

# Antimicrobial Resistance, Virulence, and Genotypic Profile Comparison of *Campylobacter jejuni* and *Campylobacter coli* Isolated from Humans and Retail Meats

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## Abstract

A total of 360 spatially and temporally related *Campylobacter* isolates, including 168 from clinical human cases (*Campylobacter jejuni*  $n = 148$ ; *Campylobacter coli*  $n = 20$ ) and 192 from retail meats (*C. jejuni*  $n = 114$ ; *C. coli*  $n = 78$ ), were analyzed for antimicrobial susceptibilities, virulence, and genotypic profiles. Ciprofloxacin-resistant *C. jejuni* was observed in 13.5% and 19% of the isolates from humans and retail chicken breasts, respectively. Antimicrobial resistance to ciprofloxacin and erythromycin was detected in *C. coli* isolates recovered from 29% and 16.6% of retail meats and 15% and 5% humans, respectively. Overall, virulence determinants were more prevalent in *Campylobacter* isolates recovered from retail meats than from humans. *C. jejuni* isolates from humans were significantly associated with the *rakR*, *dnaJ*, and *pld* genes, whereas *C. coli* isolates from retail meats were associated with the *dnaJ*, *pld*, and *virB11* virulence genes. Genotyping of 262 *C. jejuni* isolates using pulsed-field gel electrophoresis revealed a total of 186 unique *SmaI* patterns, with 14% of patterns composed of isolates recovered from retail meats and ill humans. All unique groups with indistinguishable *SmaI* patterns were further analyzed by a second restriction enzyme (*KpnI*), which revealed limited overlap between isolates from different sources. Significant association between doxycycline-resistant *C. jejuni* strains recovered from humans and different virulence genes (e.g., *cdtB*) was identified at the statistical level but not at the genotypic level. In conclusion, significant differences observed in the distribution of antimicrobial resistance profiles, virulence determinants, and genotypic diversity among *C. jejuni* and *C. coli* isolates indicate that there are sources other than retail meats that may also contribute to human *Campylobacter* infections.

## Introduction

**C**AMPYLOBACTER IS ONE of the leading causes of foodborne diarrhea illness in the United States and a significant public health concern worldwide. In humans, infections caused by *Campylobacter jejuni* and *Campylobacter coli* are well known and are generally transmitted through water, milk, and food animals (Hänninen *et al.*, 2003; Litrup *et al.*, 2007; Kwan *et al.*, 2008; Huang *et al.*, 2009). Retail meat products, including poultry, have been frequently implicated as sources of infection, and are a major risk factor for campylobacteriosis in humans (Zhao *et al.*, 2001; Ge *et al.*, 2003; Meldrum and Wilson, 2007). Besides the host factors and sources of infection, the pathogenicity and survivability of *Campylobacter*

strains is also dependent on the antimicrobial resistance mechanisms and expression of distinct virulence factors (Luo *et al.*, 2005; Young *et al.*, 2007).

The majority of *Campylobacter* infections are sporadic and self-limiting in nature, which makes it difficult to accurately determine the true incidence rate. Due to the self-limiting nature of campylobacteriosis, antimicrobials are usually not recommended for treatment except in severe cases where fluoroquinolones and macrolides are the treatment options of choice (Butzler, 2004; Yates *et al.*, 2005). Frequency of resistance to these important classes of antimicrobials has reported to be on the rise in the United States and globally (Gupta *et al.*, 2004; Rozynek *et al.*, 2008; NARMS, 2007). Additionally, multidrug-resistant *C. jejuni* and *C. coli* have been reported

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from food animals and retail meats, including poultry and swine (Zhao *et al.*, 2001; Datta *et al.*, 2003; Ge *et al.*, 2003; Gebreyes *et al.*, 2005; Thakur and Gebreyes, 2005; Thakur *et al.*, 2006; Mena *et al.*, 2008; Pointon *et al.*, 2008).

Several putative virulence factors have been identified in *Campylobacter* species that contribute to motility, intestinal adhesion, colonization, toxin production, and invasion. Adhesion of the pathogen to the intestinal epithelium is important for colonization and to increase the secretion of bacterial toxins. An important adhesion is the outer membrane protein CadF, which is conserved in both *C. jejuni* and *C. coli* and binds to fibronectin in the gut, thereby helping the pathogen in adhesion and invasion (Konkel *et al.*, 2001; Müller *et al.*, 2006). Motility is via flagella, which is coded by the flagellin gene (*flaA*), and is important for the bacteria to reach the attachment sites in the intestine (Konkel *et al.*, 2004). Heat shock proteins, including DnaJ, are associated with the thermal stress response and have been shown to play an important role in pathogenesis and colonization (Snelling *et al.*, 2005). Other important genes that have been associated with *Campylobacter* pathogenesis include *pldA* (Zheng *et al.*, 2006), the plasmid-encoded *virB11* (Bacon *et al.*, 2000), and *racR* (Datta *et al.*, 2003). Cytolethal distending toxin production, encoded by the *cdtA*, *cdtB*, and *cdtC* genes, damages host enterocytes and facilitates penetration of the intestinal epithelium (Rozynek *et al.*, 2005). Carriage of these genes is variable, and helps explain virulence differences among strains.

Recent studies have been conducted to determine the association of virulence genes and antimicrobial resistance in important bacterial pathogens such as *Escherichia coli* and *Enterococcus faecalis* (Bagger-Skjøt *et al.*, 2007; McGowan-Spicer *et al.*, 2008). Ciprofloxacin-resistant *C. jejuni* strains have been shown to have an enhanced *in vivo* fitness over isogenic susceptible *C. jejuni* strains in the chicken gut even in the absence of fluoroquinolone selection pressure (Luo *et al.*, 2005), suggesting a linkage between resistance and colonization potential. Studies have also shown that humans infected with antimicrobial-resistant *Campylobacter* species have a longer duration of diarrhea when compared with antimicrobial-susceptible strains (Nelson *et al.*, 2004; Helms *et al.*, 2005). The major objective of this study, therefore, was to determine the prevalence and association of virulence genes and antimicrobial resistance profiles at the statistical and genotypic levels in different strain types of *C. jejuni* and *C. coli* isolated from retail meats and clinically ill humans.

