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<Original Article>

Prevalence of virulence and cytolethal distending toxin (CDT) genes in thermophilic *Campylobacter* spp. from dogs and humans in Gyeongnam and Busan, Korea

Hyun-Ho Cho[1], Sang-Hyun Kim[2], Wongi Min[3], Bok-Kyung Ku[1], Yong-Hwan Kim[3,*]

[1]*Animal, Plant and Fisheries Quarantine and Inspection Agency, Anyang 430-757, Korea*
[2]*Viral infectious Disease Research Center, Korea Research Institute of Bioscience & Biotechnology, Daejeon 505-860, Korea*
[3]*College of Veterinary Medicine, Research Institute of Life Science, Institute of Animal medicine, Gyeongsang National University, Jinju 660-701, Korea*

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Abstract : The prevalence of thermophilic *Campylobacter* (*C.*) spp. in stray, breeding, and household dogs was 25.2, 12.0, and 8.8%, respectively. *C. jejuni* and *C. upsaliensis* were the predominant *Campylobacter* spp. from household dogs. *cdt*A, *cdt*B, and *cdt*C were detected by PCR in all isolates. Despite the high cytolethal distending toxin (CDT) gene prevalence, only 26 (31%) *C. jejuni* strains and one (15.3%) *C. coli* strain showed evidence of CDT production in HEp-2 cell cytotoxicity assays. Virulence-associated genes detected in the *C. jejuni* and *C. coli* isolates were *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *iam*A, *pld*A, *vir*B11, *ceu*E, and *doc*C. *cad*F, *dna*J, *fla*A, and *ceu*E were found in all *C. jejuni* and *C. coli* isolates. When detecting Guillain-Barré syndrome-associated genes (*gal*E, *cgt*B, and *wla*N), *gal*E was identified in all isolates. However, *cgt*B and *wla*N were more prevalent in *C. jejuni* isolates from humans than those from dogs. Adherence and invasion abilities of the *C. jejuni* and *C. coli* strains were tested in INT-407 cells. A considerable correlation (adjusted $R^2 = 0.678$) existed between adherence and invasion activities of the *Campylobacter* spp. isolates.

Keywords : adherence/invasion ability, *Campylobacter* spp., dog, Guillain-Barré syndrome-associated genes, virulence genes

Introduction

The genus *Campylobacter* comprises 16 species of *Campylobacter* (*C.*) *jejuni* and 12 species of *C. coli* those have been associated with diseases over 95% of *Campylobacter* infections in humans [20]. The isolation rate for *C. lari* was less than 1% among the thermophilic *Campylobacter* spp. The others, such as *C. upsaliensis* and *C. fetus* are only occasionally seen in clinical isolates [20].

Campylobacter spp. are important causative agents for gastrointestinal infections in the industrialized and developing countries [1, 8]. Clinical signs include abdominal pain, fever, malaise, nausea and vomiting. Recently *Campylobacter* enteritis has been identified as an important risk factor for the development of inflammatory bowel disease [13]. In addition, extra-intestinal *Campylobacter*-associated diseases are polyarthragia (reactive arthritis), Guillain-Barré syndrome (GBS), and Miller-Fisher syndrome [10].

Campylobcter spp. are part of the normal intestine flora of wild, domesticated animals and birds. One particular importance to humans is their colonization in animals for food production, including poultry, cattle, sheep, and swine [9]. The household dog has been identified as a risk factor for human campylobacteriosis [11, 31]. Most dogs are asymptomatic when they serve as reservoirs in shedding *Campylobacter* spp. into their feces. Therefore, contact with the pets has been shown to be a risk factor for *Campylobacter* infections in humans and actual transmission has been demonstrated [15, 29].

However, virulence mechanisms underlying *Campylobacter* infections are not fully elucidated although flagella-mediated motility, adhesion to intestinal mucosa, invasion and production of enterotoxin have been identified as virulence determinants [32]. A number of putative virulence and toxin genes have been studied, including *fla*A, *cad*F, *rac*R, *cia*B, and *iam*A genes that involved in adhesion and colonization of the host's intestine [17]; *ceu*E gene which encodes a binding-protein transport system for the siderophore enterochelin [14]; *cdt* gene cluster encoding a cytolethal distending toxin (CDT) consists of three adjacent genes (*cdt*A, *cdt*B,

*Corresponding author
Tel: +82-55-772-2345, Fax: +82-55-772-2808/2349
E-mail: yho157@gnu.ac.kr

and *cdt*C). The CDT toxin is composed of CdtB protein as the enzymatically active subunit and two hetero-dimeric subunits (CdtA and CdtC), which are responsible for the holotoxin binding to cell membrane [3].

Lipo-oligosaccharides (LOS) are thought to be a critical factor in the triggering of the Guillain-Barré syndrome (GBS) and Miller-Fisher syndrome neuropathies after *C. jejuni* infection [10]. Linton *et al.* [21] demonstrated the *wla*N gene product as a β-1,3 galactosyltransferase responsible for biosynthesis of host-mimicking LOS structure.

