyointestinalis* and *C. fetus* from the American Type Culture Collection (ATCC) were used to test the specificity and sensitivity of the reagents as done in an earlier study on isolation and identification of campylobacters (Hariharan et al., 1990).

Campylobacter jejuni and *C. coli* were differentiated from *C. laridis* by their susceptibility to nalidixic acid (30μg) and their resistance to cephalothin (30μg), and their inability to grow at 25°C. Differentiation of *C. jejuni* from *C. coli* was based on Hippurate hydrolysis test. Three to five colonies from pure cultures were suspended into 0.5 ml of deionized distilled water in polyestrene tubes (Falcon [Beckton Dickinson, New Jersey, USA]) to create a suspension. For each isolate, a single Hippurate disk (Remel, Lenexa, Kansas, USA) was placed in the suspension before the tubes were incubated at 37° C for 30 minutes. Two drops of Ninhydrin solution (Remel, USA) were added to the tubes and returned to incubator for additional 10 minutes.

Streptococcus agalactiae and *S. zooepidermicus* were included in each test as hippurate positive and negative controls respectively. Positive strains give a purple color indicating the presence of glycine. All culture plates identified as *C. jejuni* and *C. coli* were scraped from the plates with sterile cotton swab and suspended into 1.0 ml of Brucella broth with 15% glycerol in 1.5 ml volume cryovials (Nalgene Co., Rochester, New York, USA). The vials were then stored at -76°C until used.

2.2.4 Results

Campylobacters were isolated from 119 (51.5%) of the total 231 chicken ceca contents sampled. *Campylobacter jejuni* was detected in 101 (85%) of the samples whereas *C. coli* represented only 18 (15%) isolates (Table 1). Separation of these two species was based on the hippurate hydrolysis test. Campylobacters were not detected in a swab culture taken from the the liver from an older bird, whereas a swab taken from the surface of the organ showed *C. jejuni* only on Preston media. Thirty one samples (30 broilers and 1 older bird) had tiny, greyish, pin-point type colonies on primary culture on the three selective media: Butzler media with 40,000 U of colistin· Preston blood-free with amphotericin B, and Preston blood-free selective media. None of these showed growth of campylobacters when subcultured onto blood agar plates and incubated under the same cultural conditions. The only growth present was represented by yeast and *Staphylococcus* spp.

Isolation rates of campylobacters were higher on Preston media than Skirrow selective media, where contaminating fecal flora were seen frequently. The most

commonly encountered contaminants in Skirrow's selective medium were gram-positive and gram-negative bacilli, including *Proteus spp*. The most frequent contaminants seen on Preston medium were Staphylococci and yeast (Table 2). All the isolates were susceptible to nalidixic acid and also resistant to cephalothin.

Table 1. Prevalence of *Campylobacter* spp. isolated from 231 Ceca of Broiler and Older Chickens in Prince Edward Island.

Type of Birds	No. of Birds Positive	Species isolated (%)*
Broilers (175)	72	*C. jejuni* (79) *C. coli* (21)
Older Birds (56)	47	*C. jejuni* (94) *C. coli* (6)

* From a total of 119 samples from chicken ceca that showed growth of *C. jejuni* or *C. coli*.

Table 2. Growth characteristics of *Campylobacter* spp. on selective media

Type of Media	No. of Cultures[a]	Growth[b]	Contaminants[c]
Preston	10	+++	yeast, staphylococci
Skirrow	10	+	gram-ves, yeast

[a] = 10 isolates were seeded on both of Preston & Skirrow medium.

[b] = Colonies of *C. jejuni* and *C. coli* on selective media: +++ = heavy growth; + = light growth.

[c] = Based on visual examination of colonies and gram stain.

2.2.5 Discussion

The isolation of *Campylobacter* species from fecal samples is generally easy if several factors are considered. These include the type of media used and the incubation conditions. The use of selective media which include various antibiotics or combinations of antibiotics to which thermophilic campylobacters are naturally resistant, will reduce, or suppress the growth of competing flora in fecal sample in which campylobacters may be present in small number. Of the two selective media used in this study, Preston blood-free agar with cefoperazone yielded the highest number of *C. jejuni* and *C. coli* isolations in cecal samples. The major contaminants found frequently in the cecal contents examined were yeast and Staphylococci. This is in agreement with a study done by Merino et al. (1986). Skirrow selective medium (Skirrow, 1977), on the other hand did not adequately inhibit some intestinal flora (Table 2) and the growth of the organisms was less prominent compared to that on Preston medium.

Isolation rates of *C. jejuni* and *C. coli* ranges from 30% (Pokamunsky et al., 1984) to 100% (Wempe et al., 1983b). The prevalence rate of 51.5% of *Campylobacter spp.* isolated from ceca of slaughtered chickens in PEI (Table 1) was higher than the 38% prevalence reported in Canada by Lammerding et al. (1988). In this study, *C. jejuni* was the predominant species found in the cecal contents sampled representing 85% of all isolates with the rest being *C. coli*. There was no indication that these two species co-existed in the fecal samples we tested. This was further confirmed by the Hippurate hydrolysis test that discriminated *C. jejuni* from *C.coli*. Furthermore, the majority of the samples grown in Skirrow and Preston media were either positive or

negative for the Hippurate hydrolysis test. A few samples that gave weak reactions in this test were retested for confirmation. *C. laridis* was not found in any of the intestines sampled. *C. laridis* differs from *C. jejuni* and *C. coli* in being resistant to nalidixic acid. Even though this organism has been implicated in serious outbreaks of enteritis involving contaminated water (Borczyk et al., 1987), it is rarely found in poultry.

CHAPTER 3. ANTIMICROBIAL SUSCEPTIBILITY TESTING AND EXAMINATION OF PLASMIDS

3.1 Introduction

Studies on antimicrobial resistance in *Campylobacter* species include surveys of resistance patterns of clinical isolates and studies focusing on the genetic determinants that encode the resistance phenotypes. Campylobacters can harbor a large group of resistance determinants, including several genes that are plasmid mediated (Taylor and Courvalin, 1988). Plasmids are small, self-replicating DNA or RNA found in many bacteria and a few eucaryotes like yeast. They carry genes that the bacterial cell needs only under special conditions, such as the resistance factors (R factors) that determine resistance of their host to antibiotics or heavy metals.

Data that may indicate trends or changes in antimicrobial resistance patterns, or the introduction of new resistance determinants from other organisms are scarce. While antimicrobial usage is becoming more frequent, there is a potential that there will be more drug resistant isolates of *Campylobacter spp.* However, many laboratories throughout the world continue to check for antimicrobial resistance in campylobacters and provide valuable information on the resistance in these organisms. Most commercially available methods of antimicrobial susceptibility testing do not perform well with campylobacters. This necessitates the use of more laborious methods like the agar dilution and broth microdilution. Disk diffusion tests have been used with success,

however, by several workers (Taylor et al., 1986; Adesiyun et al., 1992; Cabrita et al., 1992) to detect drug resistance in *C. jejuni* and *C. coli*.

3.2 Materials and methods

3.2.1 Disk diffusion testing

To evaluate the drug resistance patterns of the isolates, disk susceptibility testing was performed on all isolates against eight commonly used antimicrobials. The following eight antibiotics that were tested, concentrations of the drug are given in parentheses: ampicillin (10μg), cephalothin (30μg), chloramphenicol (30μg), enrofloxacin (5μg), erythromycin (15μg), kanamycin (30μg), nalidixic acid (30μg) and tetracycline (30μ). The antibiotic disks were obtained from Oxoid, Canada.

