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Prevalence of virulence-associated genes and antimicrobial resistance of *Campylobacter jejuni* from ducks in Gyeongnam Province, Korea

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Abstract

Total 99 strains of *Campylobacter spp*. were isolated from 117 cases of duck's fecal samples. Among 99 strains of *Campylobacter spp.* isolates, 93 strains (93.9%) were *C. jejuni* and 6 strains (6.1%) were *C. coli*. Prevalence of virulence and GBS associated genes of 72 *C. jejuni* isolates was determined by m-PCR. Among the 10 kinds of virulence associated genes, *cad*F, *dna*J, *fla*A and *ceu*E genes were detected in all of *C. jejuni* isolates from ducks, *rac*R, *pld*A, *iam*A, *cia*B, *vir*B11 and *doc*C genes were 87.5%, 84.7%, 77.8%, 48.6%, 13.9% and 11.1%, respectively. Antimicrobial susceptibility test was performed on 72 *C. jejuni* isolates. The rate of resistance were 62.5% for oxytetracycline, 55.6% for kanamycin, 54.2% for enrofloxacin, 50% for ciprofloxacin, 37.5% for tetracycline and nalidixic acid, 18.1% for ampicillin, 15.3% for streptomycin, and 6.9% for ofloxacin. All isolates were susceptible to erythromycin. The adherence (intracellular and extracellular bacteria) abilities of the 20 isolates to INT-407 cells were between $4.21\pm1.27\times10^4$ CFU/well and $1.053\pm0.451\times10^6$ CFU/well from the isolates of cj-55 and cj-52, respectively, and that can be expressed as 0.1033% to 5.2655% to the infecting inoculum. The invasion (intracellular bacteria) abilities of the 20 isolates to INT-407 were between $1.00\pm1.73\times10^3$ CFU/well and $8.47\pm5.16\times10^4$ CFU/well from the isolates of cj-13 and cj-47, respectively, and that can be expressed as 0.0050% to 0.4235% to the infecting inoculums. The average CFU/well of 20 campylobacters isolated from ducks for adherence to and invasion were $2.646\pm2.886\times10^5$ and $3.03\pm2.7\times10^4$ respectively, and that was 1.3230±1.2139% and 0.1516±0.1343% of the starting viable inoculum. There was considerable correlation (R^2=0.627) between the adherence and invasion ability of *C. jejuni* isolates for INT-407 cell.

Key words : *Campylobacter* spp., Ducks, Virulence and GBS associated genes, Antimicrobial resistance, Adherence and invasion

INTRODUCTION

The thermophilic *Campylobacter* (*C.*), especially *C. jejuni* and *C. coli* are more associated with human gastrointestinal disease, especially *C. jejuni* and *C. coli* which accounts for 95% of all clinical isolates in the UK (Matsuda and Moore, 2004). Campylobacteriosis can cause symptoms including abdominal pain and fever with watery to bloody diarrhea (Lecuit et al, 2004). Occasionally, postinfectious sequelae follow *C. jejuni* infection and include reactive arthritis and Guillain-Barre´ syndrome (Nawaz et al, 2003). Recently, *C. jejuni* has been associated with immunoproliferative small intestine disease, which is a rare type of mucosa-associated lymphoid tissue lymphoma (Lecuit et al, 2004). The main source of transmission through the food chain is the

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consumption and handling of contaminated poultry, but the underlying reasons why poultry are particularly susceptible to colonization by *C. jejuni* are unknown (Friedman et al, 2004). *C. jejuni* has also been recovered from nonavian livestock, unpasteurized milk, and contaminated water (Blasser, 1997). Domestic birds such as ducks are carriers of *Campylobacter* spp. and serve as major sources of infection to human (Waldenström et al, 2002).