The aim of this study was to investigate the isolation rates of *Campylobacter* spp. from household, stray, and breeding dogs in Gyeongnam and Busan areas. Furthermore, prevalence of putative virulence genes was detected by PCR in the isolates of *Campylobacter* spp. from dogs and humans. The adherence/invasion ability of *Campylobacter* bacteria was evaluated by a gentamicin-treated INT407 cell assay. The expression of CDT activity in the culture supernatants of *Campylobacter* spp. isolates was tested for cytotoxicity assay with HEp-2 cells. The prevalence of GBS-associated genes (*gal*E, *cgt*B, *wla*N) that are involved in the LOS biosynthesis in *C. jejuni* was detected by PCR with the isolates from dogs and humans.

Materials and Methods

Source of samples

A total of 460 fecal samples (1 g) were collected from three groups of dogs in Gyeongnam and Busan areas from January 2009 to April 2010 for isolation of thermophilic C*ampylobacter* spp.

Group 1: A total of 274 fecal specimens (1 g) from household dogs were collected at local veterinary clinics located at Gyeongnam and Busan areas. Group 2: one gram of fecal samples from 103 stray dogs was collected from municipal animal shelter in the western Gyeongnam areas. Group 3: one gram of fecal sample from 83 breeding dogs was collected in the western Gyeongnam areas.

Ten mL of Preston selective enrichment broth (Oxoid, Germany) containing each one gram of fecal sample in ice box was carried to laboratory within 8 h and used for isolation immediately.

Thermophilic *Campylobacter* spp. isolation

The broth emulsified by brief vortexing was incubated at 42°C for 2 days in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2). One loopful of broth was streaked to modified CCDA Preston agar (Oxoid CM 739 plus selective supplement SR155; Oxoid) and the agar plates were incubated same culture condition indicated above. Typical *Campylobacter* colonies on modified CCDA Preston agar were transferred to blood agar plate and the plates were incubated at the same culture condition for 48~72 h.

Three colonies of presumptive *Campylobacter* bacteria grown on blood agar plate were identified at species level on the basic of phase contrast microscope (spiral shape and corkscrew movement), catalase, oxidase, rapid H_2S production, growth at 25°C and 42°C, hippurate hydrolysis and susceptibility to nalidixic acid and cephalothin. *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were used as reference strains.

Multiplex polymerase chain reaction (mPCR) was used to differentiate major species of campylobacters. The genomic DNA of *Campylobacter* spp. isolates was extracted using a DNA isolation kit (QIAamp DNA mini; Qiagen, Germany) by the manufacturer's instruction. Identification of *Campylobacter* spp. was performed by using MultiPerfect *Campylobacter* spp. PCR kit (G&P Life Science, Korea) containing specific primers for *C. jejuni, C. coli, C. lari, C. fetus,* and *C. upsaliensis*.

Forty-six *C. jejuni* and 4 *C. coli* isolated from humans with diarrhea in Seoul areas during 2008~2009 were kindly provided by Korea National Institute of Health for comparison. All isolates were stored in liquid nitrogen at −150°C.

Detection of CDT genes by PCR

Genomic DNA was amplified by mPCR to confirm the presence of CDT genes (*cdt*A, *cdt*B and *cdt*C). All PCR reactions were performed in a total volume of 25 μL, containing 12.5 μL GoTag Green Master Mix, 2× (Promega, USA), 1 μL upstream and downstream primer (GenoTech, Korea), 2 μL DNA template, and 8.5 μL nuclease-free water. Primer and PCR condition are listed in Table 1. PCR was performed in a DNA Thermal Cycler (Eppendorf, Germany) and PCR amplicons were analysed by horizontal electrophoresis using 1.5% agarose gel, with a 100 bp molecular weight ladder,

Table 1. PCR primers and condition used for detection of CDT genes

Target gene	Primers	Size (bp)	Sequence (5' to 3')	Condition			Reference
*cdt*A	GNW	165	GGAAATTGGATTTGGGGCTATACT	94°C	15 min	denaturation	Pickett *et al.* [26]
	IVH		ATCACAAGGATAATGGACAAT	94°C	1 min		
*cdt*B	VAT2	495	GTTAAAATCCCCTGCTATCAACCA	42°C	2 min	30 cycles	
	WMI-R		GTTGGCACTTGGAATTTGCAAGGC	72°C	3 min		
*cdt*C	WMI-F	555	TGGATGATAGCAGGGGATTTTAAC	72°C	5 min	extension	
	LPF-X		TTGCACATAACCAAAAGGAAG	8°C	ever		

CDT genes: cytolethal distending toxin genes.

Table 2. Primers used for detection of adherence, invasion and GBS-associated genes by PCR