Two to five colonies from 48 hour-blood agar cultures were suspended in 0.5 ml deionized distilled water in 17 x 77 mm polystyrene tubes (Falcon, New Jersey, USA). Turbidity of the suspension was adjusted to 0.5 McFarland by comparing the tube with standard turbidity tubes (Oxoid). A sterile swab was dipped into the standardized culture suspension, and excess broth was rinsed by pressing and rotating the swab firmly against the side of the tube just above the fluid level. The swab was then streaked evenly in three directions over the entire surface of the Müller-Hinton blood agar (Oxoid) to obtain a uniform inoculum. The inoculated plates were allowed to dry for 5-10 minutes. Antibiotic impregnated disks were then applied on the plates using a mechanical disk dispenser (Oxoid). No more than four antibiotic disks were applied on each plate. Within 10-15 minutes after the disks were applied, the plates

were inverted and incubated at 37°C in an anaerobic jar under microaerophilic conditions of 85% N_2, 10% O_2 and 5% CO_2 obtained by the use of gas generating kit (Oxoid) with catalyst. Following 48 hours of incubation, the plates were examined and the zones of inhibition were measured on the agar surface using a ruler. Any zone of inhibition around the discs was reported as sensitive, whereas lack of an inhibition zone was interpreted as resistant. Cultures with a light growth which made the zones difficult to evaluate were re-tested. For each test *Escherichia coli* ATCC strain 25922 was tested in parallel as a standard control to monitor the potency of the disks.

3.2.3 Growth of bacteria for plasmid DNA studies

Two *C. jejuni* strains from older birds that exhibited resistance to tetracycline (30μg) in disc diffusion tests were used for plasmid DNA extraction. A tetracycline-sensitive strain of *C. jejuni* was included in each test. Two human strains of *C. jejuni* ATCC 43502 and ATCC 29428 were also included in the tests as tetracycline-resistant and tetracycline-sensitive reference strains respectively. *Agrobacterium tumefaciens* ATCC 33970 which contains a high molecular weight plasmid DNA of 90-120 kbp was initially grown on blood agar plates for 48 hours at 30°C. Colonies from blood agar plates were removed to 10 ml Müller-Hinton broth containing 64μg of tetracycline per ml and incubated for 48 hours at 37°C in an anaerobic jar containing 85% N_2, 10% O_2 and 5% CO_2 obtained with gas generating Campy Pak kit (Oxoid). Colonies from tetracycline-sensitive *C. jejuni* were grown on Müller-Hinton broth without antibiotic. *A. tumafaciens* was grown in 10 ml Müller-Hinton broth without tetracycline in

Erlenmeyer flask and incubated at 30 °C. The cultures were placed on a platform shaker (Orbit Shaker, Lab-line Instruments, Illinois, USA) and subjected to extensive shaking at 100 rpm. Following incubation, bacterial growth suspension was transferred into 1.5 ml microcentrifuge tube and centrifuged for five minutes at 14,000 x g in microcentrifuge (Eppendorf Microcentrifuge model 5414). A cycle of five centrifugation repetitions were done on each sample to increase the pellet size. The pellet of cells was either stored at 4°C overnight or used the same day for plasmid DNA isolation. Bacterial cultures on Müller-Hinton agar were also used for plasmid DNA isolations.

3.3 Plasmid DNA extraction protocols

3.3.1 Rapid TELT mini-prep

The plasmid DNA was isolated using a modification of Brent (1990). The bacterial growth was scraped off the agar plates using a disposable inoculating loop and suspended in 200 µl of TELT lysing solution (appendix A) in a 1.5 ml volume microcentrifuge tube. The samples were mixed for 15 sec with a vortex (Genie 2 Vortex, Fisherbrand, New York, USA) and stored at 4°C overnight. The next day, plasmid DNA was extracted by the addition of phenol:chloroform:isoamyl (25:24:1) solution, mixed with a vortex for 15 sec and centrifuged at 14,000 x g for 5 min. The supernatant fluid was decanted into another clean microcentrifuge tube and the phenol:chloroform:isoamyl extraction repeated. The plasmid DNA was precipitated by the addition of 200 µl cold (-20°C) isopropanol and kept at room temperature for 2 min. The DNA precipitate was collected by centrifugation at 14,000 x g for 5 min at room

temperature and washed with 200 µl of cold (-20°C) 100% ethanol. The pellet was allowed to air dry at room temperature, resuspended in 50 µl of TE buffer (appendix B) with 2 µl ribonuclease A (RNase A) (appendix C). The pellets were placed on ice for 30 min until used for electrophoresis.

3.3.2 Rapid mini-prep with heat

The procedure used was a modification of the protocol of Kado and Liu (1981). The bacterial growth was removed from the agar plates using a disposable inoculating loop and suspended in 100 µl of sodium dodecyl sulphate (SDS) lysing solution (appendix D) in a 1.5 ml microcentrifuge tube. The samples were thoroughly mixed using a vortex for 15 sec and the tubes were heated for 30 min at 65°C in a water bath. While the tubes were in the water bath, 100 µl of phenol:chloroform:isoamyl were added to each sample. The solution was emulsified by shaking briefly, and centrifuged at 14,000 x g for 5 min at room temperature. The upper aqueous phase was transferred into a clean microcentifuge tube and the tubes were kept at 4°C until required for electrophoresis.

3.3.3 Promega™ mini-prep

This procedure is based on a 15-minute miniprep DNA extraction kit (Magic™ miniprep kit, Promega, USA). Plasmid DNA extraction was performed with modification of the manufacturer's instructions: a 5 ml volume of bacterial growth were transferred into a sterile 15 ml falcon tube and centrifuged at 4000 x g for 8 min in a

Beckman model TJ-6 centrifuge (USA). The supernatant was discarded and the cell pellet suspended in 200 µl of Cell Resuspension Solution. The suspension was transferred into a clean microfuge tube. Bacterial cells were lysed by the addition of 200 µl Cell Lysis Solution to the tube. The solution was mixed by inverting the tube several times until the cell suspension appeared clear. The lysis process was halted by the addition of 200 µl of Neutralization Solution to the tube and mixed the solution by inverting the tube several times. The DNA was pelleted by centrifuging the tubes at 14,000 x g for 5 min at room temperature. The upper aqueous phase was carefully removed with a micropipette into a new microfuge. DNA was precipitated by the addition of 1 ml DNA Purification Resin to the tube, and the tubes were mixed by inverting several times. For each sample, one Magic Minicolumn was prepared by removing the plunger from a 3 ml Falcon disposable syringe. The syringe barrel was then attached to the luer-lock extension of each Minicolumn. The resin-DNA mixture was pipetted into the syringe barrel, the plunger was slowly inserted and gently pushed the slurry into the Minicolumn. To avoid shearing of DNA, the syringe was detached from the Minicolumn and the plunger removed from the syringe. The barrel was reattached to the Minicolumn and 2 ml of Column Wash Solution was pipetted into the syringe. The plunger inserted into the syringe, and the solution was pushed gently through the Minicolumn. The syringe was removed and the Minicolumn transferred to a microfuge tube. The Minicolumn was centrifuged at 14,000 x g in a microcentrifuge (Eppendorf Microcentrifuge model 5414, USA) for 20 seconds to dry the resin. The Minicolumn was then transferred to a new microfuge tube. To increase DNA yield, 50

µl of preheated TE buffer (65-70°C) was added to the Minicolumn and left to stand 1 min at room temperature. Plasmid DNA was eluted by centrifuging the Minicolumn at 14000 x g for 20 sec. For each sample, 20 µl DNA was used to load the gel.

3.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using the Horizon™ 11 x 14 cm horizontal gel electrophoresis apparatus (Bethesda Research Laboratories). A 0.7% agarose gel was prepared Tris-borate-EDTA (TBE) electrophoresis buffer (appendix F). Similarly, a 1% agarose gel was prepared by the addition of 0.8 g of reagent grade agarose to 80 ml TBE buffer. The mixture was heated to boiling in order to dissolve the agarose. The agarose was left to cool to about 50°C in water bath. A 14-tooth well-forming comb was inserted into the slots of the gel tray and approximately 55 ml of molten agarose was poured onto the gel tray, giving a 4 mm thick gel. The agarose gel was left to solidify for approximately 20-30 minutes, the comb was removed and the gel was covered with TBE electrophoresis buffer. The gel was used immediately to load the DNA samples. In another microcentrifuge tube, 18 µl of the DNA sample was mixed with 2 µl of 10X sample loading dye (appendix D). The mixture was deposited in the gel wells using a micropipette. For the Promega™, 20 µl of DNA sample were used to load the gel. For the supercoiled DNA marker, 5 µl were mixed with 2 µl of 10X gel loading buffer and used in each gel. The samples were electrophoresed at 78 V at room temperature for 3 1/2 - 4 hours. The gel was stained with ethidium bromide

(appendix G) for 30 min at room temperature and destained in distilled water overnight.

