ABSTRACT OF DISSERTATION

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The Graduate School

University of Kentucky

1998

DETECTION AND ANALYSIS OF CYTOLETHAL DISTENDING TOXIN (CDT) GENES IN *CAMPYLOBACTER JEJUNI* AND *CAMPLYOBACTER COLI* ISOLATES BY POLYMERASE CHAIN REACTION AND DETERMINATION OF THE PREVALENCE OF *CDT* GENES AND CDT ACTIVITY IN CAMPYLOBACTERS ISOLATED FROM CHICKEN CARCASSES

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December 1, 1998

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirenments for the degree of Doctor of Philosophy
at the University of Kentucky

By

Aysegul Eyigor

Lexington, Kentucky

Director: Dr. Bruce E. Langlois, Professor of Food Microbiology

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Campylobacter jejuni (*C. jejuni*), a common cause of human enteritis, produces a toxin called cytolethal distending toxin (CDT), which kills cultured cells in a manner unlike other well-studied bacterial toxins. Currently available nucleotide sequence data have revealed the presence of considerable amino acid sequence divergence in Cdt proteins both between and within species boundaries. However, knowledge of the prevalence and homogeneity of *cdt* genes in *C. jejuni* and *Campylobacter coli* (*C. coli*) isolates is incomplete. Therefore this study was designed to determine *cdt* gene prevalence and homogeneity in *Campylobacter* sp. isolated from chickens, and to determine the level of active CDT production in these isolates. Initially, four PCR primer pairs were identified, which can be used to detect *Campylobacter* species *cdt* genes. Collectively they amplified *cdt* genes in all but one of the 37 *C. jejuni* and *C. coli* strains tested. Restriction analyses of the *cdt* PCR products with *Eco*RI and *Stu*I showed that all of the *C. jejuni*, but none of the *C. coli*, products were cut once by each of these enzymes.

Secondly, isolates from chicken carcasses, the primary source of *C. jejuni* and *C. coli* in human infections were analyzed to determine if these strains commonly carry *cdt* genes and also if they produce active CDT. Of the 105 isolates, 70 (67 %) were identified as *C. jejuni* and 35 (33 %) as *C. coli*. PCR tests amplified portions of the *cdt* genes from all 105 isolates. Restriction analysis of the PCR products indicated that there appear to be species specific differences between the *C. jejuni* and *C. coli cdt* genes, but that the restriction patterns of the *cdt* genes within strains of the same species were almost invariant. Quantitation of active CDT levels produced by the isolates indicated that all *C. jejuni* strains except four (94 %) had mean CDT titers greater than 100. Only one *C. jejuni* strain appeared to produce no active CDT. *C. coli* isolates produced little or no toxin activity when assayed on HeLa cells. Results indicate that *cdt* genes are essentially universally present in *C. jejuni* and *C. coli* isolates. In addition, the genes appear to be highly conserved within each species, and that there appear to be significant differences between the CDT amounts produced by *C. jejuni* and *C. coli*.

Dyegnil Eyigor

December 1, 1998

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I would like to dedicate this dissertation to

my loving grandmother, Fikriye (Cik) Senses.

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INTRODUCTION

C. jejuni and *C. coli* are recognized as one of the most common causes of bacterial enteritis in the United States (Advisory Committee on Microbiological Safety of Food, 1993; Blaser and Reller, 1981; National Advisory Committee on Microbiological Criteria for Foods, 1995; Tauxe, 1992).

The most frequent source of *Campylobacter* species in human disease in the United States is the consumption of improperly handled or undercooked contaminated chicken (Bryan and Doyle, 1995; Deming et al., 1987; Graves et al., 1998; Harris et al., 1986; Hopkins and Scott, 1983; Jones et al., 1991; Park et al., 1981; Stern et al., 1985; Tauxe, 1992). It is well documented that *C. jejuni*, *C. coli*, and to a lesser extent *C. lari* inhabit the chicken intestinal tract without causing apparent health problems for the chickens (Advisory Committee on Microbiological Safety of Food, 1993).

C. jejuni and *C. coli* have been reported to produce a number of apparently different toxin activities. The best characterized of these toxins is cytolethal distending toxin (CDT), first shown by Johnson and Lior (1988) to be produced by several *Campylobacter* sp. CDT causes a variety of cultured eucaryotic cell lines to become slowly distended and die (Johnson and Lior, 1988). Johnson and Lior (1988) screened several hundred *Campylobacter* isolates for CDT production and their results suggested that only about 40% of *C. jejuni* and *C. coli* isolates produce CDT. They also reported that there was no apparent correlation between production of CDT and biotype, serotype, or strain source (Johnson and Lior, 1988).

The *C. jejuni cdt* genes have been cloned and sequenced (Pickett et al., 1996) and Whitehouse et al. (1998) have recently shown that *C. jejuni* CDT causes sensitive cells to

become blocked in the G_2 phase of their cell cycle, indicating that CDT has a novel mechanism of action for a bacterial toxin. While the role of CDT in *Campylobacter*-caused disease has not been defined, Okuda et al. (1997) have shown that a CDT produced by *Shigella dysenteriae* is capable of causing diarrhea in a suckling mouse model.

Cdt genes have also been found in some *Escherichia coli* isolates (Comayras et al., 1997; Johnson and Lior, 1987; Johnson and Lior, 1988; Peres et al., 1997; Pickett et al., 1994; Scott and Kaper, 1994), in some *Shigella* sp. isolates (Johnson and Lior, 1987; Okuda et al., 1995; Okuda et al., 1997) and in *Haemophilus ducreyi* (Cope et al., 1997). In all cases there are three adjacent or slightly overlapping genes, *cdtA*, *cdtB*, and *cdtC*, the expression of all of which is apparently required for activity (Okuda et al, 1997; Pickett et al., 1994; Scott and Kaper, 1994). The predicted amino acid sequences for the three Cdt proteins produced by these varied organisms indicate that considerable amino acid sequence divergence has occurred, particularly within the CdtA and CdtC sequences.

Rationale for this work. Pickett et al. (1996) performed PCR experiments to verify the presence of a *cdtB* gene in representatives of selected *Campylobacter* species by using two degenerative primers based on two variant *E. coli cdtB* genes (Pickett et al., 1994; Scott and Kaper, 1994). They were able to show that all of the species tested, *C. jejuni*, *C. lari*, *C. upsaliensis*, *C. hyointestinalis*, *C. coli* and *C. fetus*, apparently contain *cdtB* genes. These experiments also illustrated these primers' ability to amplify *cdtB* gene sequences from a variety of sources. In that study, they tested the performance of only one primer pair and in only one strain from species other than *C. jejuni* and *C. coli*. In the same study, Pickett et al. (1996) reported that 100% (20/20) of the *C. jejuni* strains

tested produced at least some CDT, suggesting that CDT production may be more universal in *Campylobacter* than originally reported (Johnson and Lior, 1988). However, since only a few campylobacters (20 *C. jejuni* and 12 *C. coli* isolates) from animal sources were tested, it was not clear whether CDT production was prevalent in strains isolated from humans with disease or whether CDT is in fact produced by essentially all *C. jejuni* strains and that the previous report was incorrect.

In this study we proposed:

1. To develop PCR tests for detection and analysis of *cdt* genes in both *C. jejuni* and *C. coli* strains.

2. To determine whether *Campylobacter* isolates from chicken sources commonly carry *cdt* genes.

3. To evaluate whether these isolates produce active CDT.

LITERATURE REVIEW

General background.

Bacteria belonging to the genus *Campylobacter*, previously classified as microaerophilic *Vibrio*, have been known as potential pathogens for more than 70 years (Butzler and Skirrow, 1979). From these microaerophilic vibrios, which were regarded as only animal pathogens, *V. fetus* was indicated as a cause of abortions in cattle and sheep (Mc Fadyean and Stockman, 1913; Smith, 1913; Smith and Taylor, 1919), *V. jejuni* as a cause of winter dysentery in cattle (Jones et al.,1931), and *V. coli* as a cause of swine dysentery (Doyle, 1944).

In 1946, Levy reported the possible association of a vibrio-like organism in a human outbreak of acute gastroenteritis in Illinois. Ten years later, King (1957) studied the characteristics of microaerophilic vibrios isolated from human and animal infections, and reviewed 15 reported cases of human infections with *V. fetus* and 4 cases where the infection was caused by "related vibrios". The vibrios isolated from different sources were morphologically similar, but certain "related vibrios" isolated from the blood cultures of patients with gastroenteritis, differed from *V. fetus* in that they grew at 42°C but not at 25°C. King (1962) also noticed that in certain cases, contact with animals was a possible source of infection.

During the 1950s, King's hypothesis could not be confirmed due to the lack of isolation and identification methods for studying fecal samples from humans with gastroenteritis. In 1972, Dekeyser et al. combined a fecal filtration technique used in veterinary medicine with a selective plating, and isolated "related vibrios" from two patients with acute enteritis. Using the same method, Butzler et al. (1973) isolated the

"related vibrio" as the only suspected pathogen in 5.2% of 800 children with diarrhea and in 4% of 100 adult patients. The organism was also isolated from 1.3% of non-diarrheal subjects. Since its first isolation from diarrheal stools, *C. jejuni*, which was then known as a *Vibrio* species (see taxonomy of campylobacters), has become recognized as one of the leading causes of bacterial gastroenteritis in humans (Tauxe, 1992).

Taxonomy of campylobacters.

Campylobacters were first assigned by Smith and Taylor (1919) to the genus *Vibrio* on the basis of morphological criteria. Later, Sebald and Véron (1963) suggested that *V. fetus* was different from classical *Vibrio* species based on their comparison of the DNA base compositions of several *Vibrio* species and *Vibrio fetus*. They discovered that the mol % G + C (guanine + cytosine) content of *V. fetus* was lower (mean 34.3 %, range 32-35 %) than that of other vibrios (mean 47.2 %, range 42-52 %). In addition, *V. fetus* was different from classical *Vibrio* sp. phenotypically. *V. fetus* did not ferment carbohydrates and needed a microaerobic atmosphere for growth (King, 1962). Based on these evidences, Sebald and Véron (1963) proposed the removal of *V. fetus* from the genus *Vibrio* and reclassified it as a new genus *Campylobacter* ("curved rod" in Greek), with *C. fetus* as the type species. In 1973, Véron and Chatelain published a comprehensive study of the genus, *Campylobacter* and classified it at the species level. In Bergey's Manual of Determinative Bacteriology, Smibert assigned the genus *Campylobacter* and *Spirillum* to the family of *Spirillaceae* (1984), when the phylogenetic relationships of these organisms were still largely unknown (Vandamme and De Ley, 1991). More recently, studies focusing on phylogenetic analyses of campylobacters

(Romaniuk et al., 1987; Vandamme and De Ley, 1991) have led to the revision of the taxonomy of the *Campylobacter* genus. Presently, there are 15 *Campylobacter* species and six subspecies in the family *Campylobacteraceae* (Vandamme and De Ley, 1991). They are assigned to rRNA superfamily VI (Vandamme and De Ley, 1991) which includes the genus *Helicobacter* (Goodwin et al, 1989), and a number of other taxa. In the *Campylobacteraceae* family, the most frequently identified pathogen from diarrheal cases in humans is *C. jejuni* (ACMSF, 1993; NACMCF, 1995; Tauxe, 1992). The closely related species *C. coli* causes less human disease. The relative isolation frequencies of these two species from humans with gastroenteritis has varied in different reports, but *C. coli* appears to represent between 3% (Griffiths and Park, 1990) and 10% (ACMSF, 1993) of *Campylobacter* isolates. Other species such as *C. lari* (Nachamkin et al., 1984; Tauxe et al., 1985) and *C. fetus* (Guerrant et al., 1978), which can also cause human disease, appear to do so less frequently than *C. jejuni* and *C. coli* (Table 1).

General features of campylobacters.

Campylobacters are Gram negative, spiral rods, 0.5 to 5 μm long and 0.2 to 0.9 μm wide. They are motile with a single polar flagellum at one or both ends of the cells. The cells move quickly across the microscopic field with a corkscrew-type motion. The rods have one or more spirals, and in older cultures cells may become spherical or coccoid, which are usually considered as non-viable. Growth temperatures range from 30°C to 42°C. They are oxidase positive. Carbohydrates are neither fermented nor oxidized. Their energy is taken from tricarboxylic acid cycle intermediates and amino acids. All of the campylobacters causing gastroenteritis in humans are microaerophilic

Table 1. Campylobacters of medical importance for humans

Campylobacter[a]	Known source(s)	Disease
C. fetus subsp. *fetus*	Cattle, sheep	Septicemia, gastroenteritis, abortion, meningitis
C. hyointestinalis	Pigs, cattle, hamsters, deer	Gastroenteritis
C. concisus	Humans	Periodontal disease, gastroenteritis
C. sputorum bv. sputorum	Humans, cattle, pigs	Abcesses, gastroenteritis
C. curvus	Humans	Periodontal disease, gastroenteritis
C. upsaliensis	Dogs, cats	Gastroenteritis, septicemia, abcesses
C. coli	Pigs, poultry, bulls, sheep, birds	Gastroenteritis, septicemia
C. jejuni subsp. *jejuni*	Poultry, pigs, bulls, dogs, cats, water, birds, mink, rabbits, insects	Gastroenteritis, septicemia, meningitis, abortion, proctitis, Guillain- Barré syndrome
C. jejuni subsp. *doylei*	Humans	Gastroenteritis, gastritis, septicemia
C. lari	Birds (including poultry), river water, sea water, dogs, cats, monkeys, horses, fur seals	Gastroenteritis, septicemia

[a] All campylobacters listed belong to rRNA superfamily VI, rRNA homology group I (On,1996).

(Smibert, 1984; Vandamme and De Ley, 1991; Ursing et al., 1994).

In 1994 the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Campylobacter* and Related Bacteria agreed in principle to minimum requirements (specified by phenotypic characteristics and molecular data) for the description of new species of the family *Campylobacteraceae*. This proposal is reported by Ursing et al. (1994) (Table 2).

Detection of campylobacters.

Pre-enrichment and enrichment. These steps are required for successful isolation of campylobacters from samples with low numbers of cells, such as in food, milk, or environmental samples (Griffiths and Park, 1990). Also, for samples that are heavily contaminated, such as chicken skin or sewage, Humphrey (1989) determined that pre-enrichment in a selective medium at 30 or 37°C for 2 to 3 h is advantageous. This step suppresses growth of the contaminants and allows injured cells to repair before they are submitted to higher incubation temperatures (Griffiths and Park, 1990).

Media for isolation. After the first selective isolation medium was developed by Skirrow (1977), many other formulations consisting of nutrient base, blood or charcoal, antibiotic supplements, and other selective agents (Table 3), have been suggested and used. A single selective medium can not be used to isolate all *Campylobacter* species because of their differences in resistance to antibiotics and selective agents (Griffiths and Park, 1990).

Temperature requirements. The thermophilic campylobacters *C. jejuni*,

Table 2. Phenotypic characteristics of *Campylobacter* species causing human gastroenteritis[a]

Campylobacter	Catalase	Nitrate reduction	H_2S production[b]	Hippurate hydrolysis	Indoxyl acetate hydrolysis	Growth at (°C) 15	25	42	Susceptibility to[c] Nalidixic Acid	Cephalothin	G+C content (mol%)
fetus subsp. *fetus*	+[d]	+	-	-	-	-	+	-	R	S	33-35
hyointestinalis	+	+	+	-	-	-	+	+	R	S	33-36
sputorum bv. sputorum	-	+	+	-	-	-	-	+	S	S	30-31
jejuni subsp. *jejuni*	+	+	-	+[e]	+	-	-	+	S	R	30-33
jejuni subsp. *doylei*	V	-	-	V	+	-	-	-	S	S	30-31
coli	+	+	-	-	+	-	-	+	S	R	30-33
lari	+	+	-	-	-	-	-	+	R	R	30-32
upsaliensis	W	+	-	-	+	-	-	+	S	S	32-36
concisus	-	+	+	-	-	-	-	+	R	R	37-41
curvus	-	+	+	-	+	-	-	+	S	ND	45-46

[a] Adapted from Vandamme and De Ley, 1991.

[b] On Triple Sugar Iron Agar.

[c] Susceptibility to antibiotics was determined by using 30μg discs.

[d] +, positive reaction; -, negative reaction; W, weak reaction; V, variable reaction; ND, not determined; S, susceptible; R, resistant.

[e] Hippurate negative strains have been isolated (Totten et al., 1987).

Table 3. Composition of most commonly used selective agar media for campylobacters associated with enteritis

Medium[b]	Base	Selective agent	Supplements
Skirrows	Blood Agar Base No. 2 (Oxoid CM 271)	Vancomycin 10 mg/l Polymyxin B 2500 iu/l Trimethoprim 5 mg/l	Lysed Horse Blood (50 ml/l)
Preston	Nutrient Broth No. 2 (Oxoid CM 67)	Rifampicin 10 mg/l Polymyxin B 5000 iu/l Trimethoprim 10 mg/l Cycloheximide 100 mg/l	Lysed Horse Blood (50 ml/l)
Modified CCDA	Blood Free Selective Agar Base (Oxoid CM 739)[c]	Cefoperazone 32 mg/l	
Butzler Medium Virion	Columbia Agar (Oxoid CM 331)	Cefoperazone 15 mg/l Rifampicin 10 mg/l Colistin 10 000 iu/l Amphotericin B 2 mg/l	Sheep Blood (50-70 ml/l)

[a] Adapted from Griffiths and Park, 1990.

[b] References for media: Skirrows: Skirrow, 1977; Preston: Bolton and Robertson, 1982; Modified CCDA: Hutchinson and Bolton, 1983; Butzler Medium Virion: Goosens et al., 1986)

[c]Contains the following (g/l): Nutriet Broth No 2., 25; charcoal, 4; casein hydrolysate, 3; sodium desoxycholate, 1; ferrous sulphate, 0.25; sodium pyruvate, 0.25; and agar, 12.

C. coli, *C. lari*, and *C. upsaliensis* grow at 37°C and 42°C, but not below 30°C. In contrast, *C. fetus* can grow at 25°C and 37°C, but not at 42°C. This fact has considerable importance to the food industry, because it means that the thermophilic campylobacters, which cause diarrhea, may survive, but are unlikely to grow during cold storage at 4°C (Park et al., 1991).