## Materials and Methods

### Isolate source

*Campylobacter* isolates in this study were obtained from a baseline surveillance project conducted in Iowa from June 2001 to June 2002 (Hayes *et al.*, 2003). A total of 814 retail meat samples were collected, consisting of 209 samples each of chicken breast, pork chop, and ground beef and another 187 ground turkey. *Campylobacter* was isolated using standard bacteriological methods and speciated by polymerase chain reaction (PCR) (Zhao *et al.*, 2001; Thakur and Gebreyes, 2005). In addition, 148 *C. jejuni* and 20 *C. coli* isolates from clinically ill human patients collected in Iowa during the same period were included for comparison. A total of 262 *C. jejuni* and 98 *C. coli* were recovered for analysis.

### Antimicrobial susceptibility testing

We used agar dilution to determine the minimal inhibitory concentrations (MIC) for four antimicrobials, following the Clinical and Laboratory Standards Institute protocol (CLSI, 2006), and *C. jejuni* ATCC 33560 as the quality control organism. Clinical and Laboratory Standards Institute interpretative criteria were used for ciprofloxacin (Cip, 4 µg/mL), erythromycin (Ery, 32 µg/mL), and doxycycline (Dox, 8 µg/mL) (CLSI, 2006). For gentamicin (Gen), the breakpoint level of 8 µg/mL was used following the National Antimicrobial Resistance Monitoring System criteria (NARMS, 2007). Multidrug resistance (MDR) in this study was defined as isolate exhibiting resistance to two or more antimicrobials at the same time.

### Virulence gene characterization

Genomic DNA used for PCR was purified using the UltraClean Microbial DNA Kit following the manufacturer's instructions (MoBio Laboratories, Carlsbad, CA). The presence of virulence and toxin genes, including *flaA*, *cadF*, *rakR*, *cdtA*, *cdtB*, *cdtC*, *dnaJ*, *pld*, and *virB11*, were determined as described previously (Datta *et al.*, 2003; Zheng *et al.*, 2006). The primers were developed based on conserved sequences that were available in the GenBank database. A separate set of primers was developed for virulence genes where difference in the sequences were noticed between *C. jejuni* and *C. coli*. All PCR amplifications were performed in total reaction volumes of 50 µL consisting of 0.1 µg of template DNA, 0.25 mM concentration of deoxynucleotide triphosphate, 1.5 mM MgCl<sub>2</sub>, 50 pmol of each primer, and 0.2 U of AmpliTaq Gold (Perkin-Elmer, Foster City, CA). PCR cycling conditions included an initial denaturation of 94°C for 10 minutes followed by 30 cycles of 94°C for 30 seconds, annealing at primer-specific pair for 30 seconds, followed by extension at 72°C for 20 seconds. A final extension step was done at 74°C for 6 minutes. The final PCR products were run on 1.5% agarose gel mixed with ethidium bromide for analyzing the result.

### Pulsed field gel electrophoresis

We used the U.S. Centers for Disease Control–standardized protocol for genotyping *Campylobacter* species by pulsed-field gel electrophoresis (PFGE) with slight modifications (Ribot *et al.*, 2001). Fresh *Campylobacter* cells were suspended in phosphate-buffered saline (0.01 mol/L phosphate, pH 7.4; 0.85% NaCl) and adjusted to a reading of 0.42 ± 0.02 by Dade Microscan Turbidity Meter (Dade Behring, West Sacramento, CA). Next, genomic DNA embedded in agarose was digested with *Sma*I restriction enzyme (Boehringer Mannheim, Indianapolis, IA). The digested DNA was then separated by electrophoresis in 0.5×TBE (Tris-Borate 15 EDTA) at 14°C and 6V/cm for 17 hours using a CHEF DR-III apparatus (Bio-Rad Laboratories, Hercules, CA) with pulse times of 6.7–38.4 seconds. *Salmonella enterica* serovar Braenderup H9812 was used as the reference marker. Gels were stained with ethidium bromide for 30 minutes and photographed under UV transillumination (Bio-Rad Laboratories). *Campylobacter* isolates showing identical patterns were further digested with a second restriction enzyme *Kpn*I (Boehringer Mannheim). This was done to determine whether the use of a second restriction

enzyme could further discriminate between the clusters created by the *Sma*I restriction enzyme.

#### Statistical and data analysis

The frequency of antimicrobial resistance patterns and MIC levels comparison between *Campylobacter* isolates from different sources were compared using the  $\chi^2$ -test (Minitab, PA) and Fisher's exact two-tailed test when applicable. Significant association of antimicrobial resistance and virulence gene profile for the two *Campylobacter* species and isolation source was determined using the odds ratio test (Egret version 2.0.3; Cytel, Cambridge, MA). Results with a Type I error of  $p \leq 0.05$  was considered statistically significant. Analysis of PFGE data was performed using Bionumerics 4.0 software (Applied Maths, Kortrijk, Belgium) after normalization of the three *Salmonella* control lanes on each gel. The banding patterns were compared using the Dice coefficient and unweighted pair group method using arithmetic averages with 0.45% optimization and 0.85 position tolerance for *Sma*I and 0.41% optimization and 1.5% position tolerance for *Kpn*I respectively. Isolate relatedness was determined using the unweighted pair group method using arithmetic averages.