Target gene		Primers	Size (bp)	Sequence (5' to 3')	References
Adherence and invasion-associated genes	*cia*B	*cia*B-403 *cia*B-1373	986	TTTTTATCAGTCCTTA TTTCGGTATCATTAGC	Datta *et al.* [9]
	*iam*A	*cia*3f *cia*5r	518	GCACAAAATATATCATTACAA TTCACGACTACTATGAGG	Müller *et al.* [22]
	*cad*F	*cad*F-F2B *cad*F-R1B	400	TTGAAGGTAATTTAGATATG CTAATACCTAAAGTTGAAAC	Konkel *et al.* [17]
	*vir*B11	*Vir*B11F *Vir*B11R	709	GAACAGGAAGTGGAAAAACTAGC TTCCGCATTGGGCTATATG	Bacon *et al.* [4]
	*doc*C	*doc*C1 *doc*C2	1835	TGAGCTACGCTATCATTG GCTTACGCTATGGGTTGG	Müller *et al.* [22]
	*pld*A	*pld*A-84 *pld*A-981	913	AAGCTTATGCGTTTTT TATAAGGCTTTCTCCA	Datta *et al.* [9]
	*ceu*E	JEJ1 JEJ2	794	CCTGCTCGGTGAAAGTTTTG GATCTTTTTGTTTTGTGCTGC	Gonzalez *et al.* [14]
	*rac*R	*rac*R-25 *rac*R-593	584	GATGATCCTGACTTTG TCTCCTATTTTTACCC	Datta *et al.* [9]
	*dna*J	*dna*J-299 *dna*J-1003	720	AAGGCTTTGGCTCATC CTTTTTGTTCATCGTT	Datta *et al.* [9]
	*gal*E	*gal*E-F *gal*E-R	584	GATGATCCTGACTTTG TCTCCTATTTTTACCC	Nawaz *et al.* [23]
GBS-associated genes	*cgt*B	DL39 *cgt*Brev	584	GATGATCCTGACTTTG TCTCCTATTTTTACCC	Linton *et al.* [21]
	*wla*N	*wla*N-DL39 *wla*N-DL41	584	GATGATCCTGACTTTG TCTCCTATTTTTACCC	Linton *et al.* [21]

GBS-associated genes: Guillain-Barré syndrome-associated genes.

then stained with 0.5 μg/mL ethidium bromide.

PCR detections of virulence- and GBS-associated genes

PCR was performed as generally same as described in the above CDT case. Primer sets and PCR condition are listed in Table 2.

CDT cytotoxicity assay with HEp-2 cells

HEp-2 cells were seeded into 24 well tissue culture plates (Nunc, Germany) at a density of 2×10^4 CFU (colony forming unit) per well in 0.5 mL. Two-fold serial dilutions of culture filtrates were prepared in MEM (Invitrogen, USA) and 0.5 mL of each dilution was added to the HEp-2 cells and incubated for 3 days at 37°C in an atmosphere of 5% CO_2.

Cell-free bacterial culture supernatants from all the strains were prepared according to the method described by Florin and Antillon [12] with minor modifications. Briefly, each strain was harvested from CCDA plates and inoculated into MEM cell culture medium. The volume of medium used was adjusted so that the OD_{600} of the bacterial suspension was 0.125 (2×10^8 CFU/mL). Bacterial strains suspended in MEM tissue culture medium were lysed by sonication (4/30 sec bursts with 30 sec intervals between each burst). Cell debris and intact bacteria were then removed by centrifugation at $3,000 \times g$ for 20 min at 5°C and sterilized by filteration (0.22 μm). Finally, the filtered supernatant was stored at 20°C until use. The morphological changes of cells were examined with an aid of inverted microscope at the time of incubation between 24 h and 72 h.

Adhesion and invasion assay in INT-407 cells

The adhesion and invasion assays were performed by the method of Konkel *et al.* [17] with slight modifications. Briefly, *C. jejuni* strains were grown microaerobically on blood agar with 5% sheep blood for 48 h at 37°C. Bacteria were harvested from the plates with PBS (phosphate buffered saline) and adjusted spectrophotometrically to approximately 1×10^7 CFU/mL. The CFU containing of approximately 200 times higher than the cell number was inoculated into duplicated wells of a 24 well tissue culture plated containing semiconfluent monolayers of INT-407 cells. The infected monolayers were incubated for 3 h at 37°C in a 5% CO_2 humidified atmosphere to allow for bacterial adherence and internalization.

For determination of adherence, the cells were washed 3 times with PBS and the cell monolayer was lysed with 1% Triton X-100 (Sigma, USA) and the total bacteria associated with the cells (intracellular and extracellular bacteria) were enumerated by plating serial dilutions of the lysates on blood agar with 5% sheep blood. In order to measure bacterial invasion, the infected cells were washed 3 times with PBS and incubated in fresh PBS containing 1% fetal bovine serum (FBS) and 200 μg/mL gentamicin for 2 h to kill the remaining viable extracellular bacteria. In preliminary experiments, 200 μg/mL of gentamicin killed all the extracellular bacteria within 2 h after the exposure. Quantification of viable intracellular bacteria was performed by washing the infected eukaryotic cells twice with PBS and subsequently lysing with 1% Triton X-100. Following serial dilution in PBS, the released intracellular bacteria were enumerated as described in the adherence assay. Non-invasive *E. coli* DH5α was used as negative control for all the analyses. The correlation between the adherence to and invasion of the INT-407 cells for *Campylobacter* spp. isolates was analyzed by a linear regression using SPSS (SPSS, USA).

Results

Prevalence of thermophilic *Campylobacter* spp.