Campylobacter spp. infections respond to macrolide antibiotics such as erythromycin, fluoroquinolone antibiotics like ciprofloxacin, and also to tetracyclines, cephalosporins, penicillins and sulphonamides (Lehtopolku et al, 2010). Globally, the occurrences of drug resistance to several major antimicrobials useful in the treatment of Campylobacteriosis are increasing and multiple drug resistance patterns to several classes of antimicrobials are emerging. Engberg et al(2001) suggested increased proportion of drug resistance of Campylobacter isolates especially fluoroquinolones. It was postulated that introduction of fluoroquinolones in veterinary practice in the 1990s has led into increase in *Campylobacter* isolates that are resistant to fluoroquinolones. Several countries have since then reported increased drug resistance of *Campylobacter* isolates from humans and animals (Steinbrückner et al, 2001). These findings indicate the potential risk of drug resistant *Campylobacter* spp. could subsequently be transferred to humans through food chain.

Several potential markers of bacterial virulence have been identified. One of these is the *cad*F gene, which encodes a 37 kDa protein belonging to the group of outer-membrane proteins (OMPs) that functions as an adhesin responsible for certain steps of invasion (Konkel et al, 1999). Another interesting region, designated an invasion-associated marker (*iam*), has been identified in some *C. jejuni* and *C. coli* strains (Carvalho et al, 2001). The virulence of *Campylobacter* species is also associated with *fla*A, *rac*R and *dna*J the expression of adherence and colonization, *vir*B11, *cia*B, *doc*C and *pld*A as pathogenic genes responsible for the expression of invasion (Bang et al, 2003; Datta et al, 2003).

Guillain-Barre' syndrome (GBS) characterized by limb weakness and areflexia, is a typical post-infectious autoimmune diseases (Yuki et al, 2001). Lipo-oligosaccharide (LOS) is a major cell-surface structure expressed by *C. jejuni*. The similarity of structure between human gangliosides and *C. jejuni* LOS, cause Guillain-Barre' and Miller-Fisher Syndrome neuropathies. There are several different GBS associated genes, especially the gene products of *wla*N and *cgt*B both were characterized as β-1,3 galactosyltransferase and *gal*E (UDP-galactose 4-epimerase) were responsible for biosynthesis of LOS (Linton et al, 2000).

The ability of *C. jejuni* to adhere and invade to the epithelial cells of the gastrointestinal tract is important to the development of *Campylobacter* spp.-mediated enteritis. However the pathogenic mechanism of *Campylobacter* spp. has not been clearly understood. The adherence to and invasion of *C. jejuni* into host cells has been studied in a variety of cell lines (Konkel et al, 1992, Gilbert and Slavic, 2005). Human embryonic intestine (INT-407) cells have been widely used to assess the ability of enteric bacteria to adhere to and invade the epithelium (Monteville et al, 2003).

Despite the increased importance of *Campylobacter* spp. in public health in the world, also the duck's meats were increased in consumption as health food in Korea, there is a little of information on prevalence, virulence and antimicrobial susceptibility of isolates from ducks. Therefore the aims of this study were to determine the prevalence of thermophilic *Campylobacter* spp., the drug resistance patterns, the prevalence of putative virulence genes and GBS associated genes were examined for *C. jejuni* isolates from duck's fecal samples in Gyeongnam Province, Korea. And the adherence and invasion properties of *C. jejuni* isolates to the INT-407 cells were examined.

MATERIALS AND METHODS

Sample collection

A total of 117 fecal samples of each ducks were collected from six farms in Gyeongnam area from June 2009 to January 2010. Approximately 5 g of fecal samples were transferred to 15 mL conical tubes (Becton

Dickinson, NJ, USA) containing the brucella broth with 5% activated charcoal. All samples were transported to the laboratory under chilled conditions and processed immediately.

Bacterial isolation

Approximately 1 g of fecal sample was emulsified on 5 mL of the brucella broth with 5% activated charcoal and one loopfull of the emulsion was inoculated onto modified CCDA-Preston agar (Oxoid, Hampshire, England) with selective supplement SR155 (Oxoid, Hampshire, England). The inoculated plates were incubated for 72 h at 42°C in a microaerophilic atmosphere of 5% O_2, 10% CO_2 and 85% N_2. On each plate, three *Campylobacter*-like colonies were identified. Suspected colonies were confirmed as *Campylobacter* spp. following the standard criteria, suggested by Luechtefeld et al. (1980). *Campylobacter* isolates were frozen in laked horse blood (Oxoid, Hampshire, England) at −70°C in preparation for antimicrobial susceptibility testing and final identification by the use of the multiplex-PCR procedure. *C. jejuni* ATCC 33560, *C. coli* ATCC 33559 were used as control strain.