## Results

### Antimicrobial resistance phenotypes

Antimicrobial resistance and the distribution of MIC<sub>50</sub>, MIC<sub>90</sub>, and MIC ranges to ciprofloxacin, doxycycline, eryth-

romycin, and gentamicin for the two *Campylobacter* species and their sources are shown in Table 1. Antimicrobial resistance to ciprofloxacin (Cip<sup>R</sup>) was observed in 13.5% and 19% of the *C. jejuni* isolates from humans and chicken breasts, respectively. None of the *C. jejuni* isolates were Ery<sup>R</sup>. However, 14.2% of the *C. coli* isolates, including those isolated from humans ( $n = 1$ ) and retail meats ( $n = 13$ ), displayed Ery<sup>R</sup>. Cip<sup>R</sup> *C. jejuni* from chicken breast had a higher MIC<sub>90</sub> (16  $\mu\text{g}/\text{mL}$ ) than those recovered from humans (0.25  $\mu\text{g}/\text{mL}$ ). The Ery MIC<sub>90</sub> distribution in *C. coli* from retail meats was  $>64 \mu\text{g}/\text{mL}$  compared to 8  $\mu\text{g}/\text{mL}$  from humans. Overall, the MIC<sub>50</sub> and the MIC<sub>90</sub> values to both Ery and Cip for *C. coli* were consistently higher compared with *C. jejuni* isolates across all sources. Within the retail meat types, significantly more *C. coli* isolates obtained from ground turkey displayed resistance to Cip, Ery, and Dox than isolates recovered from chicken breast ( $p < 0.01$ ). All tested *Campylobacter* isolates demonstrated susceptibility to gentamicin. We found a significantly higher frequency of pan-susceptible *C. jejuni* than *C. coli* from chicken breast ( $p < 0.001$ ).

MDR was more common in *C. coli* than in *C. jejuni* from all sources, with Ery<sup>R</sup>-Dox<sup>R</sup> (11.2%) being the dominant phenotype observed among overall *C. coli* isolates. Among *C. jejuni* isolates recovered from humans, the Cip<sup>R</sup>-Dox<sup>R</sup> MDR pattern (8.7%) was found to be the most prevalent (Table 2). Overall, *C. coli* isolates from ground turkey tended to be more MDR than *C. jejuni* including the Cip<sup>R</sup>-Dox<sup>R</sup> (54.5%) and the Cip<sup>R</sup>-Ery<sup>R</sup>-Dox<sup>R</sup> (27%) than from any other meat type or humans.

TABLE 1. COMPARISON OF ANTIMICROBIAL RESISTANCE PHENOTYPES AMONG *CAMPYLOBACTER* ISOLATES RECOVERED FROM HUMANS AND RETAIL MEATS

Antimicrobials and source	<i>Campylobacter jejuni</i>				<i>Campylobacter coli</i>			
	MIC <sub>50</sub> <sup>a</sup>	MIC <sub>90</sub> <sup>b</sup>	MIC range <sup>c</sup>	Resistant, n (%)	MIC <sub>50</sub>	MIC <sub>90</sub>	MIC range	Resistant, n (%)
Ciprofloxacin ( $\geq 4 \mu\text{g}/\text{mL}$ ) <sup>d</sup>								
Chicken breast <sup>e</sup>	0.25	16	0.06 to $>64$	21(19)	0.25	32	0.06–64	14 (21.5)
Ground turkey	0.25	0.25	0.125–0.25	0	32	64	0.25–64	9 (82)
Pork chops	0	0	0	0	0.25	0.25	0.25	0
Humans	0.25	0.25	0.06 to $>64$	20(13.5)	0.25	32	0.125–64	3 (15)
Doxycycline ( $\geq 8 \mu\text{g}/\text{mL}$ )								
Chicken breast	0.5	32	0.06 to $>32$	41(37)	8	32	0.125 to $>32$	36 (55)
Ground turkey	12	32	0.125–0.25	2(50)	32	32	0.25–32	10 (91)
Pork chops	0	0	0	0	16	32	16–32	2 (100)
Humans	32	32	0.125 to $>32$	94(56)	16	32	0.125 to $>32$	12 (60)
Erythromycin ( $\geq 32 \mu\text{g}/\text{mL}$ )								
Chicken breast	2	4	1–8	0	4	$>64$	0.5 to $>64$	9 (14)
Ground turkey	2	4	2–4	0	4	$>64$	4 to $>64$	2 (18)
Pork chops	0	0	0	0	$>64$	$>64$	$>64$	2 (100)
Humans	2	4	1–8	0	8	8	0.5 to $>64$	1 (5)
Gentamicin ( $\geq 8 \mu\text{g}/\text{mL}$ )								
Chicken breast	1	1	0.5–2	0	1	1	0.25–1	0
Ground turkey	1	1	1	0	0.5	1	0.5–1	0
Pork chops	0	0	0	0	1	1	1	0
Humans	1	2	0.5–2	0	1	2	0.5–2	0

<sup>a,b</sup>MIC<sub>50</sub> and MIC<sub>90</sub> indicate the concentration ( $\mu\text{g}/\text{mL}$ ) at which 50% and 90% of the isolates tested were susceptible to the antimicrobial, respectively.

<sup>c</sup>Indicates the MIC level for *Campylobacter* isolates at a particular antimicrobial concentration.

<sup>d</sup>Antimicrobials and associated resistance breakpoints ( $\mu\text{g}/\text{mL}$ ).

<sup>e</sup>Represents the number of *Campylobacter* isolates tested from different sources—*C. jejuni*: humans ( $n = 148$ ), chicken breast ( $n = 110$ ), and ground turkey ( $n = 4$ ); *C. coli*: humans ( $n = 20$ ), chicken breast ( $n = 65$ ), ground turkey ( $n = 11$ ), and pork chops ( $n = 2$ ).

MIC, minimal inhibitory concentrations.