The isolates of *Campylobacter* spp. were identified by using Multi-perfect *Campylobacter* spp. PCR kit. The mPCR could amplify 800 bp band for *Campylobacter* spp., 502 bp band for *C. coli,* 251 bp band for *C. lari,* 161 bp band for *C. jejuni*, and 86 bp band for *C. upsaliensis* (Fig. 1). A total of 44 strains of *C. jejuni,* 9 strains of *C. coli,* 6 strains of *C. upsaliensis* and 1 strain of *C. lari* were isolated from dogs in Gyeongnam and Busan areas (Table 3). The rates of isolation for the thermophilic *Campylobacter* spp. were 8.8% (24/274) in household dogs, 12.0% (10/83) in breeding dogs, and 25.2% (26/103) in the stray dogs. In the fecal samples of household dogs, the isolation rate for *C. jejuni* was 54.2% (13/24), *C. upsaliensis* 25.0% (6/24), *C. coli* 16.7% (4/24) and *C. lari* 4.2% (1/24). In the fecal samples of stray dogs, the isolation rate for *C. jejuni* was 88.5% (23/26) and for *C. coli* was 11.5% (3/23). In the fecal samples of the breeding dogs, the isolation rate for *C. jejuni* was 80.0% (8/10) and for *C. coli* was 20.0% (2/10). *C. upsaliensis* and *C. lari* were not isolated in the stray and breeding dogs.

CDT gene detection and cytotoxicity assay

The prevalence of CDT genes was detected by mPCR with 87 *C. jejuni* and 13 *C. coli* strains isolated from dogs and humans. The *cdt*A, *cdt*B and *cdt*C genes were detected in the all isolates (Table 4). Cytotoxicity assay was performed by microscopic examination of HEp-2 cells treated with the prepared culture supernatants to observe formation of small polymorphic, rounded cells, and elongated cells with irregular vacuoles formation in the peri-nuclear cytoplasm, those are associated with CDT activity. As a result, a total of 27 strains of *Campylobacter* spp. displayed cytotoxicity, among them, 26 (31.0%) of *C. jejuni* and 1 (15.3%) of the *C. coli* isolates showed evidence for CDT production in the HEp-2 cell cytotoxicity assays (Table 5). Among the 26 *C. jejuni* isolates that were positive to cytotoxicity assay, 15 strains were human origin, and the remaining was dogs origin isolates. Only one isolate of *C. coli* isolated from dog showed a cytotoxicity of HEp-2 cells.

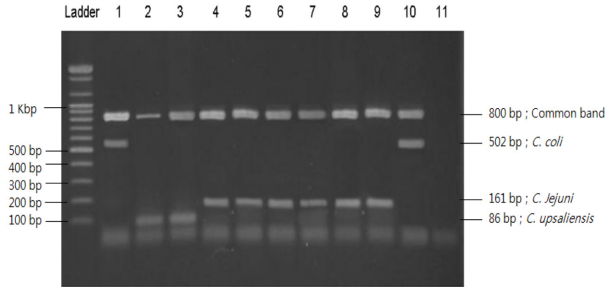

Fig. 1. Identification of thermophilic *Campylobacter* (*C.*) spp. by multiplex polymerase chain reaction (m-PCR). Ladder; 100 bp marker, Lane 1; *C. coli* (ATCC 33559), Lane 2; *C. upsalienesis* (ATCC 43954), Lane 3; *C. upsalienesis* (isolate), Lane 4; *C. jejuni* (ATCC 33560), Lane 5~9; *C. jejuni* (isolates), Lane 10; *C. coli* (isolate).

Detection of virulence-associated genes

Thirteen virulence-associated genes were detected in 87 strains of *C. jejuni* and 13 strains of *C. coli* isolates from humans and dogs by using mPCR method. Four virulence-associated genes (*cad*F, *dna*J, *fla*A, and *ceu*E) were detected in the all isolates of *C. jejuni* and *C. coli* from dogs and humans (Table 6). The *rac*R gene was detected as 90~100% in *C. jejuni* isolated from dogs and humans, and in *C. coli* from humans, however, it was not detected in *C. coli* isolated from dogs. The *cia*B gene was detected as 40~87.5% in *C. jejuni* isolated from dogs and humans, and in *C. coli* from humans, however it was not detected in *C. coli* isolated from dogs. The *iam*A gene was detected as 80-100% in *C. jejuni* isolated from dogs and humans, and 50% in *C. coli* isolated from dogs, however it was not detected in *C. coli* isolated from dogs. The *pld*A gene was detected as 40~90% in *C. jejuni* isolated from dogs and humans, and in *C. coli* isolated from humans, however it was not detected in *C. coli* isolated from dogs. The *vir*B11 was detected as only 6.5% in C. *jejuni* isolated from humans. The *doc*C was detected as 19.5~82.0% in *C. jejuni* isolated from dogs and humans, however, it was not detected in *C. coli* isolates.

Detection of GBS-associated genes

Three GBS-associated genes (*gal*E, *cgt*B, *wla*N) were detected by mPCR method. These 3 GBS-associated genes were detected in total 87 strains of *C. jejuni* and 13 strains of *C. coli* isolates from humans and dogs. The *gal*E gene was detected in all *C. jejuni* and *C. coli* isolated from dogs and

Table 3. The isolation rates of thermophilic *Campylobacter* spp. isolated from dogs faeces in Gyeongnam and Busan areas

Species	Household dogs (n = 274)	Stray dogs (n = 103)	Breeding dogs (n = 83)	Total (n = 460)
	n (%)	n (%)	n (%)	n (%)
C. jejuni	13 (54.2)	23 (88.5)	8 (80.0)	44
C. coli	4 (16.7)	3 (11.5)	2 (20.0)	9
C. upsaliensis	6 (25.0)	–	–	6
C. lari	1 (4.2)	–	–	1
Total	24 (8.8)	26 (25.2)	10 (12.0)	60 (13.2)