Other growth requirements. Growth of campylobacters requires complex media including amino acids and growth factors. Tenover and Patton (1987) reported marked growth differences between *C. jejuni* and *C. coli*, and within *C. jejuni* strains with regard to their amino acid requirements in a chemically defined medium. Most campylobacters require an atmosphere of 7-10% oxygen and 5-10% carbon dioxide to grow. This specific atmosphere and addition of 'protective' mixtures to media (Table 3) create an environment with the correct oxidation-reduction potential (Eh), which help campylobacters to maintain their viability and to grow (Park et al., 1991).

Sensitivity of campylobacters. Campylobacters are sensitive to chlorination, however, the 1-5 ppm free chlorine in the chill water used in commercial poultry processing appears to be insufficient to eliminate *C. jejuni* from chicken carcasses (Waldroup et al., 1992). Campylobacters are also sensitive to heat, and are killed by pasteurization and by the 60°C poultry scalding temperatures (Sorqvist and Danielsson-Tham, 1990). In general, campylobacters can not withstand dehydration. Doyle and Roman (1982) reported that *C. jejuni* with $>10^7$ initial population of cells in 50μl brucella broth or skim milk inoculated onto sterile glass cover slips was not recovered after 24 h incubation in anhydrous environment (relative humidity <1% obtained by anhydrous calcium sulfate) at 25°C. However, they could survive in large numbers for more than 6

weeks in appropriate humidity at 4°C. This may be one explanation why campylobacters can survive better on chicken carcass surfaces than on red meats at refrigeration temperatures.

Identification of campylobacters. The *Campylobacteraceae* family is comprised of relatively slow growing, fastidious, and biochemically unreactive species with a complex taxonomy. There are conventional phenotypic tests for species identification, where reproducibility is reported as a problem due to differences in strains, methods, varying inoculum size, age of the culture, atmospheric conditions and test medium used (Griffiths and Park, 1990). There are also other tests that are used for identification to the genus or species level, such as latex or lectin agglutination, serological tests, cellular acid profiling, protein profiling, identification by nucleic acid probes, and PCR. Since each test has its own advantages and disadvantages, one should select and use several suitable methods for a particular purpose (On, 1996).

Epidemiology of *Campylobacter* enteritis in humans.

Incidence. In the United States, the *Campylobacter* isolation rate seems to be increasing. The national *Campylobacter* surveillance reported an annual isolation rate of 5 to 6 /100,000 for the years between 1982 and 1989 (Tauxe et al., 1988). An incidence of 36 to 37.5/100,000 from a surveillance in Colorado was reported by Hopkins and Olmsted (1985). More recently, isolation rates of 14/100,000 in Georgia and 58/100,000 in California were reported by the Foodborne Disease Surveillance Network (CDC, 1996). The estimated incidence of *Campylobacter* infection appears to be 1% of the

population per year (Tauxe, 1992). In addition, *Campylobacter* infections were reported to be more common than *Salmonella* infections, if they were sought with equal frequency (Tauxe, 1992). In the future, more precise incidence reports may be available by the recently established FoodNet (CDC, 1996), which currently works as an "active" surveillance system in seven sites across the United Sates. This system will gather data from active laboratory-based surveillance, surveys of clinical laboratories, physicians and the population, and case-control studies all over the United States (CDC, 1996).

Common source outbreaks versus sporadic cases. National foodborne and waterborne disease surveillance systems reported 57 *Campylobacter* outbreaks between 1978 and 1986 (Table 4). *C. jejuni* was the indicated cause in 42 of these outbreaks (Tauxe, 1992). During 1988-1992, national foodborne disease surveillance system reported 27 *Campylobacter* outbreaks (Bean et al., 1996). There are several differences between the epidemiology of *Campylobacter* outbreaks and sporadic cases. First, the majority of the *Campylobacter* infections occur as sporadic cases, and they are poultry related (Deming et al., 1987, Graves et al., 1998; Harris et al., 1986; Hopkins and Scott, 1983). In contrast, most reported outbreaks are due to consumption of contaminated milk or water (Tauxe, 1992). The sporadic cases are not recognized by any of the surveillance systems that focus only on outbreaks, which results in underreporting of overall cases, as well as underreporting of the causative agents. Eating or cross-contamination with raw or undercooked chicken were reported as significant risk factors for *Campylobacter* enteritis in over 50% of the cases in two case control studies in Seattle, WA (Harris et al., 1986; Seattle-King County Department of Public Health, 1984) and in over 70% of the cases in another case control study in Athens, GA (Deming et al., 1987). Second, when the

Table 4. Reported common-source outbreaks of *Campylobacter* infections by vehicle of transmissions, United States, 1978 to 1986[a]

Vehicle	Number of outbreaks	Number of ill persons
Food	45	1,308
Raw milk	26	829
Poultry	3	27
Egg	1	26
Other	6	87
Unknown	9	339
Water	11	4,983
Community water supply	7	4,930
Other	4	53
Travel associated	1	150
Total	57	6,441

[a] Adapted from Tauxe, 1992.

outbreak surveillance results (1978-1986) and reports from sporadic cases are compared, a difference in their seasonal distribution is observed; outbreaks peak in May and October whereas sporadic cases peak in the summer months (Tauxe, 1992).

Reservoirs. *Campylobacter* species live as commensals in the gastrointestinal tract of a wide variety of wild and domestic animals, including cattle, sheep, swine, goats, dogs, cats, rodents, and poultry (Blaser et al., 1984). Studies also report that, several different serotypes can be present in a cattle herd (Blaser et al., 1984). Garcia et al. (1985) examined more than 500 specimens from 100 slaughtered beef cattle for *C. jejuni* and *C. coli*. Fifty animals were found positive for *C. jejuni* and one was found positive for *C. coli*. Of those animals infected, *C. jejuni* was isolated from gall bladders (33%), large intestines (35%), small intestines (31%), liver (12%), and lymph nodes (1.4%).

Risk of carcass contamination with intestinal contents in pigs is reported to be higher than in cattle, due to the special dressing procedure applied in pig slaughter. During epidemiological studies at 6 different slaughterhouses, 182 of the 300 (60.7%) samples collected from normal slaughtered pigs were found positive for *C. jejuni* (Franco and Williams, 1994). Pigs appear to carry *C. coli* more commonly than *C. jejuni*. For example, Munroe et al. (1983) reported 115 of 118 pig isolates as *C. coli*.

Domestic pets, such as healthy cats and dogs, can carry campylobacters at rates up to 45% and 49%, respectively (Bruce et al., 1980), and can apparently transmit these bacteria to humans. In a case-control study conducted by Deming et al. (1987), contact with cats was indicated as a risk factor in 30% of the cases. Laboratory animals, such as Syrian hamsters, can also carry *Campylobacter* species, but these are probably not of concern to the general public (Stern, 1992).

Campylobacter species are frequently present in the intestinal flora of commercially raised birds and wild birds such as pigeons, seagulls, crows, ravens, and quail (Shane, 1992). Chickens may harbor up to 10^6 to 10^7 CFU *C. jejuni* /g feces in their intestinal tracts without any ill effect to the host (Oosterom, 1983; Stern, 1984). Beery et al. (1988) reported that the mucus filled crypts in the cecum, large intestine, and cloaca as the principal sites of localization for *C. jejuni* in chicks.

Prescott and Gellner reported that the majority of the birds were carriers of *C. jejuni* from 28 out of 60 flocks (1984). In contrast, Stern reported that colonization prevalence could differ depending on the flock and the bird's age (1992). At present, there is cumulative evidence indicating a correlation between contamination of poultry with *C. jejuni* and gastroenteritis in humans. Studies comparing the *C. jejuni* strains originating from human infections and chickens revealed that the most prevalent serotypes in poultry were the same as or similar to the serotypes isolated from human enteritis (Munroe et al., 1983; Rosef et al., 1984; Oosterom et al., 1985).

Campylobacters are often present on retail poultry meat. Prevalence rates including fresh or frozen chicken carcasses and parts range from 0 to 100%, with a median of 62% positive (Bryan and Doyle, 1995). *C. jejuni* populations may range from $>10^3$ to 10^6 per eviscerated (Oosterom et al., 1983; Gill and Harris, 1984) and up to 10^7 (Hood et al., 1988) per uneviscerated (New York dressed) chicken carcass.

Campylobacter contamination in poultry, and modes of transmission to humans.

Fowl in hatcheries and on farms. The epidemiology and origin(s) of *C. jejuni* colonization in poultry and its mode of transmission within commercial poultry flocks is

an active research area. The factors responsible for the introduction and spread of campylobacters at the farm level were studied in turkeys (Acuff et al., 1982), in ducks (Kasrazadeh and Genigeorgis, 1987), and in chickens (Kazwala et al., 1990). Results of these studies indicate that vertical transmission of *Campylobacter* by eggs was not likely. Newly hatched turkey poults, chicks and ducklings started excreting *C. jejuni* in their feces when the first two species became 15 d (Acuff et al., 1982) to 19 d (Kazwala et al., 1990) old, and ducklings as early as fourth day of age (Kasrazadeh and Genigeorgis, 1987). All three studies indicated drinking water and containers contaminated with fecal droppings of rodents and poults, contaminated brooder house and litter, footwear and clothing of farm staff to be the likely sources for *C. jejuni* contamination.

Pre-slaughter and slaughter. Dust generated when birds are grabbed and shackled, and defecation during electrical stunning, cause contamination of feathers and skin near the cloacal opening (Patterson, 1973; Oosterom, 1983). Even if a bird or a flock is free of *Campylobacter* until slaughter, carrier birds from other flocks can cause cross-contamination during scalding, defeathering, and chilling in water tanks. Scalding water temperature is very critical for survival of *C. jejuni*. Scald baths with water temperatures of 58°C to 60°C can significantly reduce *C. jejuni* numbers (Oosterom et al., 1983; Wempe et al., 1983). After scalding, defeathering and evisceration subsequently can increase *C. jejuni* numbers on the skin (Wempe et al., 1983). During defeathering, continuously beating rubber fingers can transfer microorganisms from one bird's contaminated skin to others. Also bacteria are pushed into skin crevices and follicles (Oosterom et al., 1983). A final cross-contamination is likely to occur if carcasses are chilled by immersion chilling (Wempe et al., 1983). In contrast, if air

chilling is used, *C. jejuni* can not survive, probably due to drying of the skin surface (Oosterom et al., 1983). Finally, equipment used for weighing, segregating, holding, transporting, cutting, and any other further processes can also play a role in cross contamination (Bryan and Doyle, 1995).

Preparation and storage in kitchens. Work surfaces, utensils, and cleaning cloths come into contact with raw carcasses, with water accumulating inside fresh chicken carcasses or thaw water from frozen carcasses. If carcasses carry campylobacters, all of these can become cross-contaminated if they are not cleaned properly (Hutchinson and Bolton, 1983; De Boer and Hahne, 1990). Sufficient heating during roasting, frying and grilling can eliminate *C. jejuni* from the food (Bryan and Doyle, 1995). The cooked product should also be placed in a clean area to prevent post-contamination (De Boer and Hahne, 1990).

Clinical manifestations and infections in humans. The primary and most common clinical sign for *C. jejuni* infection is diarrhea. In industrialized nations, hospital surveys showed that approximately half of the *C. jejuni* infected patients had inflammatory and bloody diarrhea, accompanied by abdominal pain and fever (Taylor, 1992). In developing countries, the infection is most often reported as a milder, non-inflammatory, watery diarrhea (Butzler and Skirrow, 1979; Walker et al., 1986). Regardless of the pattern observed, the diarrhea is usually self-limiting, with an average duration of one week (Pitkanen et al., 1983), and does not require antimicrobial therapy.

Although rare, intestinal complications, such as appendicitis and pancreatitis, due to *Campylobacter* enteritis have been described (Pitkanen et al., 1983).

Bacteremia can be seen in malnourished and immunocompromised patients, such as patients with AIDS (Sorvillo et al., 1991). In addition, Allos and Blaser noted relapses of *C. jejuni* infections in hypogammaglobulinemic patients (1994).

Reactive arthritis has been reported after about 1% of both symptomatic and asymptomatic *Campylobacter* infections. In the majority of these cases patients were found to carry the HLA-B27 haplotype. The frequency of arthritis is uncertain and the pathogenesis is unknown (Allos and Blaser, 1994). In the last decade, several investigators have noted an association between *C. jejuni* infection and Guillain-Barré syndrome (GBS). Both serological and culture studies indicate that 20% to 40% of patients with GBS had *C. jejuni* infection 1 to 3 weeks before the onset of neurological symptoms (Kuroki et al., 1993; Mishu et al., 1993). The mechanism of the onset of GBS and the reason(s) why only some *C. jejuni* infected patients acquire it, is not clear. However, it is known that the core oligosaccharides of LPSs of certain *C. jejuni* serotypes have a structural similarity to that of ganglioside GM_1 (Prendergast et al., 1998).

***Campylobacter* pathogenesis.** There are four recognized virulence properties, which may play a role in *Campylobacter* pathogenesis: motility, adherence, invasion, and toxin production (Walker et al., 1986).

Chemotaxis and motility. Hughdahl et al. (1988) showed that *C. jejuni* is chemoattracted to mucin, L-fucose, and L-serine, and it can utilize these substances for growth. In 1992, Takata et al. indicated that chemotactic movement was important for colonization of the intestinal tract of suckling mice, after they demonstrated that flagellated but non-chemotactic strains were cleared from the intestinal tract in 48 h.

Motility was also suggested to be of significance in *Campylobacter* virulence by Black et al. (1988), after they observed that a non-flagellated *C. jejuni* strain switched to the flagellated form when passaged through human volunteers. It seems clear that *C. jejuni* colonization depend upon functional flagella.

Adhesion. Several authors have studied the role of *Campylobacter* adhesion to epithelial cell surfaces with or without subsequent invasion. De Melo and Pechere (1990) indicated that a variety of outer-membrane proteins and McSweegan and Walker (1986) suggested that lipopolysaccharide (LPS) were important in adhesion to eucaryotic cells. Doig et al. (1996) reported the production of pili by campylobacters, which was induced by bile salts in the medium. These authors reported that *Campylobacter* strains with or without pili colonized ferrets, but the strain with the pili seemed to be more virulent due to the severity of the disease it caused compared to the strain without pili (1996). The role of pili in *Campylobacter* pathogenesis has not yet been clearly established.

Invasion. Initially, inflammation and bacteremia in *Campylobacter* enteritis suggested that invasion was occurring during the disease (Blaser and Reller, 1981). Later, invasion of campylobacters was shown in in-vitro studies (De Melo et al., 1989; Everest, 1992; Grant et al., 1993). This process has not been characterized at the molecular level, and its function in pathogenesis is not yet clear.

Toxins. The production of toxin(s), and their mechanism of action by *Campylobacter* species is an active area of research.

Enterotoxin production by *Campylobacter* sp. Enterotoxin production by *C. jejuni* was first described in 1983 (Ruiz-Palacios et al.), and then confirmed by other groups (Johnson and Lior, 1984; Klipstein and Engert, 1984). These studies reported a

cell product which caused elongation in cultured Chinese Hamster Ovary (CHO) cells and intraluminal fluid secretion in the rabbit ileal loop test (RILT) model. This activity, which was detected with a GM1-based ELISA, was also reported to cross react immunologically with *Escherichia coli* heat-labile toxin (LT) and cholera toxin (CT) by the same authors. In contrast to these findings, Wadström et al. (1983) could not detect this enterotoxin activity in any of their *C. jejuni* and *C. coli* strains' sonic lysates tested on CHO and Y-1 cells, or in the RILT model. Klipstein and Engert (1984) associated these kinds of negative findings to suboptimal media and growth conditions, and to using bacterial sonicated cells in in-vitro assays. However, even in a study where care was taken to promote enterotoxin production, not of the strains was found positive for this cholera-like enterotoxin (Perez-Perez et al., 1989). In 1984, Olsvik et al. tested hybridization of *C. jejuni* DNA with degenerate gene probes for subunit A of CT, subunit B of CT, subunit A of LT and subunit B of LT, and detected no hybridization between *C. jejuni* and any of the probes. Based on this finding, Perez-Perez et al. (1992) reported that there was no homology between *C. jejuni* DNA and *E. coli* and *V. cholerae* genes encoding enterotoxin production.

It is still not clear if this activity is a non-toxic protein artifact that shares epitopes to CT, and hence is capable of binding to GM1 and cross-reacting with anti-CT antisera, or if it is expressed by a few strains, and in very restricted conditions (Ketley, 1997). Despite all the research done in this area, enterotoxin production by *Campylobacter* species remains ambiguous and its importance in pathogenicity remains unproven.

Cytotoxin production by *Campylobacter* species. In 1983, Wong et al. (1983) reported a heat and trypsin sensitive "cytopathic effect" on HeLa, MRC and HEp-2 cells

by *C. jejuni* isolates from acute gastroenteritis and septicemia. This finding was followed by similar reports from various researchers. For example, Johnson and Lior (1986) described an activity in *C. jejuni* culture filtrates that caused cytolethal effects on CHO, Y-1 and Vero cells. One year later, Guerrant et al. (1987) reported that five out of 12 *C. jejuni* culture filtrates caused death in HeLa and CHO cells. This cytotoxic effect was not neutralized with anti-Shiga-like toxins I and II (SLT I and SLT II) or with anti-*Clostridium difficile* toxins A and B (Guerrant et al., 1987). No further information is available about any of these activities.

In 1988, Moore et al. reported the detection of a cytotoxic activity neutralized by anti-B subunit of *E. coli* SLT I in only approximately 1/3 of the *Campylobacter* isolates they tested. Based on their hybridization studies, they concluded that some *Campylobacter* isolates produce low levels of SLT I which is genetically distinct from the *E. coli* SLT I. No other papers describing this activity have appeared.

In addition to all these toxic activities, a hemolytic activity (Arimi et al., 1990; Pickett et al.,1992), which was observed in aging *Campylobacter* cultures, and a hepatotoxic activity observed in mice (Kita et al.,1990 and 1992) have been reported.

Cytolethal distending toxin (CDT). CDT is the only well documented of the toxins attributed to *Campylobacter* species. This toxic activity was first described in several *Campylobacter* species by Johnson and Lior (1988) and was named CLDT. CDT appears to have replaced CLDT in more recent reports. CDT causes cellular distention, and ultimately death, in several cultured cell lines such as CHO, Vero, HeLa and HEp-2 cells. CDT is immunologically different from the so called cytotoxin, since the antiserum raised against this cytotoxin did not neutralize CDT (Johnson and Lior, 1988).