Identification of *C. jejuni* and *C. coli* by PCR

Chromosomal DNA was extracted from cultures grown on Muller-Hinton agar plate supplemented with 5% sheep blood for 48 h at 42°C under microaerobic condition (5% O_2, 10% CO_2, 85% N_2) using a Accuprep genomic DNA extraction kit (Bioneer, Korea) according to the manufacturer's instruction. Because some species of *Campylobacter* can be difficult to distinguish, commercial multiplex-PCR assay was also used for isolate identification. Extracted chromosomal DNA of *C. jejuni* was used for template DNA. Identification of *Campylobacter* isolates was performed using MultiPerfect™

Table 1. Primers for PCR used detection of virulence and GBS associated genes in *Campylobacter* spp.

	Gene	Primer	Sequence	Annealing condition	size (bp)	Reference
A	*fla*A	*fla*A-F	GGA TTT CGT ATT AAC ACA AAT GGT GC	45°C, 1 min	1728	Nachamkin et al (1993)
		*fla*A-R	CTG TAG TAA TCT TAA AAC ATT TTG			
	*cia*B	*cia*B-403	TTT TTA TCA GTC CTT A	42°C, 1 min	986	Datta et al (2003)
		*cia*B-1373	TTT CGG TAT CAT TAG C			
	*iam*A	cia3f	GCA CAA AAT ATA TCA TTA CAA	52°C, 1 min	518	Muller et al (2006)
		cia5r	TTC ACG ACT ACT ATG AGG			
	*cad*F	*cad*F-F2B	TTG AAG GTA ATT TAG ATA TG	45°C, 1 min	400	Konkel et al (1999)
		*cad*F-R1B	CTA ATA CCT AAA GTT GAA AC			
	*doc*C	*doc*C1	TGA GCT ACG CTA TCA TTG	57°C, 1 min	1835	Muller et al (2006)
		*doc*C2	GCT TAC GCT ATG GGT TGG			
	*pld*A	*pld*A-84	AAG CTT ATG CGT TTT T	45°C, 1 min	913	Datta et al (2003)
		*pld*A-981	TAT AAG GCT TTC TCC A			
	*ceu*E	JEJ1	CCT GCT CGG TGA AAG TTT TG	57°C, 30 sec	794	Gonzalez (1997)
		JEJ2	GAT CTT TTT GTT TTG TGC TGC			
	*rac*R	*rac*R-25	GAT GAT CCT GAC TTT G	45°C, 1 min	584	Datta et al (2003)
		*rac*R-593	TCT CCT ATT TTT ACC C			
	*dna*J	*dna*J-299	AAG GCT TTG GCT CAT C	46°C, 1 min	720	Datta et al (2003)
		*dna*J-1003	CTT TTT GTT CAT CGT T			
	*vir*B11	*Vir*B11F	GAA CAG GAA GTG GAA AAA CTA GC	60°C, 1 min	709	Bacon et al (2000)
		*Vir*B11R	TTC CGC ATT GGG CTA TAT G			
B	*cgt*B	DL39	TTA AGA GCA AGA TAT GAA GGT G	56°C, 1 min	561	Linton et al (2000)
		*cgt*Brev	GCA CAT AGA GAA CGC TAC AA			
	*gal*E	*gal*E-F	GAA CCA CAA ACT CCC GTT G	54°C, 30 sec	497	Nawaz et al (2003)
		*gal*E-R	ACA CTA GGA TCA CCC GCA C			
	*wla*N	*wla*N-DL39	TTA AGA GCA AGA TAT GAA GGT G	46°C, 1 min	672	Linton et al (2000)
		*wla*N-DL41	CCA TTT GAA TTG ATA TTT TTG			

A: adherence and invasion associated genes , B: GBS associated genes

Campylobacter strain PCR kit (G&P Life Science, Korea) containing primers for *C. jejuni*, *C. coli*, *C. lari*, *C. fetus* and *C. upsaliensis* by manufacturer's instruction.