3.4 Results

3.4.1 Antimicrobial susceptibility

Drug susceptibility results of the isolates are presented in Tables 3,4 and 5. The majority of the isolates were susceptible to tetracycline, erythromycin and kanamycin. None of the isolates exhibited resistance to enrofloxacin or chloramphenicol. Multiple resistance was found towards erythromycin, ampicillin, and kanamycin in one isolate from laying hens. One isolate from older chicken exhibited multiple resistance to erythromycin, kanamycin and ampicillin. Multiple resistance to both kanamycin and tetracycline was also seen in three isolates: two strains from older birds and one strain from a broiler. Typically, all the strains were susceptible to nalidixic acid and resistant to cephalothin. These two antimicrobials are routinely used to separate *C. jejuni* and *C. coli* from other campylobacters that may be present in the fecal sample. *C. jejuni* and *C. coli* are both resistant to cephalothin and susceptible to nalidixic acid.

3.4.2 Plasmids

Isolation of plasmid DNA was carried out using various protocols including the rapid lysis method of Kado and Liu (1981); a miniprep TELT method, a modification of the protocol of Brent (1990), and a Magic^{R} miniprep kit (Promega, USA). Of these three methods, only the mini-prep kit from Promega^{TM} gave consistently clearly visible DNA bands. The two tetracyline resistant isolates of *C. jejuni*, RO 28 and RO 33, had

a band located above the 16 Kbp band of the DNA marker. This band was not observed in tetracycline sensitive strain *C. jejuni* RO 13 from an older bird. The isolates had other visible bands that corresponded to the 12 Kbp band of the supercoiled DNA marker. The mini-prep kit method did recover the high molecular size tetracycline resistance (TET^r) plasmid. The size of the plasmid which was located above the 16 Kbp band of the marker DNA matches the 45 Kbp (38 Mdal) tetracycline resistance plasmid present in *C. jejuni* strain ATCC 43502. Since a smaller size plasmid DNA was used as a marker, the exact size of the plasmid in question could not be determined precisely. The plasmids found in tetracycline resistant isolates of *C. jejuni* were consistent when the procedure was done repeatedly to evaluate consistency of their size. The 120 Kbp size plasmid DNA from *A. tumafaciens* was not detectable in all the protocols tested (Fig. 1).

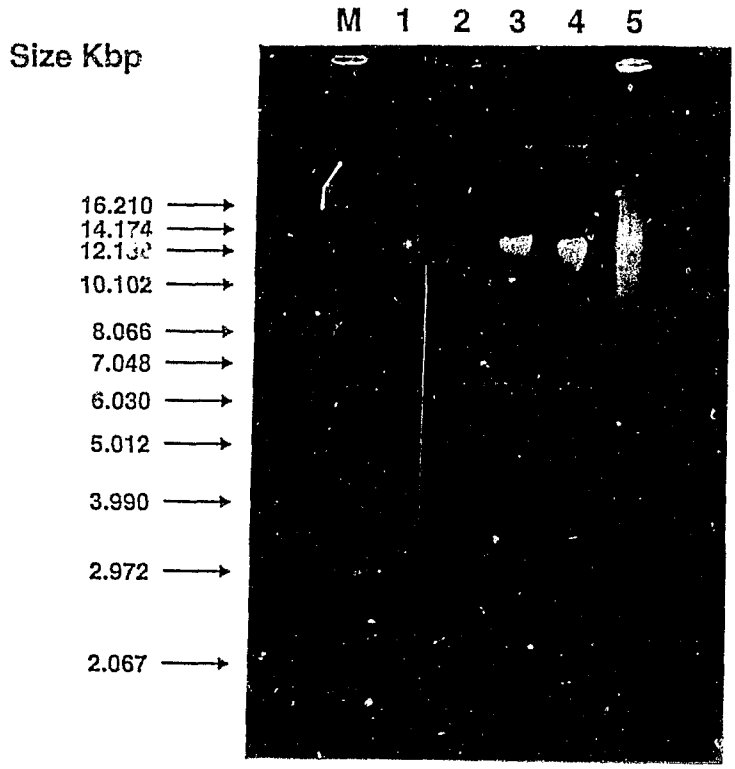


Fig. 1. Agarose electrophoresis of *C. jejuni* plasmid DNA: M, supercoiled marker DNA (16.2 - 2.0 Kbp); 1, tetracycline resistant (TET^r) *C. jejuni* ATCC 43502; 2, *A. tumafaciens* ATCC 33970 (80, 120 Kbp); 3, TET^r *C. jejuni* RO 28 chicken isolate; 4, TET^r *C. jejuni* RO 33 chicken isolate; 5, TET^s *C. jejuni* RO 13 chicken isolate.

Table 3. Drug resistance of *C. jejuni* and *C. coli* from Chickens on PEI.

DRUG (Disk potency)	No. RESISTANT ISOLATES (%)*	
	C. jejuni	*C. coli*
Ampicillin (10μg)	57 (48)	6 (5)
Cephalothin (30μg)	101 (85)	18 (15)
Chloramphenicol (30μg)	0 (0)	0 (0)
Enrofloxacin (5μg)	0 (0)	0 (0)
Erythromycin (15μg)	6 (5)	2 (1.7)
Kanamycin (30μg)	4 (3.4)	1 (0.8)
Nalidixic acid (30μg)	0 (0)	0 (0)
Tetracycline (30μg)	2 (1.7)	0 (0)

* A total of 119 isolates tested with Disk diffusion method.

Table 4. Susceptibility of *C. jejuni* and *C. coli* Isolated from Broiler Chickens to Eight Antimicrobial Agents

Isolate	Antimicrobial Agent[a]							
	ERY	CEF	CLO	ENO	KAN	AMP	TET	NAL
	Zone Diameter(mm)[b]							
BR 1	26	R	26	34	20	R	34	22
BR 2	24	R	28	36	20	R	34	22
BR 5	18	R	26	34	14	R	26	22
BR 22	20	R	22	34	22	18	34	20
BR 25	18	R	28	36	10	12	36	26
BR 27	16	R	22	36	14	12	36	26
BR 28	22	R	24	30	R	R	32	28
BR 29	20	R	28	34	18	20	28	20
BR 30	20	R	30	32	22	R	32	22
BR 33	18	R	26	36	16	14	34	28
BR 34	14	R	22	38	10	12	28	24
BR 35	16	R	26	34	22	16	32	23
BR 36	22	R	24	34	22	R	34	20
BR 41	28	R	28	36	22	R	36	20
BR 42	22	R	20	28	10	R	30	20
BR 43	28	R	26	32	20	R	32	20
BR 44	26	R	28	34	18	R	32	26
BR 45	24	R	22	34	16	R	32	20
BR 46	18	R	22	36	8	R	36	28
BR 47	28	R	26	32	22	R	32	22
BR 48	28	R	26	36	20	R	32	30
BR 49	26	R	26	36	14	R	36	20
BR 50	20	R	24	30	R	R	38	24
BR 51	20	R	28	32	12	R	34	26
BR 52	28	R	26	32	18	R	34	20
BR 54	22	R	30	34	24	R	30	22
BR 55	10	R	30	32	8	R	34	20
BR 56	22	R	26	34	10	R	30	20
BR 57	22	R	24	32	12	R	30	26
BR 58	20	R	28	34	12	R	36	28
BR 60	22	R	28	36	12	4	32	26
BR 62	22	R	24	34	22	R	36	26
BR 115	12	R	18	32	16	R	26	14
BR 116	14	R	24	36	24	R	32	22
BR 117	16	R	14	30	22	R	28	10
BR 118	12	R	22	34	14	R	28	18
BR 120	16	R	22	32	24	R	32	20
BR 122	20	R	26	36	24	22	30	20

Table 4. Susceptibility of *C. jejuni* and *C. coli* Isolated from Broiler Chickens to Eight Antimicrobial Agents