TABLE 2. ANTIMICROBIAL RESISTANCE PATTERNS OBSERVED AMONG *CAMPYLOBACTER* ISOLATES RECOVERED FROM HUMANS AND RETAIL MEATS

Source (total isolates) <sup>a</sup>	Isolates, n		Antimicrobial resistance patterns, n (%) <sup>b</sup>	
	<i>C. jejuni</i>	<i>C. coli</i>	<i>C. jejuni</i>	<i>C. coli</i>
Chicken breast (175)	110	65	Pan 56 (51), Dox 33 (30), Cip 13 (12), Cip-Dox 8 (7.2)	Pan 18 (27.6), Cip 5 (7.6), Ery 11 (17), Dox 14 (21.5), Ery-Dox 8 (12.3), Cip-Dox 7 (10.7), Cip-Ery-Dox 2 (3)
Ground turkey (15)	4	11	Pan 2 (50), Dox 2 (50)	Ery 1 (9), Cip-Dox 6 (54.5), Ery-Dox 1 (9), Cip-Ery-Dox 3 (27)
Pork chops (2)	0	2	None	Ery-Dox 2 (100)
Humans (168)	148	20	Pan 47 (31.7), Cip 7 (4.7), Dox 81 (54.7), Cip-Dox 13 (8.7)	Pan 7 (35), Cip 1 (5), Dox 9 (45), Ery-Dox 1 (5), Cip-Dox 2 (10)

<sup>a</sup>Represents the number of *Campylobacter* isolates tested from different sources—*C. jejuni*: humans ( $n=148$ ), chicken breast ( $n=110$ ), and ground turkey ( $n=4$ ); *C. coli*: humans ( $n=20$ ), chicken breast ( $n=65$ ), ground turkey ( $n=11$ ), and pork chops ( $n=2$ ).

<sup>b</sup>Number and percent of isolates displaying resistance to antimicrobial(s).

Cip, ciprofloxacin; Dox, doxycycline; Ery, erythromycin; Pan, pansusceptible.

Both the *C. coli* isolates from pork chops exhibited the Ery<sup>R</sup>-Dox<sup>R</sup> MDR pattern.

#### Virulence gene profiles

Overall, virulence determinants were found to be more prevalent in isolates recovered from retail meats than humans ( $p < 0.01$ ) (Fig. 1). The majority (95%) of *Campylobacter* isolates were positive for three or more virulence genes. Among the retail meat isolates, we found six *C. jejuni* (including five from chicken breast and a single from ground turkey) and a single *C. coli* isolate from chicken breast that were negative for all nine tested virulence determinants. All *C. jejuni* isolates from humans except one were positive for the *cadF* adhesion gene. The *cadF* gene was found significantly higher in *C. jejuni* from humans than from retail meats ( $p < 0.05$ ). Among the human isolates, *flaA* was present in a significantly higher number of

*C. jejuni* isolates than in *C. coli* ( $p < 0.001$ ). The cytolethal distending toxin gene cluster (*cdtA*, *cdtB*, and *cdtC*) was detected in both *C. jejuni* and *C. coli* from different sources, with a higher proportion of *C. jejuni* from ill humans than for all the meat types combined. We detected significant differences in the prevalence of *rakR*, *dnaJ*, *pld*, and *virB11* genes depending on the *Campylobacter* species and source. All *Campylobacter* isolates were negative for the *virB11* gene, except for *C. coli* isolated from retail meats (45%). In addition, the *rakR*, *dnaJ*, and *pld* genes were found in a significantly higher number of *C. jejuni* isolates recovered from humans as compared to retail meats ( $p < 0.01$ ). An important observation was that none of the *C. coli* isolates from humans possessed any of the above virulence determinants. However, a significantly higher number of *C. coli* isolates from retail meats were positive for the *dnaJ*, *pld*, and *virB11* genes than *C. jejuni* isolates ( $p < 0.001$ ).

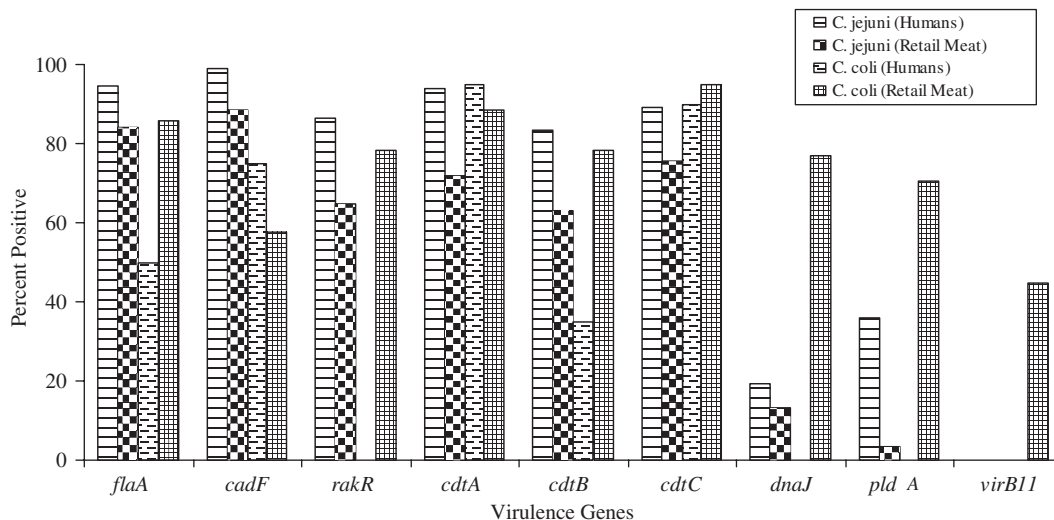


FIG. 1. Prevalence of virulence genes among the *Campylobacter jejuni* and *Campylobacter coli* isolates recovered from humans and retail meats. The number of *Campylobacter* isolates tested from different sources include *C. jejuni*: humans ( $n=148$ ), chicken breast ( $n=110$ ), and ground turkey ( $n=4$ ); *C. coli*: humans ( $n=20$ ), chicken breast ( $n=65$ ), ground turkey ( $n=11$ ), and pork chops ( $n=2$ ).

*Campylobacter* PFGE profiles

A total of 252 *C. jejuni* and 98 *C. coli* isolates were first typed using *Sma*I restriction enzyme. The *C. jejuni* isolates were grouped into 182 unique PFGE patterns (represented by a single *C. jejuni* isolate) and 43 clusters that were represented by multiple isolates (data not shown). The majority ( $n = 31$ ) of these 43 clusters were composed of isolates recovered from either ill humans or a particular retail meat. Sixty-seven unique *Sma*I PFGE patterns (represented by a single *C. coli* isolate) were identified among the 98 *C. coli* isolates, with 23 patterns had isolates from both humans and retail meats. We found three major clusters in the *C. coli* population including humans ( $n = 5$ ), chicken breast ( $n = 5$ ), and the largest cluster ( $n = 7$ ) that was comprised of isolates predominantly recovered from chicken breasts with the exception of a single ground turkey isolate.