The *C. jejuni cdt* genes have been cloned and sequenced by Pickett et al. (1996). Some *E. coli* isolates (Comayras et al., 1997; Johnson and Lior, 1987; Johnson and Lior, 1988; Peres et al., 1997; Pickett et al., 1994; Scott and Kaper, 1994), *Shigella* sp. isolates (Johnson and Lior, 1987; Okuda et al., 1995; Okuda et al., 1997), *Haemophilus ducreyi* (Cope et al., 1997), *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) (Sugai et al., 1998) are also known to possess *cdt* genes. In all cases there are three adjacent or slightly overlapping genes, *cdtA*, *cdtB* and *cdtC*, the expression of all of which is apparently required for CDT activity (Okuda et al., 1997; Pickett et al., 1994; Scott and Kaper, 1994; Sugai et al., 1998). CdtA, B and C of *C. jejuni* strain 81-176 CDT have predicted sizes of 30,116, 28,989, and 21,157 Da, respectively (Pickett et al., 1996). The predicted amino acid sequences for the three Cdt proteins indicate that considerable amino acid sequence divergence has occurred, particularly within the CdtA and CdtC sequences. For example, the predicted amino acid sequences of CdtA proteins from *E. coli* strain 9142-88 and *C. jejuni* strain 81-176 are only 34% similar (21% identical and 13% conserved amino acids [Pickett et al., 1996]). In addition, Cdt proteins from isolates from the same species may not be closely homologous, since the predicted amino acid sequences of the CdtA proteins from *E. coli* strains 9142-88 and E6468/62 are only 53% similar (37% identical and 17% conserved amino acids [Pickett et al., 1994; Scott and Kaper, 1994]).

In hybridization studies, Pickett et al. (1996) tested the degree of similarity of *C. jejuni* and *C. coli cdtB* genes within and between species, by using *cdtB* probes from each species. Their results indicated that there is a distinct species divergence in this toxin gene, since *C. jejuni* and *C. coli cdtB* probes hybridized well only to the species for

which they were specific, and hybridized poorly to the other species. A putative *cdtB* gene was also detected by PCR in single strains of *C. jejuni*, *C. lari*, *C. upsaliensis*, *C. hyointestinalis*, *C. coli*, and *C. fetus* (Pickett et al.,1996).

Pickett et al. (1996) also tested the CDT activity of sonic lysates from a variety of *C. jejuni* and *C. coli* strains in HeLa assays. Their results showed that all 20 *C. jejuni* strains produced CDT, but that the 12 *C. coli* strains appeared to produce little or no CDT in this assay (Pickett et al., 1996). This indicates that while *cdt* genes may be present in many *C. jejuni* and *C. coli* isolates, they may not show similar CDT activities in HeLa assays.

Role of CDT in pathogenesis. In 1997, Okuda et al. showed that a CDT produced by *Shigella dysenteriae* is capable of causing diarrhea in a suckling mouse model. Apart from this single report, all reported studies investigating host-CDT interactions are based on in-vitro cell culture assays. Recent studies on *E. coli* CDT showed that this toxin stopped CHO cell division (Aragon et al., 1997), and blocked HeLa cell division in the G_2/M phase of the cell cycle (Peres et al., 1997). Whitehouse et al. (1998) reported that *C. jejuni* CDT causes HeLa and Caco-2 cells to become blocked in the G_2 phase of their cycle. The role of CDT in the disease process has not yet been defined. More recently, Sugai et al. (1998) demonstrated a similar growth arrest at the G_2/M phase of HeLa cells treated with CDT produced by *A. actinomycetemcomitans*.

MATERIALS AND METHODS

Campylobacter strains.

All of the *C. jejuni* and *C. coli* strains used in this study are listed in Table 5. Some of these strains have been reported elsewhere: *C. jejuni* strains; 79-193 by Moore et al. (1988), and Pickett et al. (1996); 81-176 by Black et al. (1988); Korlath et al. (1985), and Pickett et al. (1996); 81-361 by Perez-Perez et al. (1989); 84-142 by Pickett et al. (1996); 85-360 by Perez-Perez et al. (1989) and Pickett et al. (1996); 85-452 by Hariharan and Panigrahi (1990), and Pickett et al. (1996); C31 by Guerrant et al. (1987), Perez-Perez et al. (1989) and Pickett et al. (1996); G13 by Pickett et al. (1996); D1816 by Perez-Perez and Blaser (1985), and Pickett et al. (1996); D133 by Pickett et al. (1996); Lior 19 by Lior et al. (1982). *C. jejuni* isolates CN593, W91, and SP923.1 were kindly provided by N. J. Stern. *C. jejuni* human stool isolates 79-101, 79-445, 81-160, 85-374 and *C. coli* D220, a cow isolate were kindly provided by M. J. Blaser.

C. coli strains 43473, 43482, 43485, 43488, D730, D2591,D2593, D2594, D2598, D1821, D115 and D126 were reported by Pickett et al. (1996).

All other *C. jejuni* and *C. coli* strains were isolated from chickens during the course of this study (Table 5).

Poultry samples.

Ninety-one fresh chicken carcasses were purchased from four local supermarkets in June, July, and August of 1996 and 1997. Each chicken carcass was put into a separate plastic bag and transferred to the laboratory in a cooler within one hour of purchase.

Table 5. Sources of the *C. jejuni* and *C. coli* strains used in this study

Source	Organism	
	C. jejuni	*C. coli*
Human Stool	79-101, 79-193, 79-445, 81-160, 81-176, 81-361, 84-142, 85-452, 85-374, C31, G13	43473, 43482, 43485, 43488, D730, D2593, D2598
Blood	85-360	D2591, D1821, D2594
Spinal fluid	D1816	
Cow	D133	D115, D220
Sheep		D126
Chicken Stool	Lior 19, W91, CN593, SP923.1	
Intestine mash	AE216	AE219, AE220, AE235, AE236, AE239, AE241
Carcass[a]	AEB966, AEB968, AEB9720, AED9711S, AEB969, AEB9610, AED9712, AED9716L, AED961, AED962, AED9717S, AED9717L, AED964, AED965, AED9718, AED9719, AED966, AED967, AED9720, AED9721, AED968, AED9610, AED9722, AED9723, AEA961, AEA962, AED9724, AED9725, AEA963, AEA965, AED9726, AED9727, AEA967S, AEA967L, AED9728, AEB9721S, AEA968S, AEA969, AEB9723, AEB9724S, AEA9610L, AED972, AEB9724L, AEB9725, AED974, AED977, AEB9726, AEA971, AED978, AED979, AEA972S, AEA972L, AEB975, AEB9710, AEA973S, AEA974S, AEB9711, AEB9712S, AEB9715S, AEB9716S, AEB9713S, AEB9714, AEB9718, AEB9719, AEB9716L, AEB9717, AEA975	AEB961, AEB962, AEB963, AEB965, AED963, AED969, AEA964, AEA966, AEA968S, AEA9610S, AED971, AEB971, AEB972, AEB973, AEB974, AEB976, AEB977, AEB978, AEB979, AEC971, AEC972, AEC973, AEB9712L, AEB9713L, AEB9715L, AED9711L, AED9713, AED9714, AED9715, AED9716S, AEB9721L, AEB9722S, AEB9722L, AEB9727

[a] Chicken carcass strains were named according to these guidelines: First 2 letters are initials of the individual; 3rd letter is a code for the 4 supermarkets; first and second number is year designation; third and/or fourth numbers are sequential numbering of the isolates; S (small, round) and L (large, swarming) designate the colony appearance differences.

Antibiotic solutions.

Sodium cefoperazone (C-4292, Sigma, 4 g/l). Sodium cefoperazone (0.4 g) was made up in 100 ml distilled water in a volumetric flask. The solution was filtered through a 0.45 μm filter and stored a maximum of 5 months at -70°C in plastic tubes.

Trimethoprim lactate (T-0667, Sigma, 3.75 g/l). Trimethoprim lactate (0.375 g) was made up in 100 ml distilled water in a volumetric flask, and prepared as above. This antibiotic solution was stored a maximum of one year at 4°C.

Vancomycin HCl (86,198-7, Aldrich, 2.5 g/l). Vancomycin HCl (0.25 g) was made up in 100 ml distilled water, prepared as above, and stored a maximum of 2 months at 4°C.

Amphotericin B (A-4888, Sigma). Amphotericin B (50 mg) was made up in 100 ml distilled water in a volumetric flask, prepared as above to a concentration of 0.5 g/l, and stored a maximum of one year at -20°C.

Isolation agars. Modified Campylobacter Blood-free Selective Agar Base (CCDA) with designated antibiotics was used as a primary selective medium after enrichment. Abeyta-Hunt Agar (AHA) with designated antibiotics and blood was used as a primary medium for subculturing and purification of the isolates. CCDA is comprised of CCDA agar base (Oxoid), 42.5 g; yeast extract, 2.0 g; distilled water, 1000 ml. A final pH of 7.4 ± 0.2 was obtained. After the medium was autoclaved for 15 min at 121°C and cooled to 55°C, sodium cefoperazone and amphotericin B were added for final concentrations of 32 mg/l and 2 mg/l, respectively. AHA consists of Heart infusion broth (Difco), 25 g; Bacto agar, 15 g; yeast extract, 2 g; and distilled water, 950 ml. The

medium was prepared as CCDA, and lysed horse blood was added to a final concentration of 5% in addition to the antibiotics designated for CCDA.

Storage medium. This medium was used to store the *Campylobacter* cultures for long time periods (3-4 years) at -80°C. Twenty-eight grams of Brucella broth (Difco) was dissolved in 1000 ml distilled water. The pH was adjusted to 7.4 ± 0.2 and the medium autoclaved as described previously. Two milliliters of Brucella broth was used to suspend overnight grown *Campylobacter* cultures on Brucella agar plates, and 0.7 ml of this suspension was mixed with 0.3 ml of 50% sterile glycerol (final concentration of 15%) in sterile, small, screw-cap, glass vials and stored at -80°C.

Maintenance medium. Once the *Campylobacter* isolates were purified, Brucella agar was used for routine growth of the cultures, such as streaking of the culture from -80°C stock, subculturing for confluent growth for DNA isolation, HeLa assays, and for phenoptypic identification by API Campy. Brucella agar consists of Brucella broth, 28 g; Bacto agar, 15 g; and 1000 ml distilled water. The pH was adjusted and the medium was autoclaved as described previously. The medium was cooled to 55°C, and when necessary, selective antibiotics were added to the following final concentrations: cephalothin, sodium salt (C-4520, Sigma), 15 μg/ml; vancomycin hydrochloride (86,198-7, Aldrich), 10 μg/ml; trimethoprim (T-7883, Sigma), 5 μg/ml.

Media used in HeLa cell assays for CDT activity. Incomplete Minimal Essential Medium (MEM) consists of minimal essential medium Eagle (Modified), and Earle's salts, but does not include L-glutamine 1X (Mediatech, VA). Complete MEM was prepared by adding 100 ml fetal bovine serum, 10 ml L-glutamine in 0.85% saline (200 mM or 29.23 mg/ml), 2 ml gentamicin (gentamicin sulfate, Gibco BRL; 50 mg/ml),

5ml penicillin (10,000 U/ ml) / streptomycin (10,000 U/ ml) (Hazleton, 17602A) to 1 liter of MEM (Modified) with Earle's salts in sterile conditions.

HeLa cell procedures.

Routine maintenance of HeLa cell cultures. In a biosafety cabinet, medium was decanted from a HeLa cell culture flask (75 cm^2) and 1 ml of trypsin solution (2.5% in modified Hank's balanced salt solution without magnesium and calcium) was added. The flask was incubated at 37°C for 4 min., removed from the incubator and shaken to loosen the cells, and then reincubated at 37°C for an additional 3 min. Ten milliliters of complete MEM was added to the flask and the HeLa cells were carefully suspended. This suspension was transferred to a 50 ml centrifuge tube, and centrifuged at 1000 rpm (DAMON IEC Centrifuge, level #3) at 25°C for 3 min. The supernatant was decanted and the pellet was resuspended in 6 ml of complete MEM. Twenty-five milliliters of complete MEM was added to a tissue culture flask (75 cm^2, 200 ml). It was inoculated with 50-100 µl of the HeLa cell suspension, and incubated at 37°C for 3-7 days in a 5% CO_2 atmosphere.

Preparation of HeLa cells for toxin assays. Cells were prepared as above and then counted. One hundred microliters of the original HeLa cell suspension was added to 900 µl of complete MEM. One hundred microliters of this 1:10 dilution was transferred to a tube containing 100 µL of a 0.1% trypan blue solution in saline. Both sides of a Neubauer chamber were filled with this mixture and the non-stained cells were counted in 5 squares of each side. The dilution necessary to obtain a suspension with a concentration of 16,667 cells/ml was calculated by the following formula:

$$V = \frac{V(f) \times C(d)}{\frac{N(c) \times DF \times VF}{10}} = \frac{V(f) \times 16{,}667 \text{ cells/ml}}{\frac{N(c) \times 20 \times 10{,}000}{10}}$$

V = volume of the HeLa cell suspension

V (f) = final volume desired (10 ml/ microplate)

C (d) = desired final HeLa cell concentration (16,667 cells/ml)

N (c) = number of HeLa cells counted

DF = dilution factor (20)

VF = volume factor (10,000)

The "V" of the HeLa cell suspension was diluted in "V(f)" of complete MEM and 100 µL of this suspension was dispensed to each well of a 96-well tissue culture microplate. The microplate was incubated overnight at 37°C in a 5% CO_2 atmosphere. This seeding was very sparse so that the cells would not form a monolayer within the next two days of incubation.

Toxin assay.

CDT titers were determined by the HeLa cell assays as described by Pickett et al. (1994). A *Campylobacter* culture grown at 42°C for 16-18 h on a single 10 cm agar plate was harvested into 2 ml brucella broth. The suspension was transferred into

polystyrene tubes and pelleted at 1800 x *g* for 10 min at 25°C. The supernatant was discarded, and the pellet was resuspended in 0.3 ml incomplete MEM with 0.2 % gentamicin sulfate (50 mg/ml, Gibco BRL). At this step, the absorbance at A_{600} was read from a 1:10 dilution of the culture. The culture was then sonicated twice for 30 sec. The debris was pelleted at 1800 x *g* for 15 min at 25°C and 50 μl from each supernatant was assayed. Two-fold serial dilutions of this supernatant aliquot were prepared in complete MEM in a 96 well cell culture plates (The initial dilution was 1:4 [50 μl aliquot into 150 μl MEM]). One hundred microliters of each dilution was applied to 18-hour old HeLa cells prepared as above. The results were evaluated after microaerobic incubation (5% oxygen, 10% carbon dioxide, 85% nitrogen) at 37°C for 48 h. Each strain was assayed in at least three independent assays. The toxin titer was calculated by dividing the reciprocal of the highest lysate dilution that caused at least 50% of the HeLa cells in a given well to be distended by the A_{600} of the resuspended bacteria prior to sonication. Distention of all the cells were observed until a well after which approximately 75, 50 and 25% of the HeLa cells in the next 3 consecutive wells were distended. The middle well of these 3 wells (showing 50% distention) was chosen to be the end point of the reading and designated as the well from which the titer was going to be calculated from. The titers were expressed as the geometric mean and the standard deviation of the mean.

Isolation of *Campylobacter* species.

The isolation procedure for *Campylobacter* from whole chicken carcasses was based on the method described by Hunt and Abeyta (1995), except that only CCDA was used as a primary isolation medium in the major part of the study and the plates were

incubated at 42°C in microaerobic incubators. The method described by Hunt and Abeyta (1995) uses both CCDA and AHA for primary isolation medium and both plates are incubated microaerobically at 37°C.

Two hundred milliliters of 0.1% sterile peptone water was added to the sterile polyethylene bag in which the chicken carcass had been placed, the bag was sealed, and the chicken hand-massaged for 3 min. The rinse solution was then poured through a sterile cheese cloth, and centrifuged at 16,000 x *g* for 15 min. The supernatant was discarded and the pellet was suspended in 12 ml 0.1% peptone water. For preenrichment, 3 ml of this suspended pellet was transferred to 100 ml of Hunt broth and incubated microaerobically with agitation (200 rpm) at 30°C for 3 h, and then without agitation, at 37°C for 2 h. This was followed by a 24 h microaerobic enrichment at 37°C in the same flask. Fifty microliters of sample was streaked onto CCDA, and incubated microaerobically at 42°C for 24-48h. The plates were then observed for *Campylobacter* colonies and assesed by Gram stain. Curved, gull-wing shaped, Gram negative rods were identified as probable *Campylobacter* species. One representative colony was picked from the CCDA plates where one colony type was observed for *Campylobacter* and streak-purified on AHA 2 to 4 times depending on the amount of apparent contamination with enteric bacteria present on the isolation plate. In several instances, two different colony types were observed in the primary isolation plates. In those instances, colonies representative of both types were picked and streak-purified. The purified cultures were then used in catalase and oxidase tests, for biochemical identification, and to prepare storage cultures as described above.

Identification of *Campylobacter* species.

Species identification of the chicken isolates was performed by using both API Campy (Bio-Merieux Ltd., Marcy l'Etoile, France), and by a species specific polymerase chain reaction (PCR).

Identification by API Campy. API Campy is a manual test kit in a test strip format that includes 20 selected biochemical tests optimized for the identification of *Campylobacter* species. The test strip has 20 microtubes containing dehydrated substrates divided into two parts. The first group of tests identifies the presence of selected enzyme activities or the presence of certain metabolic end products. The second group of tests is conducted by inoculation of a minimal medium (AUX, see below) and incubation in microaerobic conditions. *Campylobacter* species grow if they are capable of utilizing the corresponding substrate or if they are resistant to the antibiotic tested. After appropriate incubation times and temperatures (see below), the reactions are read according to a table and the identification is obtained using the API Campy Analytical Profile Index or the identification table.

Compositions of the sterile media and the reagents. NaCl Medium (0.85%) consists of sodium chloride, 8.5 g; demineralized water, qsp (quantum sufficit-sufficient quantity) 1000 ml. AUX Medium is comprised of ammonium sulphate, 2 g; mineral base, 83 mg; amino acids, 250 mg; vitamins and nutritional substances, 36 mg; agar, 1.5 mg; phosphate buffer, 0.04 M; qsp 1000 ml, with a final pH of 7.1. NIT1 reagent consists of sulphanilic acid, 0.8 g; acetic acid, 5N; qsp 100 ml. NIT2 reagent consists of N-N-dimethyl-1-naphthylamine, 0.6 g; acetic acid, 5N; qsp 100 ml. FB reagent is

comprised of fast blue BB, 0.35 g; organic solvants, qsp 100 ml. NIN reagent consists of ninhydrine, 7 g; 2-Methoxyethanol, qsp 100 ml.