Isolate	Antimicrobial Agent[a]							
	ERY	CEF	CLO	ENO	KAN	AMP	TET	NAL
	Zone Diameter(mm)[b]							
BR 123	12	R	10	28	18	R	28	16
BR 124	20	R	24	30	16	R	32	20
BR 126	28	R	26	32	16	R	30	24
BR 127	12	R	26	34	16	10	32	22
BR 128	12	R	22	32	18	R	28	20
BR 130	12	R	28	35	24	R	34	20
BR 131	14	R	24	30	24	R	34	22
BR 132	12	R	26	32	18	12	26	26
BR 133	20	R	28	36	22	12	26	24
BR 134	14	R	24	30	26	R	28	22
BR 135	24	R	28	30	24	10	30	24
BR 136	16	R	20	32	22	R	30	18
BR 137	14	R	16	36	16	R	26	12
BR 148	20	R	22	32	26	20	32	20
BR 151	24	R	22	30	16	18	28	24
BR 154	24	R	22	36	18	16	34	22
BR 155	24	R	26	30	20	18	34	22
BR 157	20	R	24	32	22	R	36	22
BR 158	26	R	24	32	20	R	32	28
BR 159	28	R	22	32	12	R	36	26
BR 160	22	R	22	32	16	R	32	20
BR 161	26	R	26	36	24	R	34	28
BR 162	22	R	20	30	8	14	30	22
BR 163	18	R	20	24	6	14	32	18
BR 165	20	R	28	32	20	R	36	22
BR 166	28	R	26	32	18	R	32	28
BR 167	22	R	28	30	22	R	36	22
BR 168	24	R	24	32	20	16	36	26
BR 169	28	R	24	36	22	R	32	22
BR 170	22	R	26	36	24	R	30	24
BR 172	28	R	28	34	22	20	34	24
BR 173	20	R	28	36	14	R	36	28
BR 174	22	R	24	32	18	R	36	20
BR 175	26	R	26	34	24	R	32	20

[a] ERY, erythromycin; CEF, cephalothin; CLO, chloramphenicol; ENO, enrofloxacin; KAN, kanamycin; AMP, ampicillin; TET, tetracycline; NAL, nalidixic acid.

[b] Zone diameter were measured on agar media. *E. coli* ATCC 25922 was used as a standard control strain.

Table 5. Susceptibility of *C. jejuni* and *C. coli* Isolated from Older Chickens to Eight Antimicrobial Agents

Isolate	Antimicrobial Agent[a]							
	ERY	CEF	CLO	ENO	KAN	AMP	TET	NAL
			Zone Diameter(mm)[b]					
RO 1	R	R	26	30	R	R	36	28
RO 3	26	R	24	30	12	R	30	26
RO 11	20	R	28	34	24	R	38	26
RO 13	28	R	24	36	24	R	32	22
RO 15	28	R	22	36	22	R	32	28
RO 16	26	R	22	30	18	R	34	22
RO 17	24	R	28	34	16	R	36	28
RO 18	10	R	18	36	12	R	32	16
RO 19	26	R	22	30	14	R	32	20
RO 20	20	R	24	34	14	R	38	20
RO 21	20	R	24	32	14	22	32	20
RO 22	22	R	20	36	14	R	34	22
RO 23	22	R	28	34	18	6	36	26
RO 24	12	R	26	36	22	12	36	26
RO 25	R	R	22	36	22	12	18	22
RO 26	R	R	28	30	22	12	18	26
RO 27	R	R	26	30	12	12	14	22
RO 28	16	R	26	34	R	10	R	26
RO 29	R	R	20	30	22	16	16	24
RO 30	16	R	22	36	18	20	30	22
RO 31	R	R	28	30	18	16	16	22
RO 32	R	R	30	30	19	18	16	24
RO 33	12	R	28	36	R	10	R	24
RO 34	10	R	28	36	18	8	30	24
RO 35	R	R	28	30	16	16	14	24
RO 36	26	R	20	34	10	16	30	22
RO 37	24	R	26	30	8	12	32	24
RO 38	20	R	20	30	10	14	30	20
RO 39	26	R	24	36	18	14	34	20
RO 40	22	R	24	30	16	18	30	24
RO 41	22	R	20	32	8	12	32	22
RO 42	18	R	20	30	4	10	34	26
RO 43	24	R	20	34	14	14	32	24
RO 44	22	R	24	30	16	18	32	28
RO 45	16	R	24	32	14	14	30	22
RO 46	20	R	22	32	16	16	30	24
RO 47	14	R	28	38	6	12	36	24
RO 48	20	R	20	36	14	14	30	24
RO 49	18	R	24	33	8	12	34	20

Table 5. Susceptibility of *C. jejuni* and *C. coli* Isolated from Older Chickens to Eight Antimicrobial Agents

Isolate	Antimicrobial Agent[a]							
	ERY	CEF	CLO	ENO	KAN	AMP	TET	NAL
			Zone Diameter(mm)[b]					
RO 50	20	R	20	32	12	14	36	26
RO 51	22	R	20	30	18	16	34	28
RO 52	20	R	24	30	18	18	32	28
RO 53	20	R	22	34	8	14	32	22
RO 54	24	R	22	34	10	6	30	20
RO 55	20	R	20	30	14	14	30	26
RO 56	22	R	20	30	19	16	30	28
RO 57	28	R	26	36	22	R	36	28

[a] ERY, erythromycin; CEF, cephalothin; CLO, chloramphenicol; ENO, enrofloxacin; KAN, kanamycin; AMP, ampicillin; TET, tetracycline; NAL, nalidixic acid.

[b] Zone diameter were measured on agar media. *E. coli* ATCC 25922 was used as a standard control strain.

3.5 Discussion

3.5.1 Antibiotic susceptibility

Most methods of antimicrobial susceptibility testing do not perform well with *Campylobacter* spp. This often necessitates the use of more tedious methods such as agar dilution or broth microdilution. Furthermore, no standardized breakpoint values for resistance has been established for clinically useful drugs. Since we were more interested in qualitative results (positive vs. negative), a disk diffusion method was used to screen plasmid-mediated resistance.

The resistance patterns of chicken isolates of *C. jejuni* and *C. coli* from PEI were quite different from those reported from other provinces or countries. Fifty three percent of the isolates were resistant to ampicillin. Ampicillin resistance in campylobacters is common and is in association with the production of ß-lactamase. Resistance to erythromycin was reported to be less than 1% in England (Brunton et al.,1978) and Canada (Karmali et al., 1981). We found 7% of chicken isolates to be erythromycin resistant. The 2% incidence of tetracycline resistance in PEI chicken isolates is lower than the 9% reported in clinical isolates in Alberta, (Taylor et al., 1986), and in PEI pigs (Hariharan et al., 1990). Multiple resistance to tetracycline and kanamycin were seen in two isolates from laying hens and one broiler chicken. Kanamycin resistance is often mediated by a plasmid which also encodes tetracycline resistance (Kotarski et a., 1986). Antibiotic resistance to PEI isolates of *C. jejuni* and *C. coli* was found to be fairly low. In cases where antibiotic treatment is sought, drug resistance can be a problem. Furthermore, the use of antibiotics as feed additives in the growing birds can

also favor the exchange of genes responsible for antibiotic resistance not only between different strains of Campylobacters, but also between Campylobacters and other enteric bacteria like *Clostridia spp.* and other gram positive organisms which often carry resistance genes to commonly used antibiotics.

3.5.2 Plasmid profiles

The plasmid profiles of the two tetracycline resistant isolates of *C. jejuni* RO 28 and RO 33 from chicken revealed only two bands (Fig. 1). One band located above the 16 Kbp band of the supercoiled DNA marker, and another band of smaller size found in proximity to the 12 Kbp band of the supercoiled marker. These bands correspond to the 45 Kbp (38 Mdal) tetracycline resistance plasmid from the *C. jejuni* ATCC strain 43502. Both bands were found consistently in ATCC strain of *C. jejuni* as well as chicken strains of *C. jejuni* that showed resistance to both tetracycline and kanamycin.