Table 4. Detection rate of CDT genes from *C. jejuni* and *C. coli* isolates

	Origin							
	Household Dogs		Stray dogs		Breading Dogs		Humans	
	C. jejuni (%)	*C. coli* (%)	*C. jejuni* (%)	*C. coli* (%)	*C. jejuni* (%)	*C. coli* (%)	*C. jejuni* (%)	*C. coli* (%)
*cdt*A	10/10 (100)	4/4 (100)	23/23 (100)	3/3 (100)	8/8 (100)	2/2 (100)	46/46 (100)	4/4 (100)
*cdt*B	10/10 (100)	4/4 (100)	23/23 (100)	3/3 (100)	8/8 (100)	2/2 (100)	46/46 (100)	4/4 (100)
*cdt*C	10/10 (100)	4/4 (100)	23/23 (100)	3/3 (100)	8/8 (100)	2/2 (100)	46/46 (100)	4/4 (100)

Table 5. Determination of cytotoxic activity from *C. jejuni* and *C. coli* isolates

	Origin		Total (%)
	Dogs (%)	Humans (%)	
C. jejuni	11/41 (26.8)	15/46 (32.6)	26/87 (31.0)
C. coli	1/9 (11.1)	0/4 (0)	1/13 (15.3)
Total	12/50 (24.0)	15/50 (30.0)	27/100 (27.0)

Table 6. Isolation rates of virulence-associated genes in *C. jejuni* and *C. coli* isolates from dogs and humans

Gene	Household Dogs		Stray Dogs		Breading Dogs		Humans	
	C. jejuni (%)	*C. coli* (%)	*C. jejuni* (%)	*C. coli* (%)	*C. jejuni* (%)	*C. coli* (%)	*C. jejuni* (%)	*C. coli* (%)
*cad*F	10/10 (100)	4/4 (100)	23/23 (100)	3/3 (100)	8/8 (100)	2/2 (100)	46/46 (100)	4/4 (100)
*dna*J	10/10 (100)	4/4 (100)	23/23 (100)	3/3 (100)	8/8 (100)	2/2 (100)	46/46 (100)	4/4 (100)
*fla*A	10/10 (100)	4/4 (100)	23/23 (100)	3/3 (100)	8/8 (100)	2/2 (100)	46/46 (100)	4/4 (100)
*ceu*E	10/10 (100)	4/4 (100)	23/23 (100)	3/3 (100)	8/8 (100)	2/2 (100)	46/46 (100)	4/4 (100)
*rac*R	9/10 (90)	0/4 (0)	23/23 (100)	0/3 (0)	8/8 (100)	0/2 (0)	45/46 (97.8)	4/4 (100)
*cia*B	4/10 (40)	0/4 (0)	19/23 (82.6)	0/3 (0)	7/8 (87.5)	0/2 (0)	30/46 (65.2)	2/4 (50)
*iam*A	8/10 (80)	0/4 (0)	23/23 (100)	0/3 (0)	8/8 (100)	1/2 (50)	46/46 (100)	4/4 (100)
*pld*A	4/10 (40)	0/4 (0)	22/23 (95.7)	0/3 (0)	6/8 (75)	0/2 (0)	28/46 (60.9)	2/4 (50)
*vir*B11	0/10 (0)	0/4 (0)	0/23 (0)	0/3 (0)	0/8 (0)	0/2 (0)	3/46 (6.5)	0/4 (0)
*doc*C	7/10 (70)	0/4 (0)	19/23 (82.6)	0/3 (0)	3/8 (37.5)	0/2 (0)	9/46 (19.6)	0/4 (0)

Table 7. Isolation rates of GBS genes in *C. jejuni* and *C. coli* isolates from dogs and humans

Gene	Dogs		Humans	
	C. jejuni (%)	*C. coli* (%)	*C. jejuni* (%)	*C. coli* (%)
*cgt*B	10/41 (24.4)	0/9 (0)	33/46 (71.7)	3/4 (75.0)
*gal*E	41/41 (100)	9/9 (100)	46/46 (100)	4/4 (100)
*wla*N	0/41 (0)	0/9 (0)	8/46 (17.4)	2/4 (50.0)

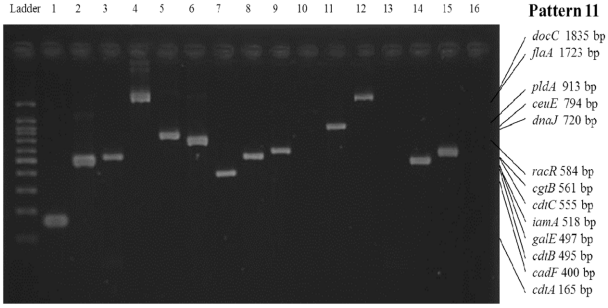

Fig. 2. Representative pattern of PCR amplifications for virulence-associated genes in *C. jejuni* D16, 17, 23 and 41 isolated from dogs. Ladder; 100 bp marker.