Test procedure. For identification with API Campy, a pure culture of the strain to be tested was grown confluently on brucella agar. The two test strips were prepared according to the supplier's instructions. The inoculum was prepared as follows: The culture was harvested from the plate with a sterile swab, and suspended in the NaCl solution. A turbidity equivalent to 6 McFarland was obtained in this suspension by comparing it to the turbidity control included in the kit. One hundred microliters of this prepared inoculum was distributed to tests URE (urease prduction) to PAL (alkaline phosphatase production) of the first strip, and test H_2S of the second strip. The URE test was overlayed with mineral oil. The first strip was incubated at 37°C for 24 h under aerobic conditions. All the remaining bacterial suspension in the NaCl medium was transferred into an ampoule of AUX medium and mixed well. The new suspension was distributed into the tubes and cupules of GLU (glucose utilization) to ERO (erythromycin sensitivity) of the second test strip, and incubated microaerobically for 24 h at 42°C. The results from the first strip were read after adding the appropriate reagents into designated test cupules, and by referring to the reading table included in the procedure booklet. The second strip including all the assimilation tests was read after 24 h, if the SUT (succinate utilization) test was positive. If this test was negative, the second strip was reincubated for 24 h before the final reading. The results were recorded and identification was obtained with the API Campy Analytical Profile Index (API). To do this, the reactions were coded into a numerical profile as follows: On the results sheet, the tests are separated into groups of three and a number, 1, 2 or 4, is indicated for each. By adding

the numbers corresponding to positive reactions within each group, a 7-digit numerical profile was obtained. This numerical profile was then searched in the API, in order to identify the genus and/or species of the isolate.

PCR procedures.

Introduction. PCR is the selective amplification of a chosen region of a DNA molecule, where two short oligonucleotides hybridize to the borders of the target DNA, one to each strand, and prime DNA synthesis by a thermostable DNA polymerase. In order to successfully amplify the target DNA sequence, a series of experiments were to be performed in order to optimize the reaction. Primer and template concentrations were optimized, and different cycle parameters were tested. After PCR, a small sample of the reaction mixture is analysed by agarose gel electrophoresis. All PCR reactions were performed in 650µl clear graduated microtubes (MH-805, Phenix, Hayward, AZ) by using Perkin Elmer DNA Thermal Cycler Model 480.

Colony PCR. This method utilizes one or more colonies from a pure bacterial culture as template in PCR. The primary aim of colony PCR is to eliminate the chromosomal DNA isolation step prior to PCR. In order to enable reaction components and DNA polymerase to react freely with the DNA template, colonies were lysed by boiling.

Initially, colony PCR was tested in the species identification of *Campylobacter* isolates, as well as in *cdt*-specific PCR. In the beginning of the studies, one colony (approximately 1 mm in diameter) from an overnight grown plate culture was used as the source of template in PCR reactions. Various boiling times (10, 15, 20, 30 min at 95°C) and two different template DNA boiling techniques were tested for cell lysis. For

example, in some cases, one colony was boiled in PCR reaction components without DNA polymerase, while in other cases the colony was boiled in water. When colonies were boiled with PCR reaction components, the tube was spun briefly and then DNA polymerase was added. When colonies were lysed in water, the tube was spun briefly to pellet the cell debris, and 1µl from the fluid overlying any pellet was used as the source of template DNA. Different primer volume to cell suspension volume ratios were tested. In the attempt to develop colony PCR, all of the parameters mentioned above were not tested for each primer pair. Specifics about particular PCRs will be mentioned under each relevant section.

PCR reagents. PCR reagents including nucleotides, *Taq* poymerase and 10x *Taq* polymerase buffer were obtained from Perkin Elmer (Norwalk, Conn.). All primers were supplied by Integrated DNA Technologies, Inc. (Coralville, Iowa). The template was either chromosomal DNA from *Campylobacter* isolates or specially prepared suspensions of *Campylobacter* cells as described above.

Species specific PCR. In this study, 3 *C. jejuni*-specific and 2 *C. coli*-specific primer pairs were tested for their ability to unequivocably distinguish *C. jejuni* from *C. coli*. These primer pairs were chosen from the available literature based on their reported successes in identifying campylobacters at the species level. Initially, primer sets C1/C4 (Winters et al., 1995) and Jej1/Jej2/Therm3 and Therm1/ Coli (Eyers et al., 1993) were tested. These primers proved unsatisfactory, which will be discussed later, and ultimately the primers VS15/VS16 (Stonnet and Guesdon, 1993) and CSF/CSR were used (Stonnet et al., 1995).

Table 6. Species specific PCR primers, and their nucleotide sequences used in this study

Primer[a]	Nucleotide sequence (5'→3')
C. jejuni specific	
C1[b]	CAAATAAAGTTAGAGGTAGAATGT
C4[c]	GGATAAGCACTAGCTAGCTGAT
Therm3[b]	TAAAGTAAGTACCGAAGCTG
Jej1[c]	GTAAATCCTAATACAAAGCT
Jej2[c]	TAAATCCTAGTACGAAGCT
VS15[b]	GAATGAAATTTTAGAATGGG
VS16[c]	GATATGTATGATTTTATCCTGC
C. coli specific	
Therm1[b]	TATTCCAATACCAACATTAGT
Coli[c]	TAAATCCTAATACGAAGCCG
CSF[b]	ATATTTCCAAGCGCTACTCCCC
CSR[c]	CAGGCAGTGTGATAGTCATGGG

[a] References for the primers: C1/C4, Winters et al., 1995;
Therm3/ Jej1/ Jej2 and Therm1/ Coli, Eyers et al., 1993;
VS15/VS16, Stonnet et al., 1995 and CSF/CSR, Stonnet et al., 1993.

[b] Forward primer

[c] Reverse primer

Primers C1 and C4. These primers were reported to be specific for *C. jejuni* strains (Winters et al. 1995). They are based on a sequence from a specific probe for *C. jejuni* (Wang et al., 1992) (Table 6). For colony PCR with C1 and C4, all PCR reactions contained 0.2 mM (each) dATP, dCTP, dGTP, and TTP, 1.5 mM $MgCl_2$, 1x *Taq* DNA polymerase buffer and 0.25 μM of each primer in a 650 μl polypropylene Eppendorf tubes. One isolated colony from an overnight grown *Campylobacter* plate culture was inoculated into the PCR reaction tube, and the tube was heated at 95°C for 10 min. The tube was allowed to cool, spun briefly, and 2.5 U of *Taq* polymerase was added. Parameters for all reactions were initial incubation of 95°C for 3 min, and then 40 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min, followed by one incubation of 72°C for 3 min.

Primers Jej1, Jej2 and Therm3. These *C. jejuni* specific primers are based on *Campylobacter* 23S rRNA genes (Eyers et al., 1993) (Table 6). PCR reactions using chromosomal DNA as template contained 0.2 mM (each) dATP, dCTP, dGTP, and TTP, 1.5 mM $MgCl_2$, 1x *Taq* DNA polymerase buffer, 0.25 μM of each primer, 0.25 μg of template DNA, and 2.5 U of *Taq* polymerase in total reaction volumes of 20 μl or 50 μl. Parameters for all reactions were 27 cycles of 94°C for 1 min, 54°C for 1 min, 74°C for 1 min. For colony PCR, one isolated colony from an overnight plate culture was inoculated into the PCR reaction tube. Ten, 15, and 20 min heating times at 95°C were used on different days. After the tube cooled, it was spun briefly, and 2.5 U of *Taq* polymerase was added.

Primers Therm1 and Coli. These primers are based on the 23S rRNA genes of *Campylobacter* strains and were reported to specifically amplify rRNA gene sequences

from *C. coli* strains (Eyers et al., 1993) (Table 6). PCR reactions, with chromosomal DNA or in colony PCR, were as described for the primers Jej1, Jej2 and Therm3.

Primers VS15 and VS16. These primers are based on two oligonucleotides in a 1189 bp DNA fragment, VS1, which was isolated from a *C. jejuni* cosmid library and which was found to contain regions specific for this bacterial species (Collection de l'Institut Pasteur, Paris, France) (Stonnet and Guesdon, 1993) (Table 6). All PCR reaction mixtures were as described previously. Parameters for all reactions were 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min as recommended by the authors. Colony PCR was performed as described for primers Jej1, Jej2 and Therm3.

Primers CSF and CSR. These primers were reported to specifically direct the amplification of a DNA fragment from the *C. coli* genome and from no other bacterial species (Stonnet et al., 1995) (Table 6). All PCR reaction mixtures were as described previously. Parameters for all reactions were 40 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min again, as recommended by the authors. For colony PCR, the procedure was as described for the primers Jej1, Jej2 and Therm3.

DNA isolation. Total bacterial cell DNA was isolated from *Campylobacter* strains in the collection of Dr. Pickett either by the method described by Silhavy et al. (1984), or by using the QIAamp Tissue Kit according to manufacturer's specifications (Qiagen, Santa Clarita, Calif.). The QIAamp Tissue Kit was also used to isolate total bacterial cell DNA from all *C. jejuni* and *C. coli* strains isolated from chicken carcasses during the course of this work.

Large scale chromosomal DNA isolation. A *Campylobacter* strain, from which the chromosomal DNA would be isolated, was grown overnight, microaerobically at 42°C on 8 plates of brucella agar. The cells were harvested in 2 ml brucella broth per plate, and suspensions belonging to the same strain were pooled. The cells were centrifuged at 5520 x *g* for 10 min at 4°C and resuspended in a solution comprised of 2.5 ml 25% glucose, 50 mM Tris-HCl (pH 8.0), and 50 mM sodium EDTA. Then, 2.5 ml of the same solution containing 2 mg/ml lysozyme was added to the resuspended cells. The cells were put on ice for 30 min, after which 0.5 ml of 10% sodium dodecyl sulfate was added, and the suspension mixed by inversion. The suspension was then incubated at 56°C for 15 min after 100 μl of 2 mg/ml proteinase K was added. This step was followed by two successive phenol extractions. The DNA was subsequently precipitated by adding two volumes of cold 95% ethanol and removed with a sterile, glass pasteur pipette. The precipitated DNA was dissolved in 1.0 ml 1X TE (10 mM Tris-HCl [pH 8.0], 1 mM sodium EDTA) and RNAse added to a final concentration of 50 μg/ml and incubated at 37°C for 1 h. This step was followed by at least two phenol-chloroform extractions, and then a final chloroform- isoamyl alcohol extraction. DNA was precipitated by adding one-tenth volume 3 M sodium acetate (pH 4.8), and two times the new volume of cold 95% ethanol. Finally, the DNA was removed with a sterile, glass pasteur pipette and dissolved in 1.0 ml 1 X TE.

Total bacterial cell DNA isolation by QIAamp tissue kit. The kit directions were followed. All the buffers used in this procedure were supplied with the QIAamp tissue kit. A loopful of bacteria grown on a single brucella agar plate was removed and suspended in 180 μl Buffer ATL by vigorous stirring. Twenty microliters of proteinase

K stock (17.86 mg/ml) solution was added, mixed by vortexing, and incubated at 55°C until the cells were completely lysed. Then, 20 μl of RNase (20 mg/ml) was added, mixed and incubated for 2 min at room temperature. Two hundred microliters of Buffer AL was added to the sample, and incubated at 70°C for 10 min. After adding 210 μl ethanol (95 %) and vortexing, the sample was applied into a QIAamp spin column which was placed on a 2 ml collection tube. The spin column was centrifuged at 6000 x *g* for 1 min and placed in a clean collection tube, and the tube containing the filtrate was discarded. The spin column was opened, 500 μl of Buffer AW was added, and centrifuged at 6000 x *g* for 1 min. This step was repeated once and finished by centrifuging the column at 10.500 x *g* for 2 min. The DNA was eluted twice with 200 μl of 70°C Buffer AE, by incubating at room temperature for 1 min, then centrifuging at 6000 x *g* for 1 min.

PCR for the detection of *cdt* genes.

All PCR reactions contained 0.2 mM (each) dATP, dCTP, dGTP, and TTP, 1.5 mM $MgCl_2$, 1x Taq DNA polymerase buffer, 0.25 μM of each primer, 0.25 μg of template DNA, and 2.5 U of *Taq* polymerase. Parameters for all reactions were 30 cycles of 94°C for 1 min, 42°C for 2 min, 72°C for 3 min. The PCR primers which were tested for their performance in detecting *cdt* genes in *Campylobacter* isolates are listed in Table 7a. The degenerate primers VAT2 and WMI1 (Fig 1) were described by Pickett et al. (1996). Both of these primers are based on highly conserved regions of the *cdtB* genes of *E. coli* 9142-88 (Pickett et al., 1994) and E6468/62 (Scott and Kaper, 1994) (Table 7a). The degenerate forward primers, A-IWG, A-IDS1, A-GNW, and A-IVH and the exact

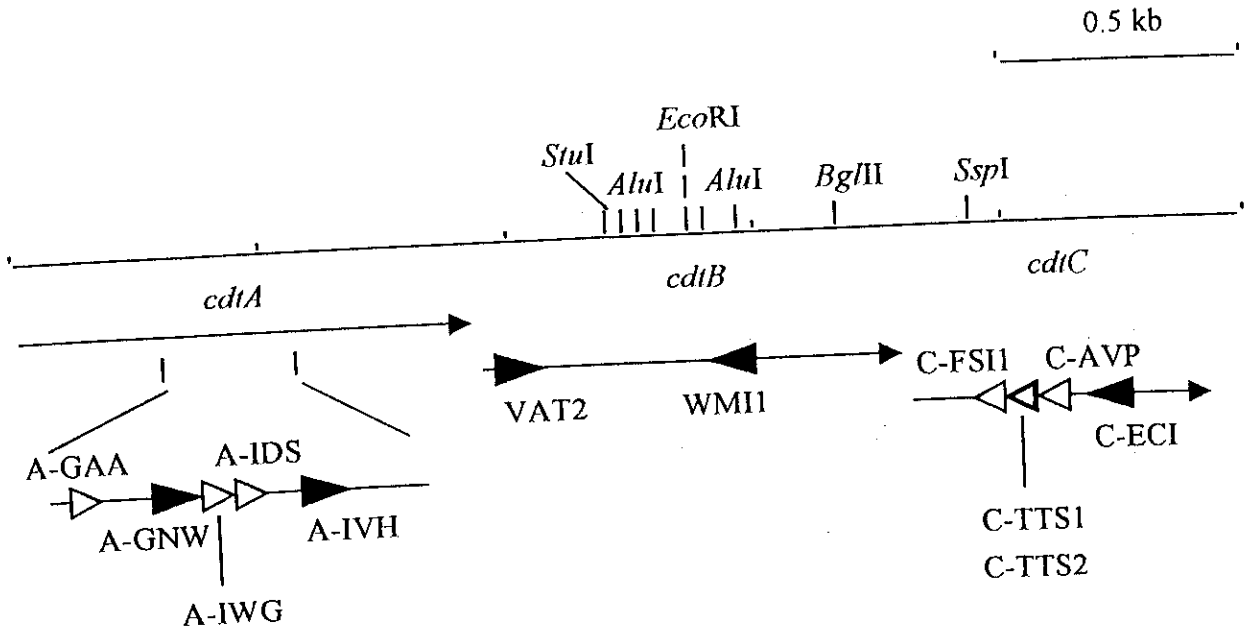

Figure 1. A partial restriction map of the *C. jejuni* 81-176 *cdt* genes and the location of the PCR primers used in this study. Arrows with small, black heads indicate the size and direction of transcription of the *cdt* genes. Large, black arrowheads indicate the location and priming direction of the primers found useful in detecting *cdt* genes. White arrowheads indicate the location and priming direction of other primers tested in this study.

Table 7a. *cdt*-specific PCR primers and their nucleotide sequences used in this study

Primer	Nucleotide sequence (5'→3')
Forward primers	
VAT2	GT(ACGT) GC(ACGT) AC(ACGT) TGG AA(CT) CT(AGCT) CA(AG) GG
A-IWG	AT(ACT) TGG GG(ACGT) TA(CT) AC(ACGT) TT(AG) AT(ACT) GA(CT)
A-IDS	AT(ACT) GA(CT) AG(CT) AA(AG) GG(ACGT) TT(CT) GG
A-GAA	GGA GCA GCT TTA ACG GTT TGG GC
A-GNW	GG(ACGT) AA(CT) TGG AT(ACT) TGG GG(ACGT) TA
A-IVH	AT(ACT) GT(ACGT) CA(CT) TA(CT) CC(ACGT) TG(CT) GA
Reverse primers	
WMI1	(GA)TT (GA)AA (GA)TC (AGCT)CC (TC)AA (TGA)AT CAT CC
C-ECI	(AG)TT (AG)AA (AG)AA (ACGT)GT (ACGT)AC (AGT)AT (AG)CA (CT)TC
C-AVP	CTA AAG AAC GAA TTT GCA CAG C
C-FSI1	GT(ACGT) GT(ACGT) GGC AT(AGT) AT(AG) CA(AG) AA
C-TTS1	(AGT)AT (CT)TG (ACGT)AC (ACGT)GC (AG)TC (ACGT)GT (ACGT)GT
C-TTS2	(AGT)AT (CT)TG (ACGT)AC (ACGT)GC (ACGT)GA (ACGT)GT (ACGT)GT

forward primer A-GAA (Fig 1) are based on the conserved regions of *cdtA* sequences of *C. jejuni* strain 81-176 (Pickett et al., 1996) and *C. coli* strain D730 (Pickett, unpublished data) (Table 7a). The degenerate reverse primers, C-ECI, C-FSI1, C-TTS1, C-TTS2, and one exact reverse primer, C-AVP (Fig 1), are all derived from the region of homology between the *cdtC* sequence of *E. coli* 9142-88 (Pickett et al., 1994) and of *C. jejuni* strain 81-176 (Pickett et al., 1996) (Table 7a).

Restriction Analyses. Restriction endonucleases were used in accordance with the specifications of the supplier (New England Biolabs, Boston, Mass.). The following restriction endonucleases were tested: *Alu*I, *Bcl*I, *Bgl*II, *Cla*I, *Dde*I, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*I, *Nsi*I, *Sau*3AI, *Sci*I, *Ssp*I, *Stu*I, *Taq*I.