The genes responsible for kanamycin resistance were found to be 4 Kbp, and therefore smaller than those responsible for tetracycline resistance (Tenover et al., 1992). Tetracycline resistance plasmid was first described by Taylor and associates (1983) with a determined size of 45 Kbp. Tenover et al. (1985) reported that the majority of the plasmids associated with tetracycline resistance in chicken were between 40-60 Kbp in size. The tetracycline-resistant chicken isolates of *C. jejuni* and ATCC strain 43502, all carried a plasmid band of about 48-51 Kbp located above the 16 Kbp band of the marker. Due to the small size of the supercoiled DNA marker used, it was not possible to determine the exact size of these bands. Of the three extraction

procedures used to isolate the TET^r plasmids, the Promega Magic™ mini-prep method gave the best yield of DNA material and in a relatively short time.

CHAPTER 4. TOXIGENICITY TESTING OF *C. jejuni* AND *C. coli*

4.1 Introduction

Enterotoxin production is an important virulence factor of *Campylobacter jejuni*. One important mechanism in inducing diarrhea is thought to be the production of potent toxins by these organism (Daikoku et al., 1990; Johnson and Lior, 1986). *Campylobacter jejuni* is known to produce at least two types of toxins: a heat-labile enterotoxin and a cytotoxin (Johnson and Lior, 1984). The enterotoxin of *C. jejuni* is similar in structure and mechanism of action to cholera toxin and the LT of *E.coli* (Klipstein and Engert, 1984; 1985; Goossens et al., 1985; Johnson and Lior, 1986). The enterotoxin produced by *C. jejuni* acts in a manner identical to that of cholera toxin (Fishman, 1990) and *E. coli* LT (Gyles, 1992), which stimulate membrane bound adenylate cyclase in intestinal epithelial cells. This stimulation results in persistent elevation of cyclic AMP, which leads to hypersecretion of electrolytes and water, usually manifested as watery diarrhea (Walker et al., 1986; Gyles, 1992). The enterotoxin of *C. jejuni* causes cytotonic changes in confluent monolayers or cells in suspension, inducing elongation of CHO cells and rounding of Y-1 mouse adrenal cells (Gubina et al., 1981; Ruiz-palacios et al., 1983; McCardell et al., 1984). The objective of the research reported here was to determine if isolates of *C. jejuni* from chickens demonstrated toxigenic effects on CHO (Chinese hamster ovary) cells.

4.2 Materials and methods

4.2.1 Propagation of tissue culture cells

Chinese hamster ovary (CHO-K1) cells were used for the detection of *C. jejuni* toxins, and were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cell monolayers were grown in 75 cm^2 tissue culture flasks and maintained in Ham's F12 media (ICN Biochemicals, Costa Mesa, California, USA) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 incubator at 37°C. The cells were dispersed by trypsinization with 0.25% trypsin - 1mM EDTA combination (ICN Biochemicals) prior to their use in tests.

Toxin assay was carried out in CHO-K1 cells using F12 media with 3% FBS. Biphasic media consisting of medium M199 (Life Technologies, Burlington, Ontario) as the liquid phase over Müller-Hinton agar (Oxoid) as the solid phase was used for the growth of bacterial cells. Sodium bicarbonate (Life Technologies, Burlington, Ontario) was added to both M199 and Ham's F12 media at a final concentration of 0.35g/l as recommended by the supplier. The pH was adjusted to 7.2 and the media were filter sterilized with 0.22 μm filter (Nalgene, Rochester, New York, USA). Stock cultures of *C. jejuni* stored at -76°C were grown for 48 hours on blood agar plates under microaerophilic conditions of 85% N_2, 10% O_2 and 5% CO_2 at 42°C in an anaerobic jar with a catalyst. A loopful (10 μl) of bacterial growth was inoculated into biphasic media consisting of 10 ml of M199 over 5 ml of Müller-Hinton agar (Oxoid) in 50 ml volume Erlenmeyer flasks. Inoculated flasks were then incubated for 48 hours at 37°C with shaking at 100 rpm on orbit shaker under a gas mixture of 85% N_2, 10% O_2 and

5% CO_2. Campylobacter cultures were harvested after 48 hours of growth by centrifugation at 3000 rpm for 30 min. Culture filtrates were obtained by filtering the supernatants through 0.22 μm cellulose acetate syringe filters (Nalgene). Cell-free filtrates were kept on ice during tissue culture cell manipulations and were tested immediately on CHO-K1 cells for the presence of toxins.

4.2.2 Detection of enterotoxin

Cell-free filtrates from biphasic media were tested on CHO-K1 cells immediately after harvesting by centrifugation (Eppendorf microcentrifuge model 5414, USA) at 4°C. Confluent monolayers of CHO-K1 cells grown in 25 cm^2 flasks were trypsinized and suspended into 80 ml of Ham's F12 media with 3% FBS. A 100 μl of samples were placed in 96 well plates to which were added a 2-fold serial dilutions of bacterial filtrates. Duplicate wells were used for each isolate. Plates were then incubated at 37°C in 5% CO_2 for 48 to 72 hours. Plates were observed using an inverted microscope after 24 hours of incubation to assess changes in cell morphology; LT^+ *E. coli* ATCC 35401 was used as an enterotoxin-positive control. Two *C. jejuni* strains 4182 and 6704 from humans, obtained from H. Lior (Laboratory Centre for Disease Control, Ottawa) were included in each test as positive and negative controls for enterotoxins, respectively. The test was interpreted as positive if elongation of more than 50% of the cells was present at filterate dilution of 1:2 or greater. The toxin titer which is the highest dilution of bacterial filtrate causing elongation of more than 50% of the cells for each well was recorded for each positive sample. Tests were performed in duplicate for each sample.

4.3 Results

Fifty six of 119 (47%) isolates of *C. jejuni* and *C. coli* strains produced toxins causing the elongation of CHO-K1 cells. The effect of the toxin on these cells was often visible after 24 hours, but it developed to its optimum after 48 hours. The toxin effect gradually reduced by one or two dilution factors after 72 hours of incubation at 37°C in a CO_2 incubator. The use of biphasic media with extensive shaking stimulated greater toxin production from the bacteria compared to Brucella broth media pH 5.6 as suggested by Saha et al. (1988). Little or no toxin was detected on CHO-K1 cells when the bacteria were grown in biphasic media without shaking during the incubation. Toxin detection also depended on the rapidity with which the tests were done and the conditions used to maintain it during the assays. A higher level of toxin production was consistently found when culture supernatants were tested for toxin production immediately following incubation and the tubes were kept on crushed ice throughout the test. Tests were run on 2-day old cultures. Filtered bacteria kept at 4°C for seven days did not cause any alteration on the cells. Moreover, cells began to form monolayers on the plate with no visible change in morphology. The occurrence of toxigenic campylobacters isolated from a poultry processing plant on Prince Edward Island is summarized in Tables 6 and 7. Toxigenic profiles were designated following analysis in CHO-K1 cells described in Materials and Methods. The degree of elongation of CHO-K1 cells for enterotoxin-positive isolates was found to vary depending on the medium used to grow the bacteria and percentage of serum in the growth media for CHO-K1 cells. Fairly consistent titers were found when the growth media for CHO-K1

cells contained 3% FBS. The heat stability of twelve campylobacter toxin-positive supernatants were tested by heating at 95°C for 10 minutes in waterbath. The effect was of the toxin was almost lost following heating.

Preliminary tests on campylobacter toxins were carried out using VERO cells. Chicken isolates of *C. jejuni* and *C. coli* did not cause consistent changes in these cells. A verotoxin-positive (VT^+) *E. coli* O157 and LT^+ *E. coli* 35401 were included in the assays as reference strains. Only the former showed consistent cytotoxicity on the monolayers. Destruction of the monolayers and rounding of the cells were seen in less than 12 hours of incubation. The cells became completely rounded within 24 hours of incubation. Poultry isolates of *C. jejuni* and *C. coli* did not cause any significant change on Vero cells.