A total of 38 *C. jejuni* isolates representing the 43 common clusters generated by *Sma*I PFGE patterns were further analyzed with *Kpn*I restriction enzyme. These 38 isolates were selected based on the source, date of isolation, and antimicrobial resistance profile. The *Kpn*I digestions produced 33 patterns from the 38 isolates and were represented by three large clusters based on 60% similarity in their banding pattern (Fig. 2). Cluster A was the largest with 21 isolates, followed by clusters B ( $n = 8$ ) and C ( $n = 9$ ). Clusters A and C were composed of *C. jejuni* isolates from chicken breast and ill humans,

with a single isolate each from a chicken breast and human having the same PFGE fingerprint but different virulence gene profiles. Cluster B was represented by isolates from chicken breast, ground turkey, and humans. We found identical subtypes shared between isolates from ground turkey and humans and between isolates from chicken breast and humans. *Kpn*I analysis of the 23 *C. coli* patterns, which included 44 isolates, revealed no common patterns among isolates recovered from retail meats and humans (data not shown). We did, however, find four clusters composed of *C. coli* isolates from chicken breast. We also found several *C. jejuni* and *C. coli* isolates were nontypeable using either *Sma*I or *Kpn*I enzymes and appears to be a case of DNA smearing rather than restriction.

*Associations between virulence genes, antimicrobial resistance, and genotypic profiles*

An important aspect of this study was to determine whether significant associations between antimicrobial resistance and virulence determinants could be ascertained among the recovered *Campylobacter* isolates (Table 3). Among Dox<sup>R</sup> *C. jejuni* strains, those from human clinical cases ( $n = 94$ ) and chicken breast ( $n = 41$ ) were significantly associated with the majority of the virulence determinants tested, with odds ratio ranging from 3.42 for *cdtB* (95% confidence interval 1.5–7.7) to 21.63 for *pld* (95% confidence interval 3.5–128). No significant

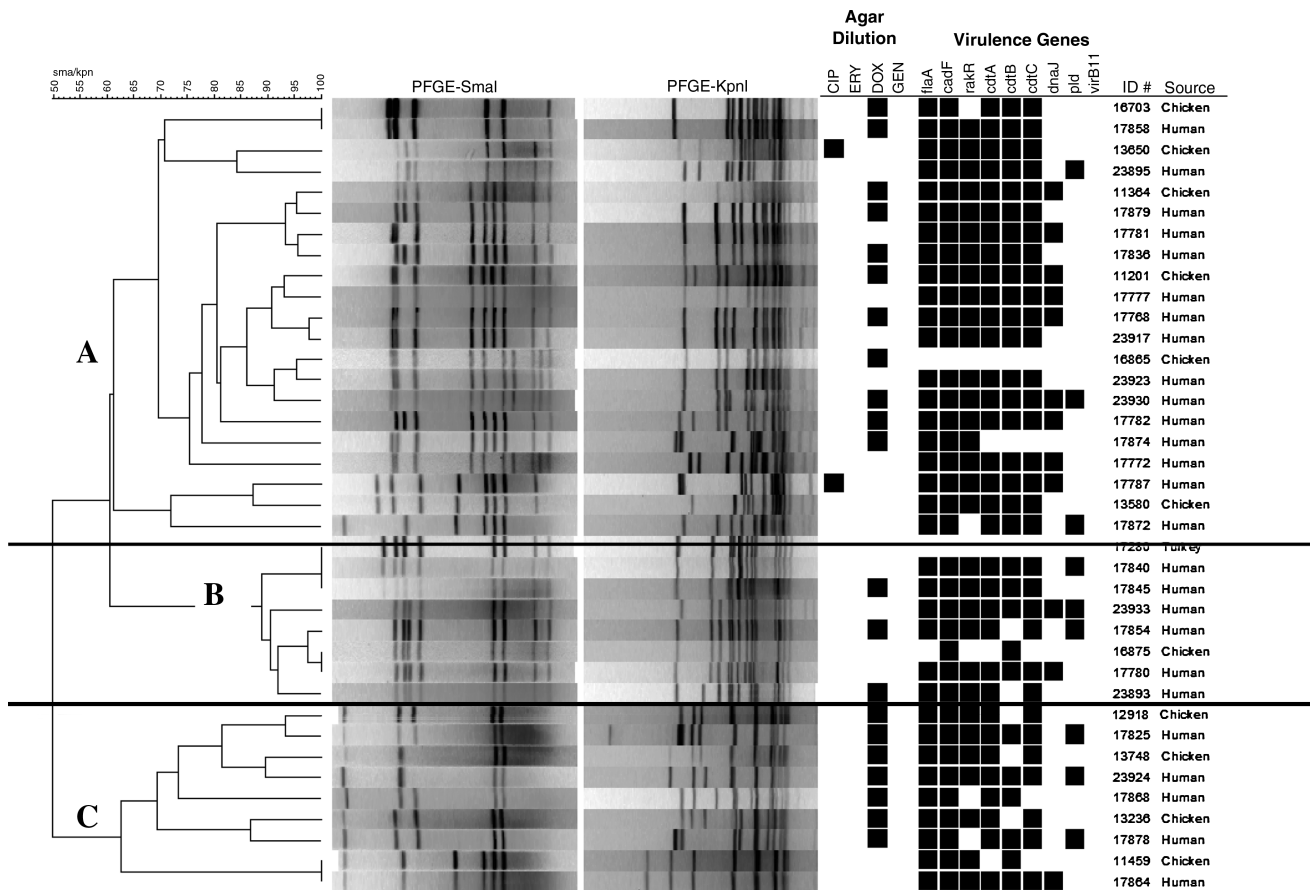


FIG. 2. Dendrogram highlighting the pulsed-field gel electrophoresis genotypic patterns, virulence, and antimicrobial resistance profile of *C. jejuni* isolates recovered from humans and retail meats.