RESULTS

Evaluation of methods for speciating *C. jejuni* and *C. coli* isolates.

Introduction. Pickett et al. (1996) reported that the *C. coli* strains they tested produced very little or no toxin (mean titers 0 ± 0 to 3 ± 4) whereas *C. jejuni* strains produced much greater amounts of toxin (mean titers higher than 100 in 18 of the 20 strains tested). This new observation was based on limited numbers of *C. jejuni* and *C. coli* strains from mostly human sources, which suggested that if more isolates from different sources were examined, some *C. jejuni* isolates that produce not much toxin and some *C. coli* isolates that produce lots of toxin might be found. In addition, Johnson and Lior (1988) reported that only about 40% of the *C. jejuni* and *C. coli* strains they tested produced CDT. One of the goals of this research was to examine more *C. jejuni* and *C. coli* isolates, in order to determine if similar toxin production patterns could be observed from these two species as reported by Johnson and Lior (1988) and Pickett et al. (1996). Since the primary source of *C. jejuni* and *C. coli* for human disease is reported as chickens, strains were isolated from chicken carcasses. However, it was essential to determine the species of the new isolates in order to draw meaningful and reliable conclusions about species-related toxin production. Since more than 100 strains were going to be isolated, a rapid test or tests were needed. A biochemical identification test by API Campy and a species-specific PCR test was used. Initially, the API Campy and species-specific PCR systems were tested with various primers to determine their performances with known *C. jejuni* and *C. coli* strains.

Preliminary results of the biochemical identification of *C. jejuni* and *C. coli* strains by API Campy. Three known *C. jejuni* strains, 81-176, D133, and Lior19, the

chicken isolates CN593, W91, SP923.1, and a chicken intestine mash isolate AE216 were successfully identified as *C. jejuni* by the use of API Campy. The known *C. coli* strain D115, and as well as chicken intestinal mash isolates, AE219, AE220, AE235, AE236, AE239, and AE241 were identified as *C. coli* by this method.

The Analytical Profile Index (API) is comprised of 7 digit numerical profiles of many campylobacters identified by the API Campy system. However in the interpretation part of API Campy system there is always a possibility that the numerical profile that is searched may not be included in the API. In addition, API Campy is based on evaluation of phenotypic tests. Therefore, the isolate tested might give an uninterpretable result, although the sample is prepared according to the manufacturer's instructions. These possible limiting factors suggested that another rapid identification method was needed. Therefore, a species-specific PCR procedure was tested. This method is highly specific and sensitive, since it is based on genotypic characteristics of the bacterium. Several primers that have been reported in the literature were available and were used to determine their suitability.

Testing of species specific PCR. Three *C. jejuni*-specific and two *C. coli*-specific primer pairs (Table 6) were tested for their performance in speciating *Campylobacter* strains. Isolated chromosomal DNA and/or colonies from a variety of *C. jejuni* and *C. coli* strains were used in these PCR reactions. The information below is presented in the order in which the primers were tested during this study. Specifics about the PCRs will be mentioned with the results for specific primers.

C. jejuni-specific PCR with primers C1 and C4. C1 and C4 (Winters et al., 1995) were the first primers we tested. They had been reported to successfully detect *C. jejuni*

directly from chicken carcasses. *C. jejuni* 81-176 was used as a positive control. Eight *Campylobacter* isolates, AE219, AE220, AE236, AE214, AE237, AE238, AE239 and AE241, five of which had been tested in API Campy and identified as *C. coli* were tested. The expected product size is 159 bp. Neither *C. jejuni* 81-176, nor the *C. coli* isolates produced a PCR product. Owing to the negative result for 81-176, use of these primers was not pursued.

C. jejuni-specific PCR with primers Jej1, Jej2 and Therm3. Primers Jej1, Jej2 and Therm3 were tested the performance for their ability to identify *C. jejuni* isolates (Eyers et al., 1993).

Colony PCR. Eight *C. jejuni* strains, 81-176, 79-193, 79-101, 84-172, 85-374, Lior19, D133, D1816, and 17 *C. coli* strains, D2594, D115, 43473, D220, D1821, D2591, D2593, D2598, 43482, 43485, 43488, AE235, AE241, AE219, AE239, AE220, AE236 were tested. Two different product sizes may be observed: 710 and 810 bp. *C. jejuni* 81-176 produced a single product of approximately 720 bp. Four of the *C. coli* strains, 43473, AE219, AE235, and AE241 produced a single 710 bp PCR product, which was close to the expected *C. jejuni* specific product size. When primers Jej1, Jej2 and Therm3 were tested, many inconsistencies in repetitions of tests with the same strain was encountered. In addition, since *C. coli* strains produced PCR products with these primers even though they were not supposed to, a decision was made not to pursue colony PCR, bit to pursue PCR using purified DNA.

PCR with isolated chromosomal DNA. Four *C. jejuni* strains, 81-176, Lior19, D133, D1816, and 11 *C. coli* strains, D2594, D730, D115, D126, D2593, 43473, D220, D2598, 43482, 43485, 43488 were tested. All *C. jejuni* strains produced an

approximately 720 bp PCR product. However, all *C. coli* strains but one produced 560 to 720 bp PCR products, as well.

In summary, 6 *C. coli* strains, 43473, D2593, D2598, 43482, 43488 and 43485 produced *C. jejuni*-sized PCR products with these primers in both colony PCR and PCR using chromosomal DNA. The inability of these primers to reliably identify *C. jejuni* strains led to the search for another *C. jejuni*-specific primer pair.

C. coli-specific PCR with primers Therm1 and Coli. Therm1 and Coli primers were tested for their ability to identify *C. coli* isolates by both colony PCR and PCR using chromosomal DNA (Eyers et al., 1993).

Colony PCR. Three *C. jejuni* strains, Lior 19, D133, D1816 and 14 *C. coli* strains, D220, D1821, D2591, D2593, D2598, 43473, 43482, 43485, 43488, AE235, AE219, AE241, AE220, AE239 were tested. The predicted product size is 390 bp. None of the *C. jejuni* produced a PCR product. Eight of the *C. coli* strains produced a single PCR product of approximately 390 bp.

PCR with isolated chromosomal DNA. Six *C. coli* strains, 43473, D2593, D2598, 43482, 43485, and 43488 were tested. All of the strains produced a single 390 bp PCR product. However, these same strains had produced a PCR product in the *C. jejuni*-specific PCR with Jej1/Jej2/Therm3 using isolated DNA as template.

Both the *C. jejuni* and the *C. coli*-specific primers tested up to this point produced data that was either inconsistent or unreliable. Some *C. jejuni* strains produced products with *C.coli*-specific primers and some *C. coli* produced products with *C. jejuni*-specific

primers. Therefore, other primer pairs were tested to speciate *C. jejuni* and *C. coli* strains.

C. jejuni specific PCR with primers VS15 and VS16. Next primers VS15 and VS16 were tested the ability to identify *C. jejuni* isolates (Stonnet et al., 1993).

Colony PCR. One *C. jejuni* strain AE216, and 7 *C. coli* strains, D126, D220, AE219, AE220, AE235, AE239, and AE241 were tested. *C. jejuni* strain AE216 produced a single 350 bp PCR product which is close to the expected product size of 358 bp. None of the *C. coli* strains produced a PCR product.

PCR with isolated chromosomal DNA. The same *C. jejuni* and *C. coli* strains used in colony PCR with 5 additional *C. coli* strains, D2598, 43473, 43482, 43485 and 43488 were tested. Only the *C. jejuni* strain produced approximately a 350 bp PCR product. No PCR product was obtained from any of the *C. coli* strains tested.

C. coli specific PCR with primers CSF and CSR. These primers (Stonnet and Guesdon, 1995) were tested to determine their ability to identify *C. coli* isolates. Colony PCR and PCR using chromosomal DNA results were also compared.

Colony PCR. The 1*C. jejuni* and 7*C. coli* strains used with the VS15/VS16 colony PCR with additional *C. coli* strains, 43473, 43485, and 43488 were tested. No PCR product was obtained from the *C. jejuni* strain. All of the *C. coli* strains produced an approximately 250 bp PCR product, which is close to the expected *C. coli*-specific 258 bp PCR product size.

PCR with isolated chromosomal DNA. Two *C. jejuni* strains, 81-176, 79-193 and 9 *C. coli* strains, D730, D2594, D2598, D126, D220, 43473, 43482, 43485, and 43488 were tested. No PCR product was obtained from the *C. jejuni* strains. All of the *C. coli* strains produced a single, approximately 250 bp PCR product.

Summary of the speciation results. Only VS15/VS16 and CSF/CSR appeared to successfully identify *C. jejuni* and *C. coli* in our initial tests. Therefore, these two primer pairs were used in all speciation studies. Chromosomal DNA was used as the template in future PCR tests, to decrease possible inconsistencies that occurred when colony PCR was used.

Development of PCR tests for the detection and analysis of *cdt* genes in *C. jejuni* and *C. coli* isolates. In this part of the study, the ultimate aim was to determine the prevalence of *cdt* genes in the chicken *Campylobacter* isolates by PCR. Pickett et al. (1996) had reported the successful detection of *cdt* genes in a variety of *Campylobacter* species by using two degenerate primers, VAT2 and WMI1, based on *E. coli cdt* sequences (Fig 1). Initially, these primers were used to detect *cdt* sequences in a small number of *C. jejuni* and *C. coli* strains. The availability of *cdt* sequence information in our laboratory on *C. coli* strain D730 enabled us to design additional primers that might be useful for detecting *cdt* sequences in *C. jejuni* and *C. coli* strains. Additional PCRprimers (Fig1, Table 7a) were sought so that *cdt* sequences could be detected in strains for which VAT2/WMI1 would not work. In addition, it is often desirable to have primers that can make more than one sized product, particularly if in the future *cdt* detection is part of multiplex PCR tests. Ten additional primer pairs were tested (Table

7b) to amplify *cdt* genes from *C. jejuni* and *C. coli* strains (Table 5). Results from these primers are given in the order in which they were tested. Both isolated chromosomal DNA and one colony were used as template to determine if colony PCR can replace PCR using isolated chromosomal DNA. If colony PCR was successful, *cdt* genes could be determined without the need of chromosomal DNA isolation. This would eliminate a time consuming step, when more than 100 strains were to be tested. The strains used and other specific information about the PCR test results performed with the specific primer pairs are explained under the relevant section below.

Cdt-specific PCR with the VAT2 and WMI1 primer pair.

PCR with isolated DNA. Nine *C. jejuni* strains, 81-176, G13, D220, Lior 19, 79-193, D1816, D133, 84-142, and AE216, and 11 *C. coli* strains, D730, D126, D2594, D115, D1821, AE219, AE220, AE235, AE236, AE239, AE241 were tested for the presence of *cdt* sequences using VAT2 and WMI1 primers. All of the *C. jejuni* strains except D1816 produced a single, approximately 500 bp (Fig. 2) PCR product which was close to the predicted product size of 495 bp (Table 7b). All of the *C. coli* strains produced an approximately 500 bp PCR product. In some of the repeated PCR assays for *C. coli* isolates, a minor second product of an approximate size of 900 bp was observed.

Colony PCR. Eleven *C. jejuni* strains, C31, 84-142, 81-160, 79-445, 79-101, 85-361, Lior 19, D133, CN593, W91, SP923.1, and 19 *C. coli* strains, D220, D2594, D126, D115, D1821, D730, D2593, D2591, 43473, 43485, 43482, 43488, D2598, AE219, AE220, AE235, AE236, AE239, AE241 were tested for the presence of *cdt* sequences using VAT2 and WMI1 primers. All of the *C. jejuni* strains produced an approximately 500 bp major PCR product. Two minor PCR products of approximately 400 and 600 bp

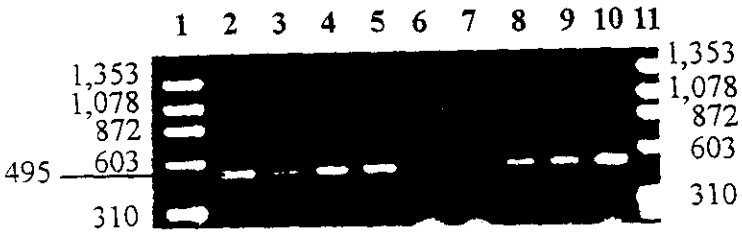

Figure 2. PCR products obtained with VAT2 and WMI1 primers and *Campylobacter* sp. template DNA. Lanes: 1 and 11, *Hae*III-digested ΦX174; 2 to 7, PCR products from reactions with template DNA from *C. jejuni* strains 81-176, 84-142, 85-360, 79-193, D1816, and AED974, respectively; 8 to 10, PCR products from reactions with template DNA from *C. coli* strains D730, D2593, and 43473, respectively. The exact size of the VAT2/WMI1 PCR product is 495 bp and is marked with a line. Standard sizes are shown in base pairs.

Table 7b. The pairing of the *cdt*-specific primers and their expected product sizes

#	Primer pair	Product size (bp)
1	VAT2 / WMI1	495
2	A-IWG / WMI1	957
3	A-IDS / WMI1	939
4	A-GAA / WMI1	999
5	A-GNW / WMI1	966
6	A-IVH / WMI1	822
7	VAT2 / C-ECI	1182[a]
8	VAT2 / C-AVP	1137
9	VAT2 / C-FSI1	1110
10	VAT2 / C-TTS1	1125
11	VAT2 / C-TTS2	1125

[a]This product size is for *C. jejuni* strains. *C. coli* strains produce 1350 bp PCR product with this primer pair.

were also observed. The specific conditions for the colony PCR were the same for all of the *C. jejuni* strains. A 1mm diameter colony was used as a source of template DNA and used to inoculate a 20 μl reaction. The colony was lysed by heating the tube at 95°C for 10 min. Fifteen of the 19 *C. coli* strains produced an approximately 500 bp PCR product. Six of these strains produced a minor product of approximately 700 bp in addition to the major product. Eleven of these 15 strains produced a *cdt*- specific PCR product in the first PCR test. Four of the remaining 15 strains gave inconsistent results with colony PCR: D1821 produced a *cdt*-specific product in one of three tests and D2591, 43473, and 43482 produced a specific product in 2 out of 3 reactions.

This primer pair successfully amplified the *cdt* sequences in all but one *Campylobacter* strains tested by PCR using isolated DNA as template. Colony PCR performed well with *C. jejuni* strains. In contrast, it did not consistently amplify *cdt* genes in some *C. coli* strains.

***Cdt*-specific PCR with the VAT2 and C-ECI primer pair.** In this PCR test, the forward primer VAT2, on the *cdtB* gene was paired with a reverse primer C-ECI, which is located on the *cdtC* gene. This primer pair was tested almost simultaneously with the 3 primer pairs mentioned after this one.

PCR with isolated DNA. Initially, 2 *C. jejuni* strains, 81-176, 79-193, and 3 *C. coli* strains, D115, D2594, and D126, were tested. Both of the *C. jejuni* strains produced approximately a 1200 bp major PCR product which was close to the expected PCR product size of 1182 bp. In addition, approximately 800, 500, 450, and 200 bp minor products were observed. All of the *C. coli* strains yielded one major PCR product of approximately 1350 bp. Two minor products with approximate sizes 900 and 600 bp

were observed in D115 and D2594. Since this primer pair performed well with chromosomal DNA template in 20μl PCR reactions, 8 additional *C. jejuni* strains, 85-452, G13, 85-360, D1816, Lior19, 79-101, 84-142, and D133, and 4 *C. coli* strains, D730, D2593, 43473, and D1821 were tested in 100 μl PCR reaction volumes. The reaction volume was increased to 100 μl to obtain sufficient PCR product to use in further restriction analysis with different enzymes. In order to optimize the PCR conditions to obtain the desired PCR product in the100 μl PCR reaction, different DNA volumes of 0.5 to 2 μl (1.25×10^{-3} to 5×10^{-3} μg) per 100 μl reaction, and different primer volumes of 0.75 to 2.5 μl (1.875×10^{-3} to 6.25×10^{-3} μM) per 100 μl reaction were tested. All of the additional *C. jejuni* and *C. coli* strains except D1816 produced 1200 bp or a 1350 bp PCR product, respectively (Fig 3). The minor PCR products mentioned above were occasionally observed in a few strains.

Colony PCR. Colony PCR results appeared to be highly inconsistent. Initially, one *C. jejuni* strain 81-176, and 5 *C. coli* strains, D115, D2594, D2593, D2591, and D2598 were tested in duplicate. An approximately 1200 bp major, and 4 minor (800, 600, 500, 300 bp) PCR products were observed in one of the two reactions with 81-176 DNA. All of the *C. coli* strains produced what appeared to be non-specific PCR products. In a repeated PCR test with the same *C. coli* strains, D115 produced an approximately 1350 bp single PCR product. Four additional *C. coli* strains, D126, 43482, 43485, and 43488 were tested, and only 43485 and D126 produced an approximately 1350 bp product. In addition, strain 43485 produced 600 and 400 bp minor PCR products. In an attempt to overcome the inconsistencies, different primer volumes (0.75 μl [375 μM] to 2.5 [1250 μM] μl in a 20μl reaction volume) and different boiling times

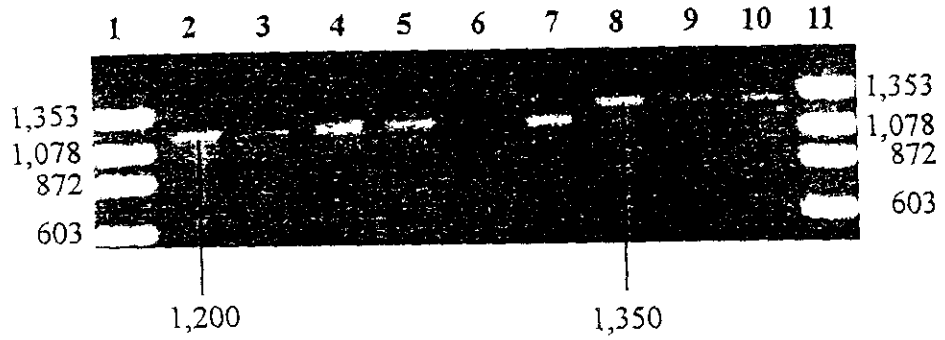

Figure 3. PCR products obtained with VAT2 and C-ECI primers and *Campylobacter* sp. template DNA. Lanes: 1 and 11, *Hae*III-digested ΦX174; 2 to 7, PCR products from reactions with template DNA from *C. jejuni* strains 81-176, 84-142, 85-360, 79-193, D1816, and AED974, respectively; 8 to 10, PCR products from reactions with template DNA from *C. coli* strains D730, D2593, and 43473, respectively. The size of VAT2/C-ECI PCR product is 1200bp for *C. jejuni* and 1350 bp for *C. coli* stains, and is marked with a line. Standard sizes are shown in basepairs.

for colony lysis (10, 15, 20, 25 min) were tested. These alterations did not eliminate the inconsistencies.