Table 6. Toxigenicity of *C. jejuni* and *C. coli* Isolated from Broiler Chickens

Isolate	Species[a]	Titer[b] on CHO-K1 Cells)
Lior 4483	1	1:4
Lior 4182	1	1:32
Lior 6704	1	-
BR 1	1	1:8
BR 2	1	1:32
BR 5	1	-
BR 22	2	-
BR 25	2	-
BR 27	2	-
BR 28	1	1:64
BR 29	2	-
BR 30	1	1:8
BR 33	2	1:4
BR 34	1	1:8
BR 35	2	-
BR 36	1	1:8
BR 41	1	-
BR 42	1	1:4
BR 43	1	1:8
BR 44	1	1:8
BR 45	1	1:2
BR 46	1	1:16
BR 47	1	1:4
BR 48	1	1:8
BR 49	1	1:16
BR 50	1	-
BR 51	1	-
BR 52	1	-
BR 54	1	-
BR 55	1	-
BR 56	1	1:16
BR 57	1	-
BR 58	1	-
BR 60	1	1:16
BR 62	1	-
BR 115	1	1:16
BR 116	2	1:8
BR 117	2	1:4
BR 118	1	1:8

Table 6. Toxigenicity of *C. jejuni* and *C. coli* Isolated from Broiler Chickens

Isolate	Species[a]	Titer[b] on CHO-K1 Cells)
BR 120	2	1:16
BR 121	1	
BR 122	1	-
BR 123	1	1:8
BR 124	1	-
BR 126	1	1:8
BR 127	2	1:16
BR 128	2	1:4
BR 130	1	-
BR 131	2	-
BR 132	2	1:4
BR 133	2	1:8
BR 134	1	1:4
BR 135	1	1:16
BR 136	2	1:8
BR 137	1	1:8
BR 148	1	1:16
BR 151	2	1:16
BR 154	1	-
BR 155	1	-
BR 157	1	1:2
BR 158	1	1:8
BR 159	1	-
BR 160	1	-
BR 161	1	-
BR 162	1	-
BR 163	1	-
BR 165	1	-
BR 166	1	-
BR 167	1	1:8
BR 168	1	-
BR 169	1	1:4
BR 170	1	-
BR 172	1	-
BR 173	1	-
BR 174	1	-
BR 175	1	1:8

Table 7. Toxigenicity of *C. jejuni* and *C. coli* Isolated from Older Birds

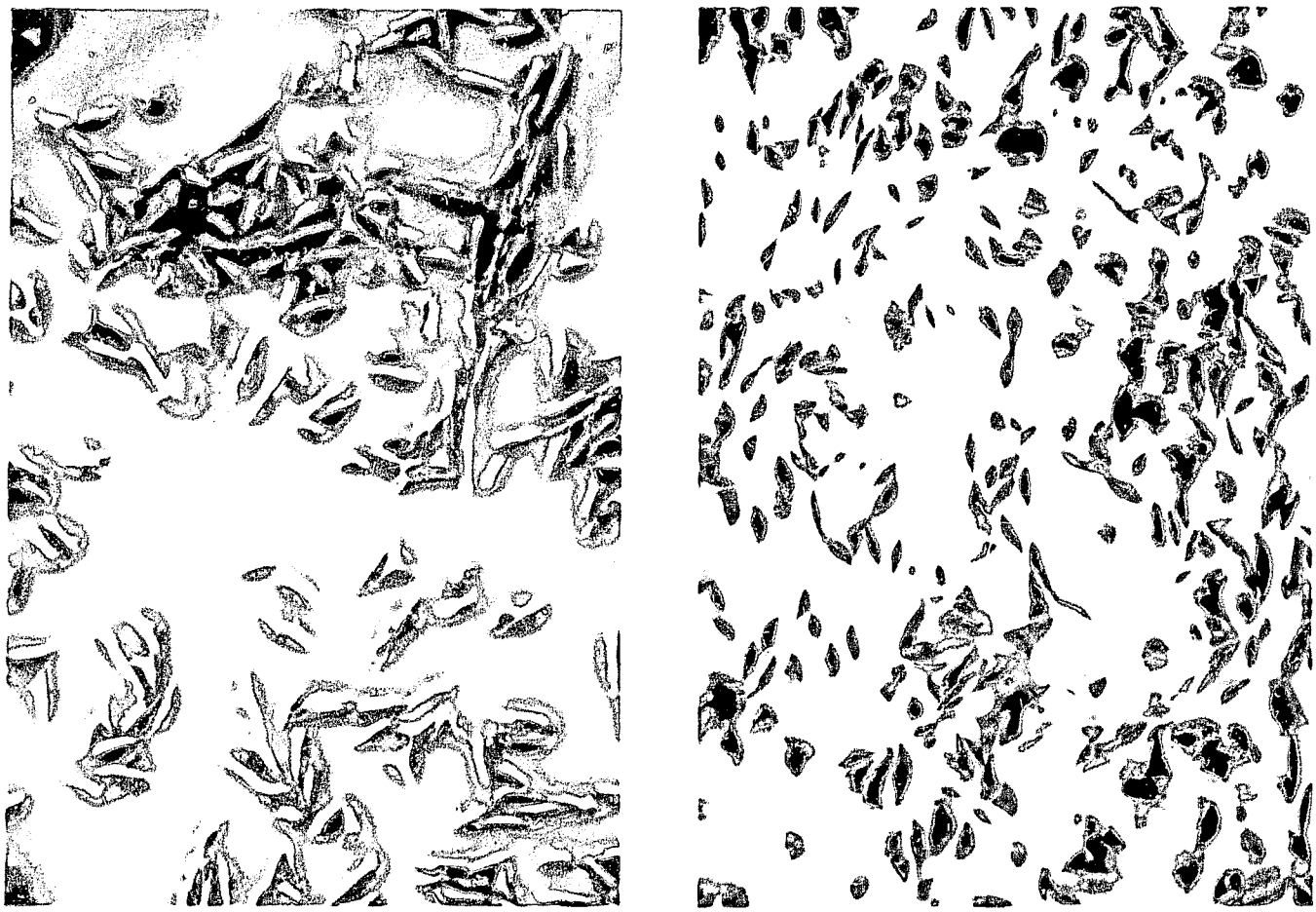
Isolate	Species[a]	Titer[b] on CHO-K1 Cells)
RO 1	2	1:4
RO 3	1	-
RO 11	1	-
RO 13	1	-
RO 15	1	1:4
RO 16	1	1:2
RO 17	1	-
RO 18	1	-
RO 19	1	-
RO 20	1	1:2
RO 21	1	1:8
RO 22	1	1:8
RO 23	1	-
RO 24	1	1:8
RO 25	1	1:4
RO 26	2	1:2
RO 27	1	-
RO 28	1	1:8
RO 29	1	1:4
RO 30	1	1:8
RO 31	1	1:8
RO 32	1	-
RO 33	1	1:4
RO 34	1	1:8
RO 35	1	-
RO 36	1	-
RO 37	1	1:4
RO 38	1	1:16
RO 39	1	1:2
RO 40	2	1:4
RO 41	1	-
RO 42	1	-
RO 43	1	1:16
RO 44	1	1:4
RO 45	1	1:2
RO 46	1	-
RO 47	1	1:2
RO 48	1	-

Table 7. Toxigenicity of *C. jejuni* and *C. coli* Isolated from Older Birds

Isolate	Species[a]	Titer[b] on CHO-K1 Cells)
RO 49	1	-
RO 50	1	1:16
RO 51	1	-
RO 52	1	-
RO 53	1	-
RO 54	1	1:8
RO 55	1	-
RO 56	1	1:16
RO 57	1	-
RO Liver	1	-

[a] As identified by the Hippurate hydrolysis test: 1= *C. jejuni*; 2 = *C. coli*.

[b] Highest dilution of cell-free filterate which caused elongation of ≥ 50% of cells. (-) indicates no toxic effect



A. B.

Fig. 2. Effect of *C. jejuni* enterotoxin on CHO-K1 cells. At 24 hrs after inoculation: **A.** TOX$^+$ *C. jejuni* BR 2, dilution 1:2. **B.** TOX$^-$ *C. jejuni* BR 52, dil. 1:2. Mag 10X (cropped). Notice the elongation of CHO-K1 cells in A.

4.4 Discussion

The pathogenesis of campylobacter diarrhea seems to involve a complex of mechanisms in which invasion of gut epithelial cells and production of enterotoxin are of primary importance. In this study we determined the frequency of toxin production in *C. jejuni* and *C. coli* strains isolated from chickens in Prince Edward Island.