TABLE 3. PERCENT DISTRIBUTION OF *CAMPYLOBACTER* VIRULENCE GENES BASED ON SOURCE, SPECIES, AND ANTIMICROBIAL RESISTANCE TO EITHER CIPROFLOXACIN OR DOXYCYCLINE

Species	Antimicrobial resistance and source <sup>a</sup>	Virulence determinants (%) <sup>b</sup>								
		flaA	cadF	rakR	cdtA	cdtB	cdtC	dnaJ	pld	virB11
<i>C. jejuni</i>	Cip <sup>R</sup> human ( <i>n</i> = 20)	95	100	95	95	75	80	15	50	0
	Cip <sup>R</sup> chicken breast ( <i>n</i> = 21)	86	100	76	86	62	91	10	0	0
	Dox <sup>R</sup> human ( <i>n</i> = 94)	95	99	93	96	83	92	17	35	0
	Dox <sup>R</sup> chicken breast ( <i>n</i> = 41)	90	88	66	81	59	85	12	2	0
<i>C. coli</i>	Cip <sup>R</sup> human ( <i>n</i> = 3)	0	67	0	100	67	100	0	0	0
	Cip <sup>R</sup> chicken breast ( <i>n</i> = 14)	93	71	79	86	79	100	71	64	43
	Dox <sup>R</sup> human ( <i>n</i> = 12)	58	67	0	100	50	100	0	0	0
	Dox <sup>R</sup> chicken breast ( <i>n</i> = 36)	81	58	75	83	75	92	72	72	50

<sup>a</sup>Represents the number of *Campylobacter* species isolated from humans and chicken breast with a particular antimicrobial resistance profile; "R" denotes resistance.

<sup>b</sup>Percent of isolates positive for tested virulence determinants.

statistical association with any virulence gene was detected in Cip<sup>R</sup> *C. jejuni* isolates either from humans or from chicken breast. We identified two clusters (based on 50% similarity) in the PFGE dendrogram generated by restricting *C. jejuni* and *C. coli* isolates (*n* = 29) with the two restriction enzymes that displayed resistance to both Cip and Dox (Fig. 3). None of the *Campylobacter* isolates in the dendrogram shared the same PFGE fingerprint. Cluster A was represented by both *C. jejuni* and *C. coli* isolates recovered from humans, chicken breast, and ground turkey. Cluster B was represented by only *C. coli* isolates recovered from the three sources as described above. Another distinct difference between the two clusters was the complete absence of *virB11*-positive isolates in cluster A. Both

the Ery<sup>R</sup> *C. coli* isolates, one each from chicken breast and ground turkey, were also restricted to cluster B.

## Discussion

*Campylobacter* is among the leading causes of bacterial foodborne gastroenteritis cases in the United States, and consumption of contaminated retail meat products is a major risk factor for humans. Subgrouping of *Campylobacter* isolates is usually done by utilizing PFGE profiles in outbreak investigations and for studying epidemiological trends. Antimicrobial resistance provides an additional level of strain information that can help describe the epidemiology of

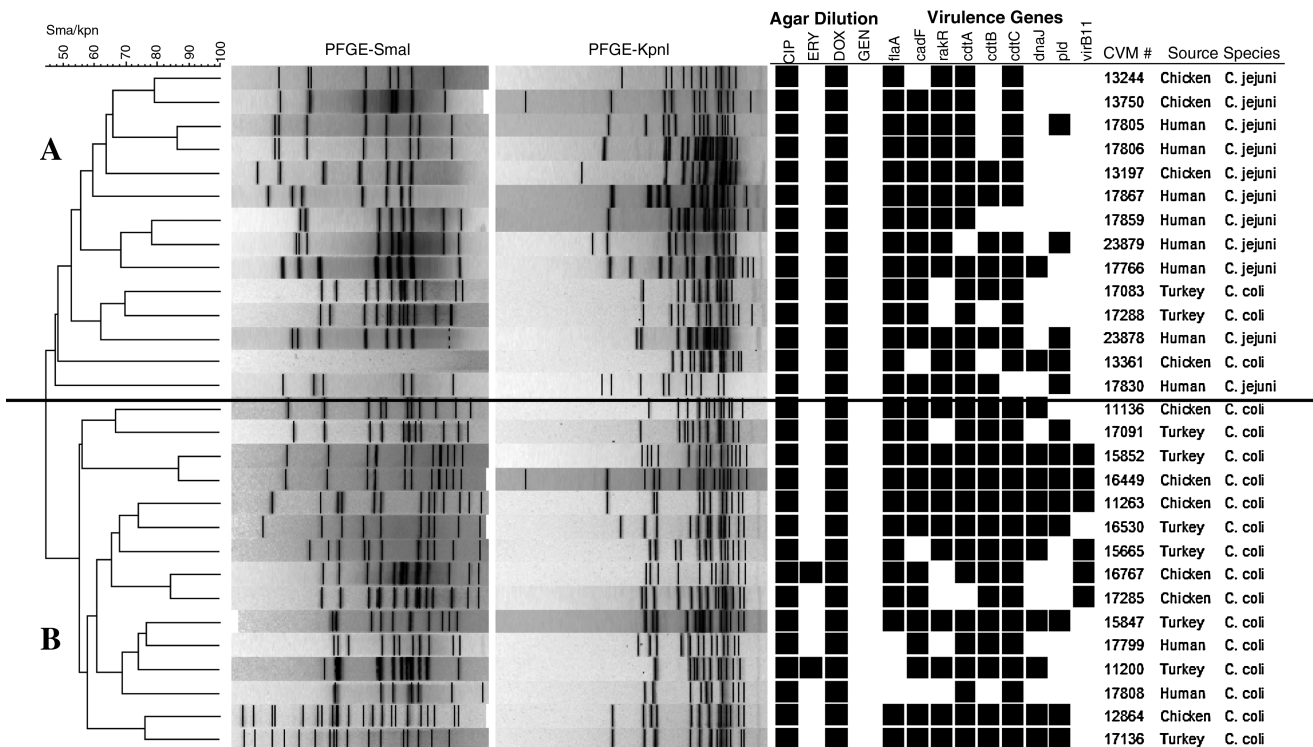


FIG. 3. Genotypic association between virulence and antimicrobial resistance profile of *C. jejuni* and *C. coli* isolates from humans and retail meats.