Summary. This primer pair successfully detected *cdt* genes in all but one of the *Campylobacter* strains tested by PCR when chromosomal DNA was used as template. Repeated inconsistencies in colony PCR were encountered. After testing the primer pairs, VAT2/WMI1 and VAT2/C-ECI, and comparing their abilities in *cdt*-specific PCR using chromosomal DNA and with colony PCR, a decision was made as to use only PCR with chromosomal DNA for the rest of the PCR primers to be tested.

***Cdt*-specific PCR with the A-IWG and WMI1 primer pair.** Three *C. jejuni* strains, 81-176, 79-193 and, D133, and three *C. coli* strains, D126, D2594, and D115 were tested for the presence of *cdt* sequences using these primers. The expected product size is 957 bp (Table 7b). All of the *C. jejuni* strains produced two non-specific PCR products. No product was obtained from the *C. coli* strains. As a result, A-IWG and WMI1 were not tested further.

***Cdt*-specific PCR with the A-IDS and WMI1 primer pair.** *C. jejuni* and *C. coli* strains used for the previous primer pair were tested. The expected product size is 939 bp (Table 7b). All of the *C. jejuni* and *C. coli* strains produced PCR products of inappropriate sizes.

***Cdt*-specific PCR with the VAT2 and C-FSI1 primer pair.** The same *C. jejuni* and *C. coli* strains used for the previous two primer pairs were tested. *C. jejuni* D133 produced a 1100 bp PCR product, which was close to the 1110 bp predicted product size (Table 7b). Both of the other two *C. jejuni* strains, 81-176, and 79-193 produced non-specific products of approximately 1400 and 2000 bp. All *C. coli* strains produced PCR

products that were not close to the expected sizes. Overall, this primer pair was judged to be not useful.

***Cdt*-specific PCR with the VAT2 and C-AVP primer pair.** Chromosomal DNA from the strains *C. jejuni* 81-176, and *C. coli* D730 was used as a template in PCR reactions. Strains 81-176 and D730 produced 1100 and 1200 bp products, respectively. These PCR products were close to the expected product size of 1137 bp (Table 7b). This primer pair was not tested further. This was partly because of having already one successful primer pair, VAT2 and C-ECI amplifying a similar region (Fig 1).

***Cdt*-specific PCR with the A-GAA and WMI1 primer pair.** Initially, chromosomal DNA from the strains *C. jejuni* 81-176, and *C. coli* D730 was used as a template in PCR reactions. A single, approximately 1050 bp PCR product was obtained from *C. jejuni* and *C. coli* strains. This product size was close to the predicted product size of 999 bp (Table 7b). Additional *C. jejuni* strains, 79-193, 84-142, 85-452, G13, D133 and *C. coli* strains, 43473, D2594, D1821, D115 and, D126 produced an approximately 1050 bp PCR product. *C. coli* strain D2594 did not produce a PCR product with these primers. *C. jejuni* 81-176, which had given a PCR product in the first PCR test, did not produce a PCR product in a repeated test. A-GAA and WMI1 could amplify *cdt* sequences in most of the *C. jejuni* and *C. coli* strains tested. However, an inconsistent result was obtained with a reference *C. jejuni* strain.

***Cdt*-specific PCR with the VAT2 and C-TTS1 primer pair.** Chromosomal DNA from one *C. jejuni* strain 81-176 was tested. From four PCR products, a very weak 1150 bp PCR product was close to the predicted 1125 bp product size (Table 7b). VAT2 and C-TTS1, therefore did not appear to be useful.

***Cdt*-specific PCR with VAT2 and C-TTS2.** *C. jejuni* 81-176 produced two weak PCR products, approximately 1100 and 745 bp in size from these primers. The 1100 bp PCR product was close to the predicted 1125 bp product size (Table 7b). VAT2 and C-TTS2 were not tested further.

***Cdt*-specific PCR with the A-GNW and WMI1 primer pair.** Eight *C. jejuni* strains, 81-176, 84-142, 79-193, 85-360, D1816, D133, 79-101, and Lior 19, and 8 *C. coli* strains, 43473, D730, D2593, D1821, D126, D2594, D115, and D220, were tested with this primer pair. The predicted product size is 963 bp (Table 7b). All of the *C. jejuni* strains except D1816, produced a single 960 bp PCR product. This strain had also been tested with VAT2 / WMI1 and VAT2 / C-ECI, and did not produce a product with those primers, either. All of the *C. coli* strains, except one, produced a 960 bp PCR product (Fig 4). *C. coli* strain D1821 produced an approximately 800 bp PCR product. These results clearly showed the ability of A-GNW and WMI1 to detect *cdt* sequences in *Campylobacter* strains. This primer pair was used further in our studies.

***Cdt*-specific PCR with the A-IVH and WMI1 primer pair.** The same strains tested with the previous primer pair were tested. The predicted product size is 819 bp (Table 7b). All of the *C. jejuni* strains except D1816, produced an approximately 820 bp, single PCR product. Again, this is the strain that was found previously not to have *cdt* sequences amplified with other primer pairs. All of the *C. coli* strains, except one, produced an 820 bp PCR product (Fig 5). *C. coli* strain D1821 produced an approximately 900 bp PCR product. A-IVH and WMI1 were therefore also successful in detecting *cdt* sequences in *Campylobacter* strains. This primer pair was used further in our studies.

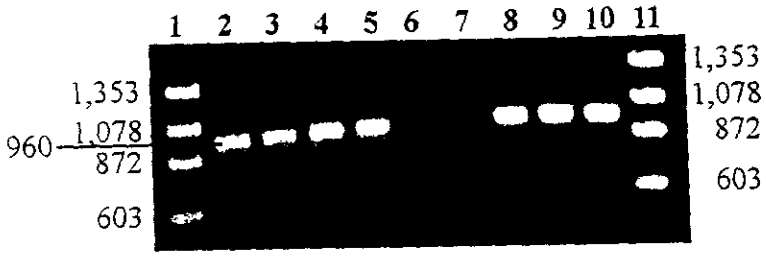

Figure 4. PCR products obtained with A-GNW and WMI1 primers and *Campylobacter* sp. template DNA. Lanes: 1 and 11, *Hae*III-digested ΦX174 size standards; 2 to 7, PCR products from reactions with template DNA from *C. jejuni* strains 81-176, 84-142, 85-360, 79-193, D1816, and AED974, respectively; 8 to 10, PCR products from reactions with template DNA from *C. coli* strains D730, D2593, and 43473, respectively. The size of the A-GNW/WMI1 PCR product is 960 bp and is marked with a line. Standard sizes are shown in basepairs.

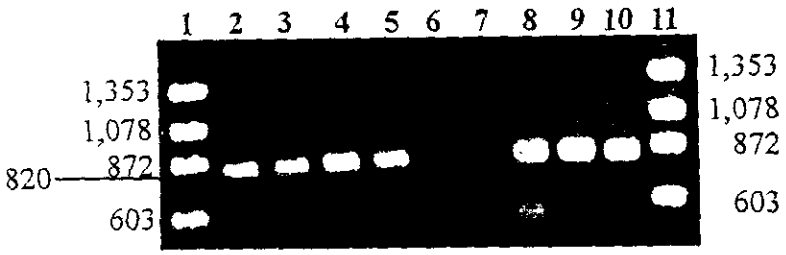

Figure 5. PCR products obtained with A-IVH and WMI1 primers and *Campylobacter* sp. template DNA. Lanes: 1 and 11, *Hae*III-digested ΦX174 size standards; 2 to 7, PCR products from reactions with template DNA from *C. jejuni* strains 81-176, 84-142, 85-360, 79-193, D1816, and AED974, respectively; 8 to 10, PCR products from reactions with template DNA from *C. coli* strains D730, D2593, and 43473, respectively. The size of the A-IVH/WMI1 PCR product is 820 bp and is marked with a line. Standard sizes are shown in basepairs.

Summary of the *cdt*-specific PCR. As a result of testing 11 primer pairs with a variety of *C. jejuni* and *C. coli* strains, 4 primer pairs (VAT2/WMI1, A-GNW/WMI1, A-IVH/WMI1, and VAT2/C-ECI) were shown to be potentially useful in detecting *cdt* sequences in *Campylobacter* strains. Therefore, a decision was made to use these primers to detect *cdt* sequences in chicken *Campylobacter* isolates. There was one *C. jejuni* strain, D1816, a human blood isolate, from which none of these 4 PCR primer pairs could amplify a *cdt* gene sequence; yet, this strain previously was shown in Southern hybridization experiments to possess *cdtB* sequences. In addition, one *C. coli* strain, D1821, another human blood isolate, produced slightly different sized PCR products with A-GNW/WMI1 and A-IVH/WMI1 than expected, but it produced the expected sized PCR products with VAT2/WMI1 and VAT2/C-ECI.

Analyses of the *cdt*-PCR products in *C. jejuni and C. coli* strains by restriction endonucleases.

In this part of the study, the objective was to determine if there was *cdt* sequence divergence, either within the same species, or between different species, that could be detected by restriction endonucleases. Any detectable *cdt* gene sequence heterogeneities, either between species or strains, might be useful in epidemiological studies in the future. The *cdt*-PCR products from several *C. jejuni* and *C.coli* strains were analyzed by digestion with selected restriction endonucleases. Where possible, nucleotide sequence information was used to guide our choice of restriction endonucleases.

Restriction analysis of VAT2 and WMI1 PCR products. Initially PCR products from *C. jejuni* strain 81-176, and *C. coli* strains D730 and D115 were digested

with *Alu*I, *Bcl*I, *Bgl*II, *Cla*I, *Dde*I, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*I, *Nsi*I, *Sau*3AI, *Sci*I, *Ssp*I, *Stu*I, *Taq*I, in order to determine which enzymes were able to cut these products. The *cdt* gene sequence data from *C. jejuni* 81-176 (Pickett et al., 1996) had indicated that there are *Alu*I, *Dde*I, *Eco*RI, *Eco*RV, *Hpa*I, and *Stu*I sites within this PCR product. Test digestions showed that three of these sites; *Eco*RI, *Stu*I, and *Alu*I (Fig 1) were likely to be useful for further analysis. 81-176 DNA was not cut with *Dde*I, *Eco*RV or *Hpa*I. PCR products from nine *C. jejuni*, 81-176, 84-142, 85-452, 79-193, 85-360, G13, D133, 79-101, and Lior 19, and 6 *C. coli*, 43473, D730, D2593, D2594, D115, and D126 were therefore digested with *Eco*RI, *Stu*I, and *Alu*I.

*Eco*RI digestion results. The restriction endonuclease (RE) fragment patterns produced by *Eco*RI digestion of the 500 bp VAT2/WMI1 PCR products were examined. Sequence data from strain 81-176 indicated that this enzyme would cut the amplified portion of at least some *C. jejuni cdtB* genes once yielding fragments of 350 and 140 bp. The 500 bp PCR products from all *C. jejuni* strains were cut once with *Eco*RI, yielding 350 and 150 bp sized fragments (Fig 6a). None of the *C. coli* PCR products was cut with *Eco*RI (Fig 6b).

*Stu*I digestion results. The 500 bp PCR products from all *C. jejuni* strains were cut once with *Stu*I, yielding 300 and 200 bp sized fragments (Fig 6a). These fragment sizes were close to the exact fragment sizes of 216 and 279 bp obtained from *C. jejuni* strain 81-176 sequence data (Pickett et al., 1996). None of the *C. coli* PCR products were cut with *Stu*I (Fig 6b).

*Alu*I digestion results. Sequence data (Pickett et al., 1996) predicted that *Alu*I would cut *C. jejuni* VAT2/WMI1 PCR products to yield a 280 bp fragment and five

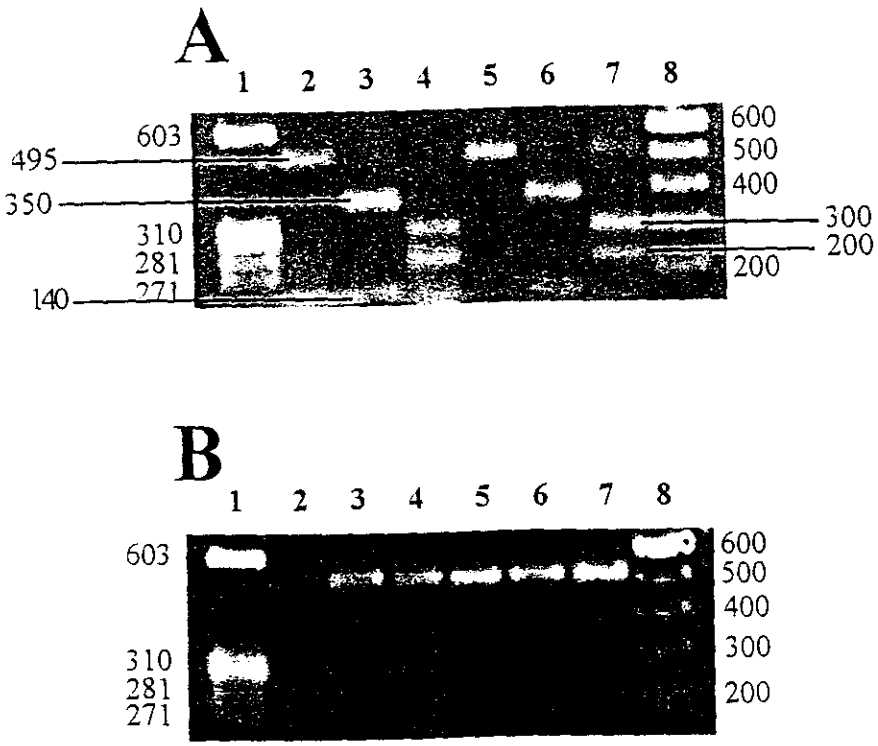

Figure 6. A. *Eco*RI and *Stu*I digestions of VAT2 and WMI1 PCR products of *C. jejuni* strains 81-176 and 84-142. Lanes: 1 and 8, *Hae*III-digested ΦX174 and 100 bp DNA ladder, respectively; lanes 2 to 4, strain 81-176 PCR product: uncut, *Eco*RI cut, *Stu*I cut, respectively; lanes 5 to 7, strain 84-142 PCR product: uncut, *Eco*RI cut, *Stu*I cut, respectively.

Figure 6. B. *Eco*RI and *Stu*I digestions of VAT2 and WMI1 PCR products of *C. coli* strains 43473 and D2593. Lanes: 1 and 8, *Hae*III-digested ΦX174 and 100 bp DNA ladder, respectively; lanes 2 to 4, strain 43473 PCR product: uncut, *Eco*RI cut, *Stu*I cut, respectively; lanes 5 to 7, strain D2593 PCR product: uncut, *Eco*RI cut, *Stu*I cut, respectively. Uncut, *Eco*RI cut, and *Stu*I cut VAT2/WMI1 PCR products are marked with lines. Standard sizes are shown in basepairs.

smaller fragments. The larger fragment in *Alu*I cuts of all *C. jejuni* strains was observed, but the smaller fragments were not resolved on the agarose gels. Sequence data from *C. coli* strain D730 (Pickett, unpublished data) indicated that *Alu*I would cut the *C. coli* PCR product four times to produce fragments of approximately 210, 110, 100, 50, and 10 bp. A 200 bp and 100 bp (likely a doublet of the 110 and 100 bp fragments) fragments in the *Alu*I cuts of the VAT2/WMI1 PCR product from four *C. coli* strains were observed, but the other bands were not resolved on the gels.

***EcoR*I restriction analysis of A-GNW and WMI1 PCR products.** PCR products from 7 *C. jejuni* strains, 81-176, 84-142, 79-193, 85-360, D133, 79-101, and Lior 19 and 8 *C. coli* strains, 43473, D730, D2593, D1821, D2594, D115, D126, and D220 were digested with *Eco*RI. The predicted sizes of the *Eco*RI digestion of the 960 bp A-GNW/WMI1 PCR product were 820 and 140 bp. *C. jejuni* strains' PCR products were cut once with *EcoR*I, yielding apparent 900 bp and 200 bp sized fragments. As expected, none of the *C. coli* PCR products was cut with *Eco*RI (Fig 7).

***EcoR*I Restriction analysis of A-IVH and WMI1 PCR products.** The same strains used in A-GNW and WMI1 digestions were tested. The predicted sizes of the *Eco*RI digestion of the 820 bp A-IVH/WMI1 PCR products were 680 bp and 140 bp. *C. jejuni* strains' PCR products were cut once with *EcoR*I, producing approximately 700 bp and 200 bp sized fragments. Again, none of the *C. coli* PCR products was cut with *Eco*RI (Fig 8).

Restriction analysis of VAT2 and C-ECI PCR products. *Bgl*II and *Ssp*I digests were performed with PCR products from 9 *C. jejuni* strains, 81-176, 84-142, 85-

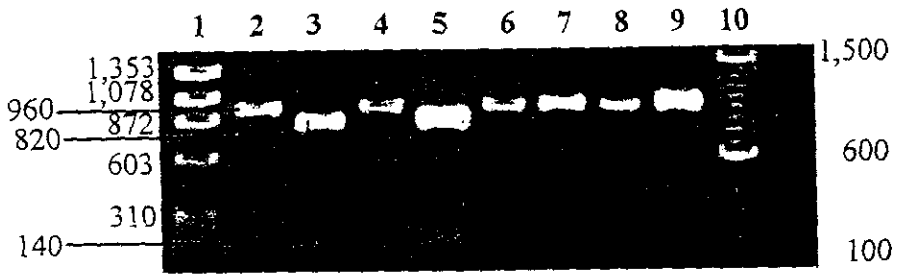

Figure 7. *Eco*RI digestions of A-GNW and WMI1 PCR products of *C. jejuni* strains 81-176 and 84-142 and *C. coli* strains D2593 and 43473. Lanes: 1 and 8, *Hae*III-digested ΦX174 and 100 bp DNA ladder, respectively; 2 and 4, uncut PCR products from *C. jejuni* strains 81-176 and 84-142, respectively; 3 and 5, *Eco*RI cuts of PCR products from *C. jejuni* strains 81-176 and 84-142, respectively; 6 and 8, uncut PCR products from *C. coli* strains D2593 and 43473, respectively; 7and 9, *Eco*RI cuts of PCR products from *C. coli* strains D2593 and 43473, respectively. Uncut and *Eco*RI cut A-GNW /WMI1 PCR products are marked with lines. Standard sizes are shown in basepairs.