The incidence of toxin-producing strains of *C. jejuni* and *C. coli* varied between 32% in Southern India (Mathan et al., 1984) to 100% in Belgium (Goossens et al., 1985) where CHO-K1 cells have been used. In both studies, strains from children with acute campylobacter enteritis were used. No such information is available in Prince Edward Island. In our study we found that 47% of the strains of *C. jejuni* and *C. coli* isolated from chickens in Prince Edward Island caused elongation of CHO-K1 cells (Tables 6 and 7). This is in contrast to the 32% incidence found in laying hens in Sweden by Lindblom and co-workers (1986). The most common method for showing the production of campylobacter enterotoxin is the CHO test. The enterotoxin raises the intracellular level of cyclic AMP, which induces cytotonic changes in the CHO-K1 cells (Guerrant et al., 1974). In this study we adopted the CHO-K1 cell tests for enterotoxin testing. We also used Vero cells in preliminary tests to detect the production of cytotoxic factors from the bacteria. Johnson and Lior (1986) claim that Vero cells are the most sensitive for campylobacter cytotoxic factors. Our chicken isolates however failed to produce any detectable effects in Vero cells except with a control strain VT' *E. coli* O157 which gave consistently high positive titers of up to 1:256. *Campylobacter jejuni* strain 4483 which was reported by Johnson and Lior (1986) to produce both

cytotonic and cytotoxic factors was also included in the tests, but no effect was seen on Vero cells. This strain caused elongation of CHO-K1 cells up to 1:4 dilutions. Campylobacter culture filtrates were reported to cause cytotoxic effects in Vero cells which consists of destruction of monolayer and rounding of cells.

Chickens are the most common reservoirs of *C. jejuni* and are generally colonised without signs of disease (Lindblom et al., 1986). We have found that 47% of 119 of *Campylobacter* strains isolated from a poultry processing plant in PEI produced enterotoxin. This is slightly higher than the frequency rates of 31% in India (Saha et al., 1988) and of 39% in Sweden (Lindblom et al., 1990), but lower than the 87% rate from Pakistan and Mexico, and 100% rate in the USA (Lindblom et al., 1990). The differences in the frequency of enterotoxigenicity reported from different parts of the world might be a result of variations in the methods of toxin detection as well as the type of media used for cultivating the bacteria. In any case, whether the different results are caused by loss of ability in some isolates to produce enterotoxin after laboratory maintenance or technical differences regarding the assay or whether this reflects the natural occurrence of enterotoxigenic *Campylobacter* strains needs further investigation. *C. jejuni* toxins are produced in limited amounts *in vitro*. The amount produced *in vivo* might of course be larger, especially when ingestion of as few as 500 cells in milk were reported to have caused acute enteritis in human (Robinson, 1981). An important aspect, therefore, is the capability of a strain to produce toxin, not the amount of toxin detected *in vitro*. Toxin production by *Campylobacter spp.* is complex. There are a number of

other toxins claimed to have been produced by thermophilic campylobacters (McCardell et al., 1984; Johnson and Lior, 1986) that need to be investigated.

CHAPTER 5. GENERAL DISCUSSION AND CONCLUSION

Campylobacter jejuni is recognized as the most common cause of enterocolitis in humans who consume undercooked poultry meats. The organism is frequently found in poultry feces and during removal of viscera may be transferred from the intestines to the surface of the skin. *Campylobacter jejuni* has frequently been isolated from raw poultry carcasses, parts and edible viscera at processing plants and at retail outlets (Wempe et al., 1983a). The use of selective growth media has allowed the culturing of campylobacters directly from highly contaminated samples such as feces and cecal contents. Direct plating is particularly advantageous where more than one strain of *Campylobacter* is present. The use of antibiotic to restrict the growth of other organisms present in the gut has greatly improved the isolation rates. However, our finding that 52% of the birds sampled in Prince Edward Island carried *C. jejuni* and *C. coli* is somewhat lower than the 85% isolation rate reported in Ontario, Canada by Prescott and Gellner (1984). This difference may be due to the lower prevalence of *Campylobacter spp.* in this geographic area. Our samples may truly reflect the prevalence of the organisms in PEI since the sampled birds originated from various farms around the Island.

The presence of *C. jejuni* contaminated foodstuff is hazardous to human health. It is estimated that about 50% of sporadic cases of campylobacter enteritis are linked to eating or handling poultry (Doyle, 1984; Rautelin, 1991). The processing methods of poultry plants almost ensure the survival and spread of *Campylobacter spp.* This is

accomplished by the use of water chilling tanks which are used to chill the carcasses after slaughter and evisceration. It would be interesting to determine the percentage of contaminated carcasses that have been packed for retail sale. Reducing the carriage rate of *C. jejuni* in poultry would have a major impact on decreasing the risk of human infections. To reduce the prevalence of microbial contamination in poultry, improvements in poultry husbandry are needed to decrease contamination of flocks. Changes should include rigorous feed sanitation, better designed buildings, motivation of the farmers to take appropriate measures to control pathogens, and better conditions for cleaning and disinfecting the farm.

Major areas that need appropriate sanitary procedures in the processing plant on PEI include:

1) Transportation - birds d stined for slaughter should be placed in thoroughly cleaned and disinfected cages. Also, overcrowding should be avoided as this may be stressful to the birds;

2) Defeathering equipment and the surrounding area should be disinfected before and after the operation; and

3) Special care needs to be taken during evisceration to prevent the spillage of the intestinal contents and their contact with other carcasses in the chilling tank.

Based on our observations, spillage of intestinal contents during evisceration represents the most likely source of contamination to the carcass. It is also imperative to implement a minimum training on health-related issues for the plant workers emphasizing the importance of sanitary handling of poultry meat and products.

Unlike *Salmonella* spp. *C. jejuni* is sensitive to desiccation (Luechtefeld et al., 1981a) and, therefore, the organism may not survive in litter for periods exceeding 10 days at 20 °C. Several factors such as availability of nutrients, relative humidity and pH also influence survival times (Lahellec, 1987). *Campylobacter jejuni* is very sensitive to ambient temperatures, it appears to colonize poorly under low temperatures (Willis et al., 1991), which may contribute epidemiologically in the colonization process with broiler chickens. Seasonal and conditional house temperatures are major factors in *C. jejuni* colonization (Blaser et al., 1983). According to Genigeorgis et al. (1986) there appears to be a very strong relationship between the seasonal environmental temperature and *C. jejuni* initial colonization with warmer temperatures favoring colonization. Disinfection procedures in chicken rearing houses can be successful only if other husbandry practices are also improved. Particular risk factors with respect to performance and disease include: poor physical condition, ambient temperature below 25 °C for the first few days, overcrowding, poor quality litter and neglected farm hygiene. A thorough decontamination of houses with complete replacement of litter is necessary to prevent transmission of infection from previous placements.

Contamination of live animals and carcasses by *Campylobacter* spp. is difficult to quantify because the pathogens are often sporadically distributed and in very low numbers on carcasses. Additionally, the pathogens may be sublethally injured during processing, thereby requiring resuscitation before enumeration. The main sources of bacterial infection associated with poultry-borne diseases include the chicks, feed and water and environmental factors. In any attempt to reduce the level of contamination,

better hygiene management, including these factors should be established. Coprophagic nature of chicks also facilitates the fast spread and colonization of *C. jejuni* in the entire flock (Lindblom et al., 1986; Pokumanski et al., 1986; Stern, 1988). Chicks should be housed in a wire-floored pen in the first few weeks of life to prevent coprophagy.

It is doubtful that the total absence or elimination of pathogenic organisms can ever be achieved. The complex causes of infections are so diverse that complete elimination of campylobacters from domestic animals is not feasible. The production of foods of animal origin should incorporate procedures to either produce a Campylobacter-free product or minimize the incidence of contamination. Important steps in reducing the incidence of *Campylobacter* spp. on chickens offered at retail outlets should include improved processing procedures at slaughter, and to reduce the available water on the carcass, either by drying or by addition of some acceptable solutes to sequester water from carcass surface. Sensitivity to low water activity suggests that campylobacters would not be a problem in foods with high solute concentrations (Shane, 1991b). Another important approach worth investigating is the placement of carcasses in a hot-air room immediately after a rinse followed by prompt packaging and refrigeration.