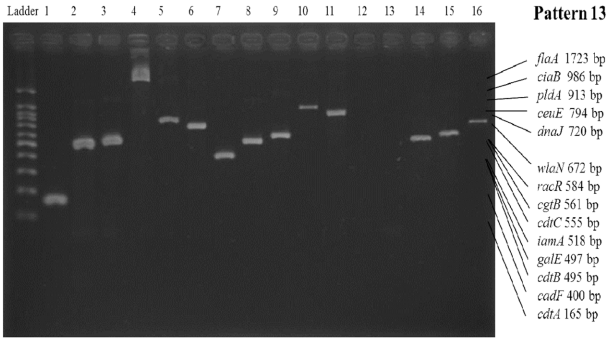

Fig. 3. Representative pattern of PCR amplifications for virulence-associated genes in *C. jejuni* H19, 28 and *C. coli* H24 isolated from humans. Ladder; 100 bp marker.

Table 8. Virulence-associated gene patterns of *C. jejuni and C. coli* isolated from dogs

Number	Patterns	*C. jejuni*	*C. coli*
1	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *dna*J, *fla*A, *gal*E	8	8
2	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *dna*J, *fla*A, *gal*E, *iam*A	1	1
3	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *dna*J, *fla*A, *gal*E, *iam*A, *rac*R	3	
4	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cia*B, *dna*J, *fla*A, *gal*E, *iam*A, *rar*R	2	
5	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *rac*R	1	
6	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *dna*J, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	1	
7	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cia*B, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *rac*R	2	
8	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cia*B, *dna*J, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	2	
9	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	1	
10	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *cia*B, *dna*J, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	3	
11	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	4	
12	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cia*B, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	20	
13	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *cia*B, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	1	
	Total	41	9

humans. The *cgt*B gene was detected as 24.4% in *C. jejuni* isolated from dogs, 71.7% in *C. jejuni,* and 75.0% in *C. coli* isolated from humans, however, it was not detected in *C. coli* isolated from dogs (Table 7). The *wla*N gene was not detected in *Campylobacter* spp. isolated from dogs, however the detection rates were 17.4 and 50.0% in C. *jejuni* and *C. coli* isolated from humans, respectively.

Occurrence pattern of virulence-associated genes in *Campylobacter* spp. isolates

Patterns of virulence-associated genes of *Campylobacter* isolates from humans and dogs were compared with 16 kinds of genes. Compared with the gene occurrence pattern among the 41 *C. jejuni* isolates from dogs, 13-gene occurrence pattern was detected in 27 strains, 8-gene occurrence pattern was detected in 8 strains, 12-gene occurrence pattern was detected in 7 strains (Table 8 and Fig. 2). However, in the gene occurrence patterns of 46 *C. jejuni* isolates from humans, 12-gene pattern was for 13 strains, 11-gene pattern was for 12 strains, 14-gene pattern was for 11 strains, 13-gene pattern was for 6 strains, and 10-gene pattern was for 3 strains (Table 9 and Fig. 3).

When compared the gene harboring patterns of 9 *C. coli* isolates from dogs, 8-gene pattern was occurred in 8 strains,

Table 9. Virulence-associated gene patterns of *C. jejuni and C. coli* isolated from humans

Number	Patterns	*C. jejuni*	*C. coli*
1	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *dna*J, *fla*A, *gal*E, *iam*A, *rac*R	3	
2	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *dna*J, *fla*A, *gal*E, *iam*A, *rac*R	12	2
3	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *dna*J, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	2	
4	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *cia*B, *dna*J, *fla*A, *gal*E, *iam*A, *rac*R	5	
5	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *dna*J, *fla*A, *gal*E, *iam*A, *rac*R, *wla*N	1	
6	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cia*B, *dna*J, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	3	
7	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	2	
8	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cia*B, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	3	
9	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *cia*B, *dna*J, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	1	
10	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cia*B, *dna*J, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R, *wla*N	2	1
11	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *cia*B, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R	4	
12	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *cia*B, *dna*J, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R, *vir*B	2	
13	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *cia*B, *dna*J, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R, *wla*N	2	1
14	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R, *wla*N	1	
15	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cgt*B, *dna*J, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R, *vir*B, *wla*N	1	
16	*cdt*A, *cdt*B, *cdt*C, *cad*F, *ceu*E, *cia*B, *dna*J, *doc*C, *fla*A, *gal*E, *iam*A, *pld*A, *rac*R, *wla*N	1	
	Total	46	4