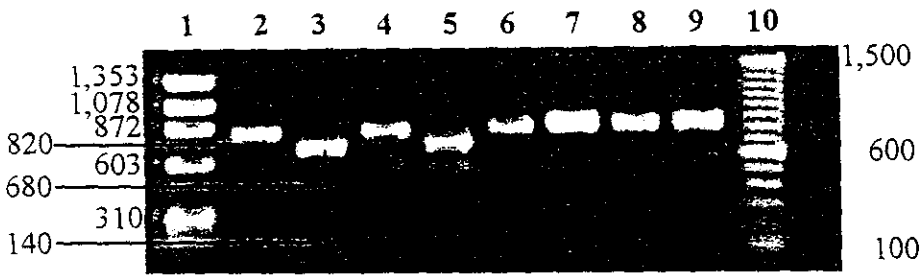

Figure 8. *Eco*RI digestions of A-IVH and WMI1 PCR products of *C. jejuni* strains 81-176 and 84-142 and *C. coli* strains D2593 and 43473. Lanes: 1 and 8, *Hae*III-digested ΦX174 and 100 bp DNA ladder, respectively; 2 and 4, uncut PCR products from *C. jejuni* strains 81-176 and 84-142, respectively; 3 and 5, *Eco*RI cuts of PCR products from *C. jejuni* strains 81-176 and 84-142, respectively; 6 and 8, uncut PCR products from *C. coli* strains D2593 and 43473, respectively; 7and 9, *Eco*RI cuts of PCR products from *C. coli* strains D2593 and 43473, respectively. Uncut and *Eco*RI cut A-IVH /WMI1 PCR products are marked with lines. Standard sizes are shown in basepairs.

452, 79-193, 85-360, G13, D133, 79-101, and Lior 19 and 7 *C. coli* strains, 43473, D730, D2593, D1821, D2594, D115, and D126.

BglII digestion results. Sequence data from *C. jejuni* strain 81-176 (Pickett et al., 1996) suggested that the VAT2 and C-ECI PCR products from *C. jejuni* strains would likely be cut once by *BglII* to produce fragments of approximately 700 bp and 500 bp. Eight of the 9 *C. jejuni* PCR products were cut once with *BglII* producing apparent 700 bp and 500 bp fragments (Fig 9). The PCR product for *C. jejuni* strain D133 was not cut with *BglII*. All *C. coli* strains' PCR products were cut once with *BglII* producing fragments with approximate sizes of 940 bp and 160 bp. Overall, *BglII* digestions were useful to demonstrate that there is nucleotide sequence difference between species in the VAT2/C-ECI region.

SspI digestion results. *C. jejuni* strain 81-176 sequence data (Pickett et al., 1996) showed that the VAT2 and C-ECI PCR products would also likely be cut once by *SspI* to produce fragments of 910 bp and 270 bp. All of the *C. jejuni* PCR products were cut once with *SspI* producing apparent 960 bp and 300 bp fragments. All *C. coli* strains' PCR products were cut once with *SspI* producing fragments of sizes 920 bp and 350 bp (Fig 9).

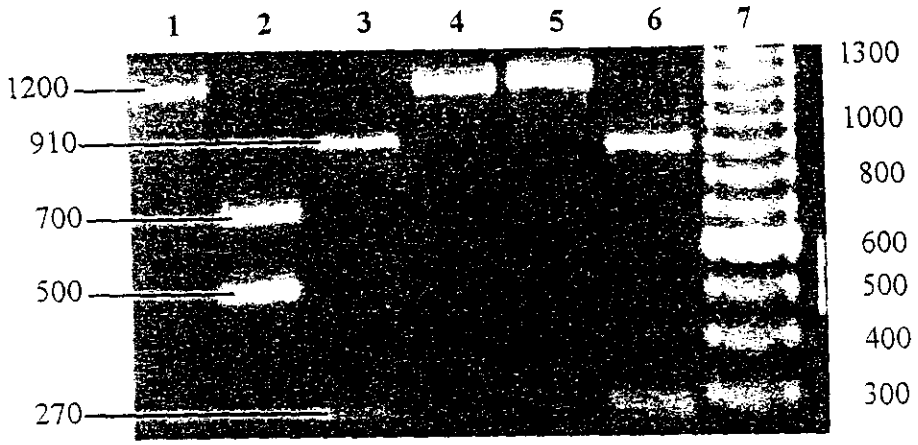

Figure 9. *Bgl*II and *Ssp*I digestions of VAT2 and C-ECI PCR products of *C. jejuni* strains 81-176 and AED974. Lanes: 1and 4, uncut PCR products of 81-176 and AED974, respectively; 2 and 5, *Bgl*II cuts of PCR products of 81-176 and AED974, respectively; 3 and 6, *Ssp*I cuts of PCR products of strains 81-176 and AED974; 7, 100bp DNA ladder. Uncut, *Bgl*II cut, and *Ssp*I cut VAT2/C-ECI PCR products are marked with lines. Standard sizes are shown in basepairs.

Introduction to isolation of *Campylobacter* sp. from chicken carcasses.

At this point, the data have summarized the speciation methods and the *cdt* specific PCR tests as well as restriction analyses of the *cdt* PCR products. These methods were now judged to be developed enough to apply to analysis of fresh chicken isolates of *Campylobacter*.

Obtaining fresh chicken carcasses from local supermarkets was planned. Approximately 100 *Campylobacter* strains would be isolated from these chickens. Then, the isolates would be speciated, their CDT production would be determined, and the presence of *cdt* genes and their variations using the PCR methods just described would be assesed.

Since our goal was to test *Campylobacter* species isolated from many different chickens, our isolation procedure was designed to produce one *Campylobacter* isolate per chicken and was not designed to collect more than one strain per chicken. However, if there appeared to be two variant, yet *Campylobacter* colony morphologies on the Abeyta-Hunt or CCDA selection agars, then a representative of both colony types would be tested further.

Identification and speciation of the *Campylobacter* isolates from chicken carcasses.

Isolation of campylobacters from chicken carcasses. One hundred and five *Campylobacter* strains were isolated from 91 fresh chicken carcasses according to the procedure in Materials and Methods.

Identification results by API Campy. All 105 *Campylobacter* isolates were tested with the API Campy biochemical identification kit. Sixty-nine (66%) of the

isolates were identified as *C. jejuni* and 34 (32%) were identified as *C. coli*. Two (2%) isolates (AED974, AEA927) could not be speciated using the API Campy identification kit (Table 8).

Two different colony types were observed in the primary isolation plates from fourteen chicken carcasses. Eight of these chicken carcasses yielded one *C. jejuni* and one *C. coli* isolate, five gave rise to two *C. jejuni* isolates, and one produced two *C. coli* isolates. The remaining 77 chicken carcasses yielded single *C. jejuni* or *C. coli* isolate.

Identification results by species-specific PCR. Our preliminary data with different species-specific PCR primers had concluded that *C. jejuni*-specific primers VS15/VS16 (Stonnet and Guesdon, 1993) and *C. coli*-specific primers CSF/CSR (Stonnet et al, 1995) correctly identified these species. Therefore, the 105 *Campylobacter* isolates were tested with both primer pairs in PCR using chromosomal DNA from each isolate. Seventy (67%) of the isolates were identified as *C. jejuni* and 35 (33%) were identified as *C. coli* (Table 8). During the course of these tests, none of the *C. jejuni* isolates produced a PCR product with the *C. coli* specific primers. Similarly, none of the *C. coli* isolates produced a PCR product with *C. jejuni* specific primers. In addition, this method identified the two (2%) isolates, AED974 and AEA927, which could not be speciated by API Campy, as *C. jejuni* and *C. coli*, respectively. A separate hippurate hydrolysis test for each of these isolates was performed, and both of them were found to be hippurate hydrolysis negative.

Summary of the speciation of the chicken isolates. Overall, the API Campy identification kit performed well in identifying the chicken isolates. However, there were

Table 8. Comparison of identification methods of *C. jejuni* and *C. coli* isolated from chicken carcasses

Isolates	Identification method PCR (%)	API (%)
C. jejuni	70 (67)	69 (66)
C. coli	35 (33)	34 (32)
Total	105	103[a]

[a]Two isolates could not be identified by API Campy.

instances where we had to repeat the test. The intrinsic factors of the isolate, such as slow growth, or the lack of confluent growth might have led to underinoculation of the test. Occasionally poor growth in the second strip was observed. In these cases the test was repeated. Some results were weak positive, and exact color formation could not be seen in some of the tests in the first strip. Some of the inoculum in the strips tended to dry out in some of the tests during incubation, which made it difficult for the strain to grow and utilize the substrate. To solve this problem, the air inside the aerobic and microaerobic incubators was kept humidified by placing a pan of water into them. Overall, both tests appeared to be reliable and the agreement between them lends credence to our species designations.

Cdt-specific PCR detection and restriction endonuclease analysis of the *cdt*-PCR products in the isolates from chickens.

Detection of *cdt* genes by PCR.

PCR with VAT2 and WMI1. VAT2 and WMI1 were used to screen for *cdt* sequences in all 105 isolates. A single product of approximately 500 bp was observed in the reactions from 104 of the isolates. This is the expected size product amplified from *cdtB* gene by these two primers (Pickett et al., 1996) (Fig 10a and 10b). One *C. jejuni* isolate, AED974, did not produce a PCR product with this primer pair (Fig 2).

This primer pair successfully detected *cdt* sequences in all but one of our isolates. Several *C. jejuni* and *C. coli* strains isolated from chickens purchased at different times were chosen. The detection performances of the following 3 PCR primer pairs on these strains were determined. The *C. jejuni* isolate AED974, which did not produce a product

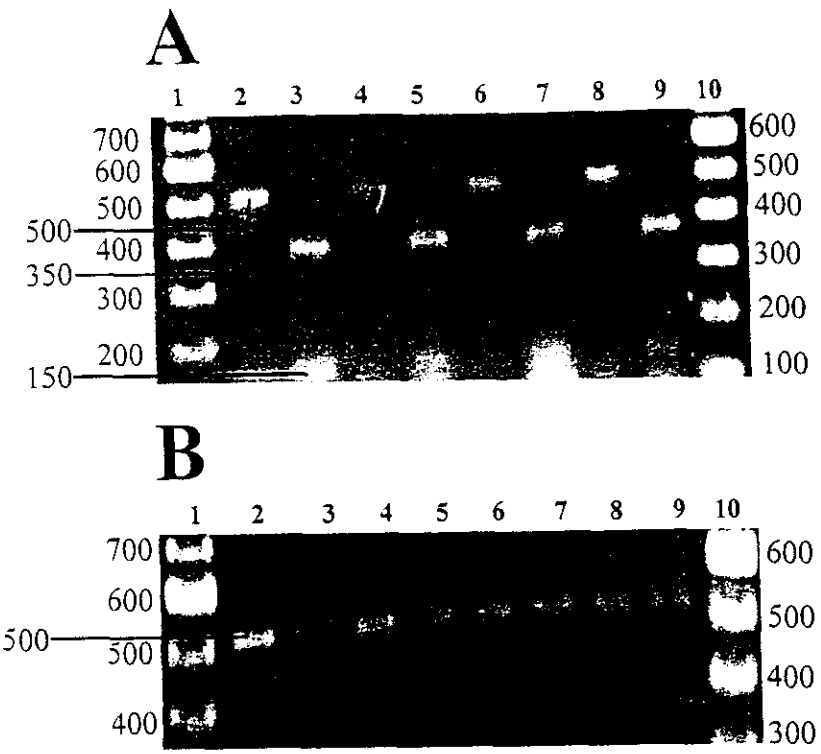

Figure 10a. PCR products and their relevant *Eco*RI digestions obtained with VAT2 and WMI1 primers and *C. jejuni* DNA. Lanes: 1 and 10, 100 bp DNA ladder; 2, uncut PCR product from 81-176; 3, *Eco*RI cut of PCR product from 81-176; 4, uncut PCR product from AEA962; 5, *Eco*RI cut of PCR product from AEA962; 6, uncut PCR product from AED962; 7, *Eco*RI cut of PCR product from AED962; 8, uncut PCR product from AED976; 9, *Eco*RI cut of PCR product from AED976. Uncut and *Eco*RI cut VAT2 /WMI1 PCR products are marked with lines.

Figure 10b. PCR products and their relevant *Eco*RI digestions obtained with VAT2 and WMI1 primers and *C.coli* DNA. Lanes: 1 and 10, 100 bp DNA ladder; 2, uncut PCR product from 43473; 3, *Eco*RI cut of PCR product from 43473; 4, uncut PCR product from AEB962; 5, *Eco*RI cut of PCR product from AEB962; 6, uncut PCR product from AED971; 7, *Eco*RI cut of PCR product from AED971; 8, uncut PCR product from AED974; 9, *Eco*RI cut of PCR product from AED974. Uncut and *Eco*RI cut VAT2 /WMI1 PCR products are marked with lines. Standard sizes are shown in basepairs.

with VAT2 and WMI1, was also chosen to see if any of the 3 primer pairs would amplify a PCR product from this strain.

PCR with A-GNW and WMI1. Only three *C. jejuni* isolates, AED973, AEB9712L, and AED974 and 4 *C. coli* isolates, AEB971, AEB979, AEB9713L, and AED9715, were tested for *cdt* sequences using these primers. A single product of 960 bp was observed in the reactions from all but one of the isolates. Nine hundred and sixty base pairs is very close to the predicted 963 bp PCR product (Table 7b). One *C. jejuni* isolate, AED974, the same one which did not produce a product with VAT2 and WMI1, also did not produce a PCR product with this primer pair (Fig 4).

PCR with A-IVH and WMI1. The same *C. jejuni* and *C. coli* isolates mentioned above were tested. An 820 bp PCR product was observed in these reactions from all but one of the isolates. Eight hundred and nineteen base pairs is the expected size for a product amplified from *cdt* genes by these 2 primers (Fig. 8). The same *C. jejuni* isolate, AED974, which did not produce a product with VAT2/WMI1 and A-GNW/WMI1, did not do so with this primer pair either (Fig 5).

PCR with VAT2 and C-ECI. Three *C. jejuni* isolates, AED973, AED974, AEB9710 and 4 *C. coli* isolates, AEB971, AEB979, AEB9713L, and AED9715 were tested. A single product of approximately 1200 bp was observed in the reactions from all *C. jejuni* isolates. This alternative PCR reaction, in which the downstream primer WMI1 used in all three of the other PCR reactions, was replaced with C-ECI , amplified a 1200 bp product from AED974 (Fig 3). This was the isolate that did not produce a product with VAT2/WMI1, A-GNW/WMI1 and A- IVH/WMI1. All of the *C. coli* isolates

produced a 1350 bp PCR product, which is the same sized product obtained from *C. coli* strains in the preliminary tests.

Summary of the *cdt*-specific PCR with chicken isolates. All of the 105 chicken carcass isolates tested were found to carry *cdt* gene sequences. Primer pairs VAT2/WMI1, A-GNW/WMI1and A-IVH/WMI1 detected *cdt* sequences in all but one *C. jejuni* isolate. However, the fourth PCR primer pair VAT2/C-ECI was apparently able to amplify a portion of the *cdt* genes from this strain.

Restriction endonuclease analysis of the *cdt*-PCR products in *C. jejuni* and *C. coli* isolates from chickens.

In this part of the study, the *cdt* PCR products obtained from the 4 PCRs were digested with selected restriction endonucleases.

Restriction analysis of the VAT2 and WMI1 PCR products.

*Eco*RI digestion results. The PCR products from all 105 isolates were digested with *Eco*RI. The 500 bp PCR products from all *C. jejuni* isolates were cut once with *EcoR*I, yielding 350 and 150 bp sized fragments (Fig 10a). None of the *C. coli* PCR products was cut with this enzyme (Fig 10b). These results were exactly the same as the results obtained from the *EcoR*I digestions of the VAT2/WMI1 PCR products of the *Campylobacter* strains used in the preliminary tests.

*Stu*I and *Alu*I digestion results. The PCR products from two *C. jejuni* isolates, AED973, and AEB9710, and 4 *C. coli* isolates, AEB971, AEB979, AEB9713L, and AED9715, were digested with *Stu*I and *Alu*I. The 500 bp PCR product from the *C. jejuni* isolates were cut once with *Stu*I, yielding approximately 300 and 200 bp sized fragments.

These fragment sizes were close to the exact fragment sizes of 216 and 279 bp obtained from *C. jejuni* strain 81-176 sequence data (Pickett et al., 1996). None of the *C. coli* PCR products was cut with *Stu*I.

In the *Alu*I digests, the 280 bp largest fragment was observed in the *C. jejuni* strains, but the five smaller fragments were not resolved on the agarose gels. *Alu*I cuts of the VAT2/WMI1 PCR products from four *C. coli* strains yielded a 200 bp and 100 bp (likely a doublet of the 110 and 100 bp fragments) visible fragments, but the other 3 fragment bands were not resolved on the gels.

These results were exactly the same as the results obtained from the *Stu*I and *Alu*I digestions of the VAT2/WMI1 PCR products of the *Campylobacter* strains used in the preliminary tests.

***EcoR*I restriction analysis of A-GNW/WMI1 and A-IVH/WMI1 PCR products.** Two *C. jejuni*, AED973, and AEB9712L, and 4 *C. coli*, AEB971, AEB979, AEB9713L, and AED9715, isolates' PCR products from both of these primer pairs were digested with *Eco*RI. These arbitrarily chosen *Campylobacter* isolates produced the digestion patterns expected. The 960 bp A-GNW/WMI1 PCR products from the *C. jejuni* strains were cut once with *Eco*RI, yielding apparent 900 bp and 200 bp sized fragments, which were close to the predicted *Eco*RI digestion sizes of 820 and 140 bp. None of the *C. coli* PCR products was cut with *Eco*RI. The *Eco*RI digestions of the 820 bp A-IVH/WMI1 PCR products from the same *C. jejuni* strains yielded 700 bp and 200 bp, which were close to the expected 680 and 140 bp fragments. Again, none of the *C. coli* PCR products was cut with *Eco*RI. These isolates produced digestion patterns

observed with the *C. jejuni* and *C. coli* strains tested in the preliminary restriction analyses.