*Campylobacter*. The antimicrobial susceptibility results of our study are comparable to others showing that resistance is generally more common in *C. coli* than in *C. jejuni*, including resistance to fluoroquinolones and macrolides (Ge *et al.*, 2003; Thakur and Gebreyes, 2005; Thakur *et al.*, 2006; Little *et al.*, 2008). A recent study from Poland reported 40% of the *C. jejuni* isolates obtained from humans and chickens to be Cip<sup>R</sup> (Rozynek *et al.*, 2008), whereas another from the United Arab Emirates reported 85.4% of *C. jejuni* from humans to be fluoroquinolone resistant (Sonnevend *et al.*, 2006). The retail meat samples in our study were collected from 2001 to 2002 when fluoroquinolones were still approved for use in the poultry industry. The impact of removal of these antimicrobials from the poultry industry on rates of resistance among *Campylobacter* in the United States in 2005 has yet to be fully determined. However, the National Antimicrobial Resistance Monitoring System 2007 executive report on retail meats highlights the steady increase in the frequency of Cip<sup>R</sup> in *C. jejuni* up from 15.2% in 2002 to 17.2% in 2007, whereas in *C. coli* it is more concerning and has gone up from 10% in 2002 to 25.9% in 2009 (NARMS, 2007).

Among the isolates from retail meat, both *C. jejuni* and *C. coli* from ground turkey samples had the highest frequency of Cip<sup>R</sup>, Ery<sup>R</sup>, and Dox<sup>R</sup>. Our results are in agreement with other studies that have reported similar findings with larger numbers of *Campylobacter* isolates from ground turkey exhibiting antimicrobial resistance (Ge *et al.*, 2003; D'lima *et al.*, 2007). In the United States, Ery<sup>R</sup> in *C. coli* isolates from ground turkey has gone up from 0% in 2002 to 22.2% in 2005 (NARMS, 2007). An increase in the percentage of Ery<sup>R</sup> isolates is a concern since macrolide drugs are important for therapeutic treatment of severe campylobacteriosis cases in humans. MDR strains that exhibited resistant to both erythromycin and fluoroquinolone were found in higher numbers of *C. coli* than *C. jejuni* in humans and retail meats, which from a public health perspective is highly undesirable. However, the significantly higher number of humans campylobacteriosis cases caused by *C. jejuni* than *C. coli* highlights the importance of virulence determinants that aid in causing infection in the host.

Variation in the pathogenicity traits of isolates observed in this study, including adherence, motility, invasion, and toxin genes, has been reported previously (Coote *et al.*, 2007; Fearnley *et al.*, 2008). *Campylobacter* strains that are *cadF* mutants have been shown to be unable to colonize the chicken gut and have significantly reduced internalization ability into INT 407 intestinal cells (Ziprin *et al.*, 2001; Monteville *et al.*, 2003). Since all the human *Campylobacter* isolates were obtained from clinical cases and carried this marker (except one), it highlights the importance of this gene in establishing the infection. However, the mere presence of this one gene is not enough to set up infection, and it is important to determine the profile of other virulence determinants. A significantly higher proportion of *C. jejuni* than *C. coli* human isolates were positive for the flagellin-coding *flaA* gene. Motility expression via the flagella is an important virulence factor for *Campylobacter* to adhere and invade the intestine (Konkel *et al.*, 2001; Malik-Kale *et al.*, 2007), and strains that carry a mutation in this gene have severely reduced motility and are unable to colonize the chicken and human gut mucosa (Konkel *et al.*, 2004). These findings may help explain why *C. jejuni* is much more common as a cause of human infections (90%–95%) than *C. coli*

(5%–10%). The *cdtB* gene encodes the active subunit of the toxin, whereas the *cdtA* and *cdtC* gene products are responsible for the binding of the holotoxin (Lara-Tejero and Galan, 2001; Lee *et al.*, 2003). Since all the three *cdt* gene products are required for the functional activity of the cytotoxin (Lara-Tejero and Galan, 2001; Asakura *et al.*, 2007), the absence of the *cdtB* gene in significant numbers among *C. coli* isolates from humans could possibly explain the reduced potential of *C. coli* to cause infections in humans. However, it is important to mention that the presence or absence of a few genes cannot be used to predict whether a particular species of *Campylobacter* is virulent or not, and that further studies have to be conducted to make valid conclusions. In addition, a negative result by PCR can be caused by sequence variation at the primer binding site and does not necessarily indicate the absence of a gene. This is a limitation of the PCR method for detecting target regions and can have an impact on the data that should be interpreted carefully.

The PCR results for the *rakR*, *dnaJ*, *pldA*, and *virB11* genes depicted an interesting profile. The *rakR*, *dnaJ*, and *pldA* genes were found in significantly higher numbers in *C. jejuni* from humans than from retail meats ( $p < 0.01$ ). All *Campylobacter* isolates, except *C. coli* recovered from retail meats (45%), were negative for the *virB11* gene. The above virulence genes were recently reported in 100% of *C. jejuni* strains ( $n = 40/40$ ) isolated from human stool samples in Bangladesh (Talukder *et al.*, 2008). This distinction could possibly be attributed to geographic location of the isolates. However, none of the *C. coli* isolates from humans in our study were positive for the *rakR* gene, which is involved in thermoregulation, and mutants have been shown to have a significantly reduced colonization potential in chickens (van Vliet and Ketley, 2001; Li *et al.*, 2008). Similar observations were made when we compared the other important virulence genes in *C. coli*. The complete absence of the *dnaJ*, *pldA*, and *virB11* genes in *C. coli* isolated from human strongly highlights the reduced potential of these strains to cause infection in humans. However, all the *C. coli* isolates from humans in this study were recovered from clinical cases; therefore, it is quite possible that the patients were suffering with a mixed *Campylobacter* infection. It is also important to highlight that the exact pathogenesis of *Campylobacter* infection in humans has not yet been fully elucidated. Therefore, it is quite possible that *C. coli* cause disease in host by a different mechanism compared with *C. jejuni*. Overall, the marked differences in the prevalence of virulence genes between *C. jejuni* and *C. coli* isolated from humans and retail meats in our study suggests that not all *Campylobacter* strains from retail meats are able to cause disease in humans.