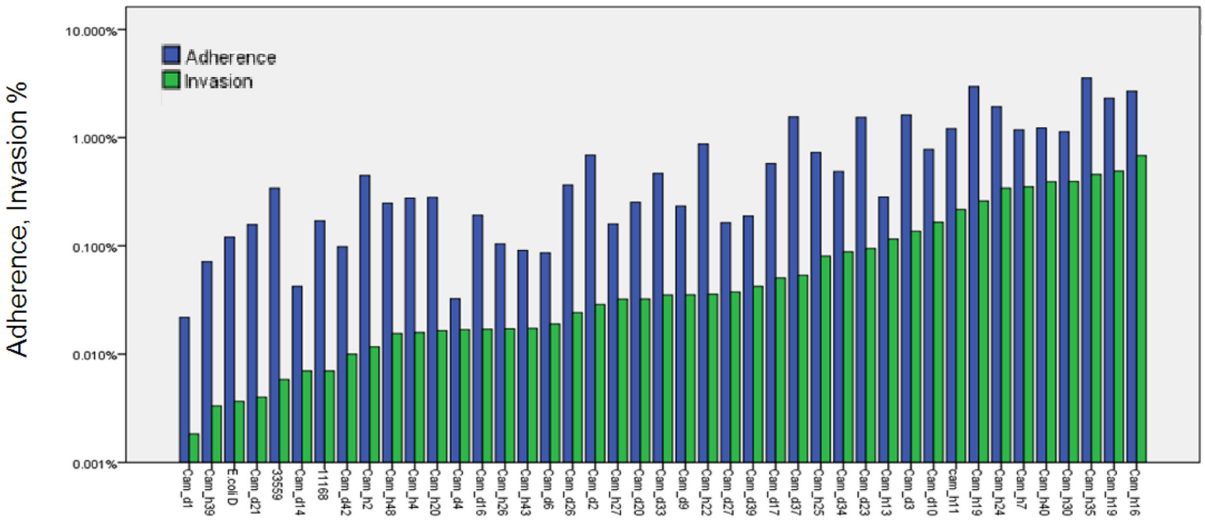

Fig 4. The adherence and invasion ability of *C. jejuni* and *C. coli* into INT-407 cells. There was a significant correlation ($R^2 = 0.678$) between the adherence and invasion ability. Cam_h; human isolates, Cam_d; dog isolates, *E.coli* D; *Escherichia coli* DH5α.

9-gene pattern was occurred in 1 strain (Table 8). However, in the gene occurrence patterns of 4 *C. coli* isolates from humans, 11-gene pattern was for 2 strains, 13-gene and 14-gene pattern was for 1 strain, respectively (Table 9).

Adherence and invasion assays

To test the pathogenic properties of *C. jejuni* and *C. coli* isolates, the adherence and invasion abilities of 40 *C. jejuni* and *C. coli* isolates from dogs and humans were analyzed with human embryonic intestine (INT-407) cells using a gentamicin resistance assay. After 3 h incubation, the 40 *C. jejuni* and *C. coli* isolates adhered to INT-407 cells between $8.5 \pm 6.0 \times 10^3$ CFU/mL and $7.098 \pm 6.025 \times 10^5$ CFU/mL, respectively, and that could be expressed as 0.0180% to 2.6822% adherence of the starting viable inoculum. Two of 40 isolates had invasion abilities lower than that of *E. coli* DH5α. The invasion abilities of the 40 isolates to INT-407 were between $3.67 \pm 2.89 \times 10^2$ CFU/mL and $1.362 \pm 0.143 \times 10^5$ CFU/mL respectively, and that can be expressed as 0.0018 to 0.6813% for the starting viable inoculum (Fig. 4). The average percentages of 20 *C. jejuni* and *C. coli* isolated from dogs for adherence to and invasion were 8.67×10^4

CFU/mL and 8.26×10^3 CFU/mL, respectively, and that was 0.4335% and 0.0413% for the starting viable inoculum (Fig. 4). The average percentages of 20 *C. jejuni* and *C. coli* isolated from humans for adherence to and invasion were 2.29×10^5 CFU/mL and 3.94×10^4 CFU/mL, respectively, and that was 1.1443% and 0.1971% for the starting viable inoculum (Fig. 4). Interestingly, *C. jejuni* and *C. coli* isolated from humans had more adherence and invasion abilities than these of dogs. *C. jejuni* and *C. coli* isolated from humans are 2.64 times more adherence and 4.77 times more invasive than that of dogs. There was a significant correlation ($R^2 = 0.678$) between the adherence ability and the invasion ability of the *C. jejuni* and *C. coli* isolates.

Discussion

This study was performed to investigate the isolation rates of *Campylobacter* spp. from dogs that assigned into 3 different groups based on their raising conditions (stray, breeding, and household dogs). For comparison of virulence gene prevalence between the isolates of *Campylobacter* spp. from dogs and humans, PCR was conducted to detect virulence-associated genes including CDT-encoding genes (*cdt*A, *cdt*B, and *cdt*C). The expression of CDT activity in the culture supernatants of *Campylobacter* spp. isolates was tested for cytotoxicity assay with HEp-2 cells. Furthermore, prevalence of GBS-associated genes (*gal*E, *cgt*B, *wla*N) involved in the LOS biosynthesis in *C. jejuni* was compared with the isolates from dogs and humans.

In present study, the isolation rate of *Campylobacter* in dog was lower than that reported by Hald *et al*. (76.2%) [15], however, it was similar to that of Tsai *et al*. (13.9%) [31]. The isolation rate was significantly higher in stray dogs (25.2%) than household dogs (8.8%). Similarly, Fernandez and Martin [11] found that *Campylobacter* spp. were isolated more frequently from stray dogs than household dogs. As with the case, *C. jejuni* and *C. upsaliensis* have been demonstrated to be the predominant species in dogs [15], especially in in the household dogs raised in Gyeongnam and Busan areas.