Restriction analysis of VAT2 and C-ECI PCR products. PCR products from three *C. jejuni* isolates, AED973, AED974, and AEB9710 and 4 *C. coli* isolates, AEB971, AEB979, AEB9713L, and AED9715, were digested with *Bgl*II and *Ssp*I.

*Bgl*II digestion results. Two of the *C. jejuni* strains, AED973, AEB9710, had PCR products that were cut once with *Bgl*II, producing 700 bp and 500 bp fragments. One *C. jejuni* strain, AED974, had a PCR product that was not cut with *Bgl*II (Fig 9). All of the *C. coli* isolates' PCR products were cut once with *Bgl*II. The 3 *C. coli* isolates AEB971, AEB979, and AEB9713L, produced fragments of sizes 840 bp and 250 bp upon digestion with *Bgl*II. One *C. coli* isolate, AED9715, produced 900 and 200 bp fragments. These measurements were taken from different gels, and when compared, all of the 3 *C. coli* strains seemed to produce approximately 99 and 250 bp fragments. These isolates produced digestion patterns observed with the *C. jejuni* and *C. coli* strains tested in the preliminary restriction analyses. *C. jejuni* isolate AED974 acted like D133, a *C. jejuni* strain used in preliminary digestion tests, since neither of these *C. jejuni* strains' PCR products was cut with this enzyme.

*Ssp*I digestion results. All three *C. jejuni* PCR products were cut once with *Ssp*I, producing 960 bp and 300 bp fragments (Fig 9). All 4 *C. coli* isolates were cut once with *Ssp*I, producing 700 bp and 400 bp fragments. These isolates produced similar digestion patterns observed with the *C. jejuni* and *C. coli* strains tested in the preliminary VAT2/C-ECI product restriction analyses.

Detection of active CDT production by the *Campylobacter* isolates from chickens.

Sixty-nine of the 70 (99%) *C. jejuni* isolates produced CDT; 65 of these produced CDT titers greater than 100 in the HeLa assay (Table 9). One *C. jejuni* isolate produced no detectable toxin in repeated HeLa assays. This strain (AED974) was the one that the PCR analysis indicated a defective *cdt* operon. The remaining four *C. jejuni* isolates produced mean titers of 87 ± 2, 56 ± 2, 57 ± 5 and 64 ± 3. All of the *C. coli* isolates had CDT titers less than 100; 30 (86%) had titers less than 5 (Table 9). Mean CDT titers for *C. jejuni* and *C. coli* isolates from chicken carcasses are shown in Table 10.

Table 9. The range of CDT titers produced by *Campylobacter* isolates from chicken carcasses

Species (total number of isolates)	Number of isolates with CDT titers[a]			
	0-100	101-400	401-800	>800[b]
C. coli (35)	35 (100)[c]	-	-	-
C. jejuni (70)	5 (7)	25 (36)	21 (30)	19 (27)

[a] Titers shown represent mean CDT titers calculated as described in Materials and Methods. The division of the mean CDT titers into four groups was done to emphasize the differences between *C. jejuni* and *C. coli* titers, and to illustrate the broad range of *C. jejuni* CDT titers between 100 and 2000 represented by these isolates and the lack of any clustering of these titers at a particular level.

[b] The highest mean CDT titer was 2164 ± 1.

[c] Numbers in parenthesis represent the percentage of isolates having CDT titers in that particular range

Table 10. Mean CDT titers for *C. jejuni* and *C. coli* isolated from chicken carcasses

Isolate	CDT titer[a]	Isolate	CDT titer[a]
C. coli		*C. jejuni*	
AEB961	12.99 ± 1.81	AEB966	191.57 ± 1.53
AEB962	4.60 ± 3.94	AEB968	182.54 ± 1.52
AEB963	3.07 ± 2.64	AEB969	279.39 ± 2.92
AEB965	12.11 ± 1.74	AEB9610	297.96 ± 2.84
AED963	4.77 ± 1.25	AED961	114.77 ± 2.04
AED969	1.00 ± 1.00	AED962	160.85 ± 2.46
AEA964	1.00 ± 1.00	AED964	419.16 ± 1.81
AEA966	2.67 ± 2.40	AED965	556.77 ± 3.12
AEA968S	2.24 ± 4.05	AED966	605.11 ± 2.85
AEA9610S	8.37 ± 1.64	AED967	87.15 ± 1.53
AED971	4.76 ± 3.95	AED968	1173.20 ± 1.52
AEB971	1.00 ± 1.00	AED9610	55.76 ± 2.15
AEB972	1.00 ± 1.00	AEA961	915.84 ± 3.16
AEB973	1.00 ± 1.00	AEA962	519.78 ± 1.22
AEB974	1.00 ± 1.00	AEA963	781.62 ± 2.06
AEB976	1.96 ± 3.21	AEA965	178.75 ± 2.94
AEB977	1.00 ± 1.00	AEA967S	743.62 ± 2.23
AEB978	1.62 ± 2.30	AEA967L	1128.64 ± 1.54
AEB979	1.63 ± 2.33	AEA968S	1491.05 ± 1.53
AEC971	1.00 ± 1.00	AEA969	658.54 ± 1.31
AEC972	1.57 ± 2.18	AEA9610L	1367.84 ± 2.55
AEC973	3.27 ± 2.92	AED972	163.07 ± 2.41
AEB9712L	1.58 ± 2.22	AED974[b]	1.00 ± 1.00
AEB9713L	1.00 ± 1.00	AED977	915.05 ± 2.84
AEB9715L	1.00 ± 1.00	AED978	541.84 ± 3.40
AED9711L	16.07 ± 11.08	AED979	170.54 ± 1.71
AED9713	21.79 ± 15.02	AEB975	181.81 ± 4.57
AED9714	1.00 ± 1.00	AEB9710	2164.05 ± 1.10
AED9715	1.00 ± 1.00	AEB9711	293.99 ± 2.91
AED9716S	1.00 ± 1.00	AEB9712S	636.06 ± 4.55
AEB9721L	1.00 ± 1.00	AEB9713S	462.85 ± 1.87
AEB9722S	1.00 ± 1.00	AEB9714	989.15 ± 2.03
AEB9722L	1.00 ± 1.00	AEB9715S	1068.93 ± 2.30
AEB9727[b]	1.00 ± 1.00	AEB9716S	825.15 ± 3.20

Table 10 continue

Isolate	CDT titer[a]	Isolate	CDT titer[a]
C. jejuni		*C. jejuni*	
AEB9716L	56.67 ± 4.56	AED9725	841.21 ± 4.13
AEB9717	63.91 ± 2.85	AED9726	402.30 ± 8.33
AEB9718	326.16 ± 1.60	AED9727	1058.04 ± 1.96
AEB9719	513.40 ± 1.10	AED9728	878.04 ± 1.98
AEB9720	156.07 ± 2.99	AEB9721S	118.72 ± 4.49
AED9711S	1243.58 ± 1.86	AEB9723	394.51 ± 4.49
AED9712	454.78 ± 2.37	AEB9724S	428.82 ± 2.04
AED9716L	226.22 ± 2.05	AEB9724L	323.89 ± 2.22
AED9717S	107.36 ± 9.98	AEB9725	819.15 ± 2.51
AED9717L	219.80 ± 2.72	AEB9726	252.57 ± 2.14
AED9718	568.66 ± 3.47	AEA971	421.20 ± 2.29
AED9719	506.80 ± 3.75	AEA972S	300.13 ± 4.79
AED9720	499.34 ± 3.46	AEA972L	472.80 ± 3.15
AED9721	723.55 ± 2.42	AEA973S	379.31 ± 2.12
AED9722	742.02 ± 4.63	AEA974S	1041.12 ± 3.15
AED9723	996.68 ± 3.32	AEA975	1257.67 ± 1.93
AED9724	879.83 ± 5.48		

[a] CDT titers were calculated as geometric mean ± SD from HeLa cell assay results as described in materials and methods.

[b] Strains could not be identified by API Campy, but successfully speciated by species specific PCR.

was previously shown in Southern hybridization experiments to possess *cdtB* sequences (Pickett et al., 1996), although both the size of the *Cla*I fragment from this strain to which the *cdtB* probe hybridized and the strength of the hybridization were atypical. These results indicate that the *cdtB* gene from strain D1816 likely differs from the *cdtB* genes of most *C. jejuni* strains at many nucleotide positions. In any case, these two strains are clearly unusual *C. jejuni* strains; the typical *C. jejuni* strain appears to have *cdt* sequences that are readily amplified by any of the four primer pairs described in this work.

At the start of this study, the primers and restriction endonucleases used were based on the available *cdt* gene nucleotide sequence from *C. jejuni* strain 81-176 (Pickett et al., 1996). While our study was ongoing, partial *cdt* sequence data from *C. coli* strain D730 became available from Dr. Pickett's laboratory (unpublished data). This sequence information enabled the use of primers based on homologies between *C. jejuni* and *C. coli cdtA* and *cdtB* genes, but not in *cdtC* gene, which was not sequenced until very recently. In addition, *cdt* sequence information was helpful in selecting appropriate restriction enzymes for analysis of the *cdt*-PCR products. *C. coli cdtC* sequence information indicates that there is considerable variation between the *cdtC* gene sequences of *E. coli* 9142-88 (Pickett et al., 1994) and *C. jejuni* 81-176 (Pickett et al., 1996) and *C. coli* D730 (Pickett, unpublished data). This may be one reason why some of the *cdtC* –based reverse primers failed, since most of them were designed based on relatively conserved regions between *E. coli* 9142-88 (Pickett et al., 1994) and *C. jejuni* 81-176 (Pickett et al., 1996), prior to obtaining *cdtC* sequences from a *C. coli* strain.

Currently, two new PCR primers that are based on conserved regions of *C. jejuni* 81-176 (Pickett et al., 1996) and *C. coli* D730 (Pickett, unpublished data) *cdtC* genes are

being tested on selected *Campylobacter* strains.

In addition,VAT2 and C-ECI product sizes differed for *C. jejuni* and *C. coli* strains. Since *cdtC* gene sequence information from a *C. coli* strain was not available when this work was done, this difference was considered to be due to differences between *cdtC* sequences of these two species. Recently sequencing of the *C. coli* D730 *cdtC* gene was completed and was found as the same size as the *C. jejuni cdtC* gene and that the primer likely does not anneal to the same region of the *C. coli cdtC* gene as it does in the *C. jejuni cdtC* genes. The region downstream of the *cdtC* gene is also going to be sequenced to see if a possible annealing site for this primer exists in an appropriate location. This may probably be the case since the product formed is cut by *Bgl*II and *Ssp*I to produce fragments of the expected sizes for amplification of the relevant *cdt* sequences.

Overall, PCR product restriction analysis results suggest that ther may be few variations in the *cdt* genes of *C. jejuni* strains. However, we did not analyze this with many restriction enzymes. The coding regions of the 3 different *C. jejuni* strains' *cdt* genes are found to be essentially remarkably similar (Newell, personal communication; Pickett, personal communication). Also extended restriction fragment length polymoprphism analysis on *C. jejuni* strains showed that they are similar (Wassenaar, personal communication). These data suggest that there is a high degree of nucleotide sequence conservation within the *cdt* coding regions of different *C. jejuni* strains. There is not enough nucleotide sequence data from different *C. coli* strains. Thus, while it is clear from analysis of all known *cdt* genes that sequence variation can be tolerated within *cdt* genes, such that the encoded variant Cdt proteins can still be functional, nonetheless

the *cdt* sequences of *C. jejuni* and *C. coli* isolates may be quite conserved. This is in contrast to what has been found for isolates of *E. coli*, where the *cdt* genes from three different strains exhibit numerous dissimilarities (Peres et al., 1997; Pickett et al., 1994; Scott and Kaper, 1994). These results also indicate that *C. jejuni* and *C. coli cdt* genes can be distinguished simply by *Eco*RI digestion of VAT2/WMI1, A-GNW/WMI1 or A-IVH/WMI1 PCR products analyzed in this work, since the *Eco*RI site present in the *C. jejuni cdtB* gene is not present in the *C. coli cdtB* gene. In addition, *Stu*I, which was tested only on VAT2/WMI1 PCR products, appears to be an alternative restriction enzyme for differentiation of *C. jejuni* and *C. coli cdt* gene sequences.

All 105 chicken isolates were assayed for CDT production. Sixty five of seventy *C. jejuni* isolates produced significant levels of CDT, yet all of the *C. coli* strains appeared to produce little or no toxin. Results obtained from these isolates confirm and extend the previous results obtained by Pickett et al. (1996), in which it was originally noticed that *C. jejuni* strains appear to have significantly greater CDT activity than *C. coli* strains when assayed on HeLa cells. These findings also continue to indicate that only occasional *C. jejuni* strains fail to produce active CDT, apparently regardless of the source. These results are in contrast to the findings of Johnson and Lior (1988), in which they reported that only 42% of *C. jejuni* and *C. coli* isolates produced CDT. In the HeLa assays (Pickett et al., 1996) sonic lysates of each culture was used, in contrast to unconcentrated culture filtrates used byJohnson and Lior (1988). CDT activity in culture supernatants is not optimal; a strain producing low CDT levels may not have detectable CDT in its culture supernatant. Whether the differences between the *C. jejuni* and *C. coli* CDTs seen in the HeLa assay reflect real differences in the amount of toxin produced, or

reflect differences in the specific activities of these two toxins, or reflect differences in their abilities to intoxicate HeLa cells, is not yet known. Regardless, it is clear that CDT production by *C. jejuni* strains appears to be nearly universal.

Since CDT production by *C. coli* strains may be significantly below that of *C. jejuni* strains, the API Campy biochemical identification tests, and the species specific PCR tests (Stonnet and Guesdon, 1993; Stonnet et al., 1995) of low or no-CDT producing *C. jejuni* strains were repeated to be sure that they were not misidentified *C. coli* strains. All of the strains identified as *C. coli* strains in this work were consistently identified as *C. coli* strains by both methods. Both *C. jejuni* strain D1816, a human spinal fluid isolate that produced a mean CDT titer of 22± 16, and four *C. jejuni* isolates from chicken carcasses with mean titers ranging from 56 ± 2 to 87 ± 2, were similarly verified to be *C. jejuni* strains by both of these methods. However, *C. jejuni* AED974, the strain that produced no detectable toxin, was identified as *C. jejuni* by species specific PCR (Stonnet and Guesdon, 1993; Stonnet et al., 1995), but gave a non-typical result with the API Campy. Nonetheless, the only possible species assignment that could be made was *C. jejuni*, since the API Campy result was closer to the expected code for *C. jejuni* than for any other species. A hippurate hydrolysis test of strain AED974 was negative. This is unfortunately inconclusive since it is well known that some *C. jejuni* strains are hippurate hydrolysis negative. Currently, hybridization studies are being pursued in our laboratory that should provide more conclusive data about whether this strain should be identified as *C. jejuni*.

Hundred percent of the chickens examined in our study were found to be contaminated with either *C. jejuni* or *C. coli* or both. Isolation rates of *Campylobacter*

from retail chicken may vary depending upon the sampling time of the year, number of samples collected, the sampling procedures, isolation methodology, and whether the sample is fresh or frozen (Bryan and Doyle, 1995; Stern and Line, 1992; Wallace et al., 1997; Willis and Murray, 1997). Other studies in which whole chicken carcasses purchased at the retail level were sampled for *Campylobacter* species using sampling procedures similar to ours (DeBoer and Hahne, 1990; Gill and Harris, 1984; Jones et al., 1991; Oosterom et al., 1983; Park et al., 1981; Stern et al., 1985; Waldroup et al., 1992) have reported isolation rates between 30% and 98%. Whether our relatively high isolation rate is a reflection of increased incidence of *Campylobacter* species on chicken carcasses, or results from a combination of optimal sampling season with optimal isolation procedure, is not clear.

Most previous studies have not differentiated between *C. jejuni* and other *Campylobacter* species, and have instead reported the total *Campylobacter* isolation rate as the *C. jejuni* isolation rate, assuming that most of the *Campylobacter* species present would be *C. jejuni*. In our study, we used two techniques for species identification. The two methods used, the API Campy identification kit and species specific PCR, were essentially in agreement, except that two isolates were not conclusively identified by the API Campy kit. However, the PCR method identified one of these isolates as *C. jejuni* and the other as *C. coli*. Taken together, the two methods indicated that 67% of our isolates were *C. jejuni* and 33% were *C. coli*. No *C. lari* isolates were obtained. These results certainly tend to confirm that *C. jejuni* is the predominant *Campylobacter* species present on fresh chicken carcasses, although the percentage of *C. coli* isolated was substantial, and it seems prudent to not dismiss the amount of *C. coli* present as

insignificant. In addition, since eight of the fourteen chickens from which two isolates were obtained yielded isolates of both species, it may not be uncommon for a single chicken carcass to be contaminated with more than one species or strain of *Campylobacter*.

This study demonstrated the possible universal presence of *cdt* genes in *C. jejuni* and *C. coli* isolates regardless of the source. But of course, the presence or prevalence of these genes in other *Campylobacter* species or in closely related genera, such as *Arcobacter* and *Helicobacter* is unknown. It would be of interest to determine whether *cdt* genes are present in these bacteria.

Whether *cdt* genes are as widespread in *C. jejuni* and *C. coli* strains isolated from live chickens, pigs, or cattle as they proved to be on strains isolated from chicken carcasses is also unknown. It may be of epidemiological value to determine this prevalence.

The HeLa assay results in this study indicated no significant toxin production by *C. coli* isolates. In future work, it might be of interest to determine whether this difference is a reflection of different cell-line sensitivities to these two toxins, or whether *C. coli* produces less CDT, or a less-active CDT.

Most importantly, it is not yet known what role CDT plays in foodborne illness. Animal model studies testing strains with defined mutations within their *cdt* genes will help elucidate the role of CDT in *Campylobacter* pathogenesis.

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Publications.

Peer Reviewed Articles.

1. Carli, K. T., K. S. Diker, and **A. Eyigor**. 1995. Sulphate-reducing bacteria in bovine feces. Lett. Appl. Microbiol. 21:228-229.

2. Akan, M., K. S. Diker, and **A. Eyigor**. 1998. Motile aeromonads in the feces and

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