Genotyping the *Campylobacter* isolates from humans and retail meats in this study highlighted the diversity in the population. Although we found clusters (*SmaI*) that were composed of isolates from different sources, the ability of the second restriction enzyme (*KpnI*) to further differentiate within these clusters highlighted the importance of using a second enzyme to make valid interpretations on the relationships shared between different *Campylobacter* strains. Although stable clones of *Campylobacter* have been described, the utility of PFGE for typing *Campylobacter* is limited, due to the high plasticity of the genome (On *et al.*, 1998). *Campylobacter* has a hypervariable genome, which can be the result of many factors, including intra-genomic and inter-genomic re-combinations and bacteriophage predation (Wassenaar *et al.*,

1995, 1998; On *et al.*, 1998; Weijtens *et al.*, 1999; Scott *et al.*, 2007). In a previous study, we genotyped the *C. coli* isolates from this study using multilocus sequence typing and found the overall population to be genotypically diverse (Thakur *et al.*, 2009). Only a minority of isolates in our study showed genotypic similarity between humans and retail meats, which is in agreement with results of recent studies and indicates the potential role of other sources of *Campylobacter* transmission for humans (Uzunović-Kamberović *et al.*, 2007; Gormley *et al.*, 2008). There were four groups of *C. jejuni* isolates with indistinguishable PFGE patterns when restricted with one or two enzymes. The detection of 100% similar PFGE patterns in isolates from humans, turkey, and chicken (Fig. 2) after using two restriction enzymes does point to the ability of these *C. jejuni* strains to infect multiple hosts. However, this does not indicate that *C. jejuni* was transmitted from either the chicken or the turkey to humans in our study. Such a conclusion can only be reached in trace-back studies conducted in disease outbreak investigations.

An important aim of this study was to determine whether there was a significant association between antimicrobial resistance and virulence profile at the statistical and genotypic levels. Contrasting information is available in the literature on the relationship between clonally related isolates and their virulence or antimicrobial resistance profiles. For example, associations between PFGE type, biotype, *in vitro* virulence properties, and source have been reported in *Campylobacter* isolates from different sources (Nadeau *et al.*, 2003; Hänel *et al.*, 2007). In our study we did find Dox<sup>R</sup> *C. jejuni* isolates from humans significantly associated with majority of the virulence genes. Antimicrobial resistance to tetracycline is coded by the *tet(O)* plasmid, which also confers resistance to doxycycline (Thakur and Gebreyes, 2005). Plasmids have been shown to code for virulence traits, and it is possible that the presence of such a plasmid is providing these isolates with virulence potential (Friis *et al.*, 2007). It is important to highlight that this statistical association was not replicated at the genotypic level for all the virulence genes. Similarly, a recent study reported the absence of any association between PFGE type and the virulence properties of *C. jejuni* strains isolated from humans and poultry (Van Deun *et al.*, 2007). Therefore, it is important to determine whether statistical association is also correlated with genotyping results before making valid conclusions.

A few studies have reported that fluoroquinolone-resistant *Campylobacter* have the ability to cause severe infection characterized by prolonged diarrhea and clinical outcome in humans as compared to antimicrobial susceptible strains (Nelson *et al.*, 2004; Helms *et al.*, 2005). Recent work on *Enterococcus faecalis* from retail foods revealed both positive and negative associations between antimicrobial resistance and virulence determinants (McGowan-Spicer *et al.*, 2008). In another study, positive statistical associations between resistance and virulence in *Escherichia coli* isolated from healthy pigs were reported with the authors concluding that antimicrobial resistance has the potential for selecting virulence in bacteria that may contaminate food products and animals (Rosengren *et al.*, 2009). No such association was detected in Cip<sup>R</sup> *C. jejuni* isolates from either humans or retail chicken in our study. A similar observation was made in *E. coli* isolates recovered from human blood and feces where no association was observed between fluoroquinolone resistance and virulence genes (Bagger-Skjøt *et al.*, 2007). It is important to

mention that association between virulence and antimicrobial resistance could be dependent on the bacterial population, strain, source, and other important factors, which must be taken into account before making valid interpretations.

In conclusion, the presence of different combinations of virulence gene determinants clearly indicates that *Campylobacter* strains with diverse pathogenic potential are circulating in the retail meat and human populations. To the best of our knowledge, this is the first study to determine whether any association exists between the virulence and resistance profile at the statistical and genotypic levels in *Campylobacter*. It is, however, difficult to define a relationship between virulence and antimicrobial resistance in *Campylobacter*. This is especially true in clinical cases since *Campylobacter* pathogenesis has not been fully elucidated. It is evident from our study that *C. jejuni* has an advantage over *C. coli* in causing clinical cases in humans primarily due to carriage of important virulence determinants that are essential for eliciting its pathogenesis. To fully understand *Campylobacter* pathogenesis, DNA microarrays and *in vivo* models will have to be developed to determine how the virulence genes work together to establish the infection. Even though the samples and isolates were temporally and spatially related, the genotypic diversity observed by PFGE typing in our study highlights the importance of conducting further detailed studies to better understand the epidemiology of *Campylobacter* in the environment and its role in transmission to humans.

#### Disclosure Statement

No competing financial interests exist.

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