CDT is a bacterial protein toxin that is widely distributed among many Gram-negative bacteria including *E. coli* strains of many serotypes and some strains belonging to *Shigella dysenteriae*, *Shigella boydii*, and various species of *Campylobacter* [2, 3, 16, 28]. Previously, Park and Richardson [25] revealed that CDT was able to stop proliferation of cell lines. The holotoxin of CDT is consist of three subunits (CdtA, B, C). The CdtB exerts DNase I-like activity, targeting the eukaryotic DNA, thus triggering a signalling pathway involving several protein kinases, hence resulting in the inhibition of the cell cycle in G2 or M phase [34]. The genes for CDT production (*cdt*A, *cdt*B, and *cdt*C) were detected as 100% in the all isolates tested in this study, which is consistent with previous reports by Bang *et al.* [5] and Asakura *et al*. [3]. However, despite the high prevalence of *cdt* genes (100%), only 26 strains (31.0%) of *C. jejuni* and 1 strain (15.3%) of *C. coli* isolates showed evidence for CDT production in HEp-2 cell cytotoxicity assays. It is well reported that cytotoxic activity of *Campylobacter* spp. is susceptible for culture passages *in vitro*, therefore decrease of toxicity may be due to repeated subculture of isolates *in vitro* [25]. For a more definitive test of cytotoxic activity, more sensitive methods should be applied to cytotoxicity assays, such as lactate dehydrogenase cytotoxicity (LDH) and 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.

Several virulence factors have been documented for *Campylobacter* spp., which could contribute to its motility, intestinal colonization and invasion. The flagellin gene of *Campylobacter* spp. has been well characterized. The *fla*A and *fla*B genes constitute the locus of flagellin. Molecular genetic research revealed that *fla*A is essential for colonization, but not the *fla*B gene [33]. Adhesion of the pathogen to the epithelium is important for colonization of *Campylobacter* spp. The protein encoded by *cad*F, which binds to fibronectin in the humans gut, SH group helping the adhesion and invasion [17, 22]. The *ceu*E gene encodes a lipoprotein component of the binding-protein-dependent transport system for the siderophore enterochelin has been identified [27]. Iron acquisition is a crucial aspect of bacterial infectivity and it has been suggested that this system may play an important role in bacterial virulence. Heat shock proteins, especially *dnaJ* are associated with the environmental thermal response of bacteria which are prominent virulence factors [9]. The *rac*R and *rac*R-dependent genes are important for growth and survival in the avian intestine, and *rac*R-*rac*S is a signal transduction system responsive to temperature [7].

In this study, *fla*A, *cad*F, *dna*J and *ceu*E genes were detected in the all *C. jejuni* and *C. coli* isolates from dogs and humans. In the previous studies, these genes were detected in almost all isolates from different sources [9, 30]. The *rac*R genes were detected in the range of 90~100% in *C. jejuni* and *C. coli* isolated from dogs and humans in this study, compared to those reports that *rac*R genes were detected in the range of 85.7~100% [4, 18]. The detection rates of the other genes, *cia*B, *pld*A, *doc*C and *vir*B11, varied among sources of isolates. The *doc*C gene was found in *C. jejuni* of human isolates (19.6%) that were lower than dog isolates (37.5~82.6%). The *vir*B11 genes were only detected in *C. jejuni* isolated from humans (6.5%).

Three GBS-associated genes (*gal*E, *cgt*B and *wla*N) were detected by mPCR method, showing that *gal*E gene was detected all *C. jejuni* and *C. coli* isolates. However, *cgt*B gene was detected with lesser rate in dog isolates (12.5~30.4% in *C. jejuni* and 0% in *C. coli*) than human isolates (71.7% in *C. jejuni* and 75.0% in *C. coli*). The *wla*N gene was not detected from *C. jejuni* and *C. coli* isolated from dogs, however, in the cases of human isolates, it was detected as 17.4 and 50.0%, respectively. The *gal*E, *cgt*B and *wla*N gene product as β-1, 3-galactotransferase are responsible for

specific LOS structure. LOS, similar with gangliosides in neurons, is thought to be critical factor in the triggering of GBS and Miller-Fisher syndrome neuropathies after *C. jejuni* infection [10, 21, 23]. The higher prevalence of these genes might be associated with GBS in humans.

Animal models that completely mimic *Campylobacter* infection in humans are not available. INT-407 cells have been used for evaluating the pathogenicity of various enteric pathogens, such as *Salmonella enteritidis*, *C. jejuni*, and enteropathogenic *E. coli* [6, 19, 24]. Even though, the process of *Campylobacter* adhering and invading to INT-407 human intestinal epithelial cells does not exactly mimic the process *in vivo* during the infection. In this study, the 40 strains of *C. jejuni* and *C. coli* isolates adhered and invade into INT-407 cells by showing variable degree of adherence. The degree of adherence and invasion resulted in this study is similar to the previous report by Biswas *et al*. [6] that 0.7416~2.1714% and 0.0012~0.4226% of the range in the adherence and invasion, respectively. Colonization and/or adherence to mucosal surfaces is the primary step of infection and it appears to be a prerequisite for invasion in most pathogens. Therefore, the relationship between adherence and invasion efficiency was analysed with *C. jejuni* and *C. coli* isolates by using statistical tests. There was a significant correlation ($R^2 = 0.678$) between the adherence and the invasion ability of the *C. jejuni* and *C. coli* isolates. Thus, the results support the notion that the adherence of *C. jejuni* and *C. coli* may facilitate invasion into host cells.

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