Prevention of plant cross contamination is important in reducing the spread of *C. jejuni* in poultry carcasses (Shane, 1992). This could be achieved by improved washing of carcasses, cleaning and disinfection of immersion chillers, before and after use, and reduction of manual handling of carcasses by introduction of automated equipment. Enhanced plant cleaning will also reduce the level of *C. jejuni* and the

extent of cross-contamination in the processing plant. Reduction of fecal contamination in the plant can be accomplished by withholding feed from broilers for at least 12 hours before slaughter. In addition, transport cages and vehicles should be decontaminated after each delivery to remove fecal material. Immersion in scalding water at 58 °C may reduce the level of *C. jejuni* surface contamination, but a lower temperature such as 52 °C frequently used in the processing industry has little inhibitory effect (Oosterom et al., 1983b; Wempe et al., 1983b). Therefore, constant monitoring of the immersion tank water temperature is very important to ensure the reduction of viable pathogens in the water. Increased numbers of *C. jejuni* can be recovered following defeathering and evisceration (Oosterom et al., 1983b). These operations play a significant role in spreading the contamination to the entire carcasses. The carcasses should be spray-washed before entry to the scalding tank to reduce the microbial load of the water. Hudson & Meade (1987) suggest that addition of quaternary ammonium disinfectant at 50 ppm to the scalding water will reduce the survival rate of *C. jejuni* at 50°C. Also, washing the carcasses with clean water when leaving the scalding tank is necessary to rinse off the microorganisms present on the skin. Evisceration should be done carefully to avoid rupture of viscera which may contaminate the equipment and the hands of workers thereby spreading the infection. Additionally, the carcasses should be spray-washed during and after evisceration to reduce cross-contamination of carcasses in the chilling tank. Luechtefeld and Wang (1981) found that storing of eviscerated turkey carcasses in tubs of chlorinated water at 50 ppm for 10 hours reduced the level of contamination from 94% to 34%. It would be interesting to investigate the

contamination level of poultry carcasses stored in tubs containing chlorinated water (50 ppm) for a few hours before packaging and refrigeration.

The occurrence of antibiotic resistance in *C. jejuni* in PEI chickens remains fairly low, perhaps due to the limited local practice of incorporating antibiotics in poultry feeds. The use of antibiotics as feed additives in the growing birds can also favor the exchange of genes responsible for antibiotic resistance not only between different strains of campylobacters, but also between other enteric bacteria like *Clostridia* spp. and other gram positive organisms which often carry resistance genes to commonly used antimicrobials.

The finding that *C. jejuni* isolates from chickens in Prince Edward Island exhibited a fairly low incidence of resistance to antibiotics commonly used for the treatment of other enteric infections of bacterial origin is encouraging. However, a large number of strains were resistant to ampicillin, whereas a small number of strains exhibited resistance to erythromycin (Table 3-5). Erythromycin is considered the drug of choice for the treatment of campylobacter enteritis in humans. However, our reported incidence of drug resistance is higher than the 3% reported by Rautelin and co-workers in Finland (1991) and it is likely to increase due to the growing use of antibiotics in poultry. Other drugs used in the treatment of campylobacter infections include tetracycline and ampicillin. A recent study by Willis et al. (1991) showed that the use of antibiotics in growing birds did not prevent them from being colonized by *Campylobacter* spp. Antibiotics may even cause the depletion of normal gut flora, which may contribute significantly to the increase of *Salmonella* and *Campylobacter*

contamination of the live birds. The results of plasmid profiles indicate that resistance to tetracycline in the isolates tested maybe plasmid mediated.

Campylobacter jejuni is thought to cause diarrhea through the production of toxins. Toxin production by this organism can be detected using freshly trypinized CHO cells. There is little information available regarding the toxigenic properties of *C. jejuni* isolated from Atlantic Canada. Chickens are the most common carriers of *C. jejuni* (Harris et al., 1986). We found that 47% of campylobacters isolated from a poultry processing plant in PEI produced enterotoxins (Table 6 and 7). This finding is slightly higher than that reported in some other countries (Saha et al., 1988; Lindblom et al., 1990).

Understanding the relative prevalence of *C. jejuni* contamination in chickens is crucial to the development of control measures to reduce the contamination of the pathogen on poultry meat and thus lower the risk of infection to consumers. There are three means by which Campylobacter infections from poultry can be controlled. The first is through the education of the consumer. The easy destruction of campylobacters during cooking makes control of the organism relatively easy, but proper hygienic practices in the home kitchen are still necessary to prevent cross-contamination to other foods. Re-contamination of cooked poultry and other meats is also common. The second method of control is to eliminate campylobacters from the intestinal tracts of birds. This may be possible as the organism cannot survive the dry conditions of the incubator, cannot penetrate eggs and is sensitive to disinfection and drying in the poultry house between lots of birds (Shane, 1991b). Studies have shown that infection in young birds

occurs as a result of contaminated drinking water introduced into the flock (Engvall, 1986; Kazwala et al., 1990). The use of chlorinated water and proper cleaning of the drinkers in poultry rearing facilities will likely reduce the rate of infection. The third method of control of campylobacteriosis is at the slaughter plant. Intestinal carriage rates among poultry delivered to processing plants is often high. Immersion scalding, defeathering, evisceration and continuous waterbath chilling serve to disseminate infection to all carcasses processed. Thorough cleaning and de-contamination of potentially contaminated areas such as scalding tanks, feather and offal channels is required. To reduce the spread of the bacteria onto other processed birds, care should be taken to prevent spillage of intestinal contents during evisceration. Proper treatment and disposal of water effluents from poultry slaughterhouses would reduce environmental contamination. Control of *Campylobacter jejuni* will therefore lie, as with *Salmonella*, in the application of appropriate food hygiene practices.

Pasteurization will effectively eliminate milkborne outbreaks and adequate cooking should prevent poultry-related infections.

APPENDIX A

Lysis solutions

SDS lysis solution

3% sodium dodecyl sulphate (SDS) 50mM Tris

adjust pH to 12.6 with 1N NaOH

adjust volume to 100 ml with deionized distilled water

APPENDIX B

TELT buffer

1M Tris-HCl	pH 8.0	5 ml
0.5M Na_2-EDTA	pH 8.0	12.5 ml
250mM LiCl		1ml
Triton X-100		4 ml

APPENDIX C

TE buffer

1M Tris-HCl	pH 8.0	1 ml
0.5M Na_2-EDTA	pH 8.0	2 ml

adjust volume to 100 ml with deionized distilled water

APPENDIX D

10X sample loading buffer

Glycerol	5 ml
Na_2-EDTA	0.37g
Sodium dodecyl sulphate (SDS)	0.1g
Bromophenol blue	0.01g

adjust volume to 10 ml with deionized distilled water

APPENDIX E

Ribonuclease A (Rnase A)

Bovine pancreas RNAse 4.3mg

50mM Na-acetate pH 5.0 860 µl

heat at 80°C for 10 minutes. Store at -20°C in 20 µl aliquots.

APPENDIX F

10X Tris-borate-EDTA electrophoresis buffer

Tris-base 108.0g

Boric acid 55.6g

0.5M Na_2-EDTA pH 8.0 40 ml

adjust volume to 1l with deionized distilled water.

Note: this is a 10X concentration solution, dilute in 1:10 with deionized distilled water.

APPENDIX G

PROMEGA™ Mini-prep Kit (1992)

Cell resuspension solution:	50mM Tris-HCl; 10mM EDTA; 100μg/ml RNase A.
Cell Lysis solution	0.2M NaOH; 1% SDS
Neutralization solution:	2.55M Potassium acetate
TE Buffer:	10mM Tris-HCl, pH 7.5; 1mM EDTA
Column wash solution:	200mM NaCl; 20mM Tris-HCL pH 7.5; 5mM EDTA.

APPENDIX H

Ethidium Bromide Dye

Ethidium Bromide	1 g
Distilled Water	100 ml

Wrap the container in Aluminum foil to protect from light and store dye in dark area.

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