Detection of virulence and GBS associated genes

A pair of specific primers used for PCR amplification of virulence associated and GBS related gene from *C. jejuni* isolates. Primer construction has been based on the follow nucleotide sequence that previously reported (Table 1) and the primers were manufactured from Bioneer, Korea. All PCR amplifications were performed in a mixture (25 μL) consisting of 12.5 μL GoTaq® Green Master Mix, 2X (dATP, dCTP, dGTP, TTP, each at 400 μM and 3 mM $MgCl_2$; Promega, Madison, USA), 1 μL template DNA and 0.2 μL of a 20 pM solution of each primer. Distilled water was added to make 25 μL. Primer sequences, annealing temperatures and lengths of products are listed in Table 1. Reactions were carried out in single tubes in a DNA thermal cycler (Eppendorf, Germany).

Gel electrophoresis and detection of amplicon

PCR products were separated by electrophoresis using 1.5% agarose gel (Promega, Madison, U.S.A) and 1X Tris-Boric acid-EDTA buffer, at 1 V/cm for 120 min. Amplicons were visualized after staining the gel with ethidium bromide (0.5 ug/mL, Bioneer, Korea) for 30 min. PCR products were identified under the 254 nm UV transilluminator (Bio-Rad, USA).

Antimicrobial susceptibility test

Seventy-two isolates were subjected to antimicrobial susceptibility test using the agar dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009). The tested antimicrobial agents were ampicillin (Amresco, USA), streptomycin, kanamycin (Duchefa, Netherlands) tetracycline, oxytetracycline, nalidixic acid, ofloxacin, enrofloxacin, ciprofloxacin and erythromycin (Sigma-aldrich, USA). Breakpoint concentrations published by the CLSI were used for the definition and determination of resistance of *Campylobacter* isolates. The breakpoint values of the MIC for resistance were as follows: for streptomycin, kanamycin, nalidixic acid, ≥64 μg/mL; for ampicillin, ≥32 μg/mL; for tetracycline, erythromycin, norfloxacin, 16≥ μg/mL; for oxytetracycline, ofloxacin, ≥8 μg/mL; for ciprofloxacin, 4 μg/mL; and for enrofloxacin, 2≥ μg/mL. Control strains *C. jejuni* (ATCC 33560) were included on each plate. Isolates resistant to two or more of the antibiotics were considered multidrug resistance.

Adhesion and invasion assays

The adhesion and invasion assays were performed by the method of Konkel et al.(1998) with some modifications. Briefly, twenty-four hours prior to adherence and invasion assays, 10^5 cells/mL were seeded at 1 mL per well in 24 well plates (NUNC, Roskilde, Denmark) to allow for attachment. *C. jejuni* isolates were grown microaerobically on Blood agar with 5% sheep blood for 48 h at 37°C. *C. jejuni* isolates were harvested from the plates with pre-warmed DMEM medium and adjusted spectrophotometrically to approximately 2×10^8 bacteria/mL (OD=0.125, 650 nm). The multiplicity of infection (MOI) was approximately 200 times higher than the cell number, and adjusted bacterial cells were inoculated into 24-well tissue culture plate containing approximately 80% confluent monolayers of INT-407 cells. Tissue culture plates were centrifuged at 1,500 rpm at room temperature for 15 min to bring the bacteria in contact with the 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 invasion. For determination of adherence, the cells were washed 3 times with 1 mL of pre-warmed DMEM medium per well to remove non-adherent bacteria. The cell monolayer was lysed with 1% v/v Triton X-100 (Sigma-Aldrich, Auckland, New Zealand) and the total bacteria associated with the cells were enumerated by plating serial dilutions of the lysates on Blood agar with 5% sheep blood and counting the resultant colonies. In order to measure bacterial invasion, the infected cells were washed 3 times with PBS and incubated in fresh DMEM containing 5% fetal bovine serum (FBS) and

200 μg/mL gentamicin for 2 h to kill the remaining viable extracellular bacteria. Following serial dilution of lysates, the released intracellular bacteria were enumerated as described for the adherence assay.

Statistical analysis

The data of isolation ratio, cell adherence and invasion assay was analysed by T-test using Microsoft Office Excel 2007. The correlation between the adherence to and invasion of the INT-407 cells by *Campylobacter* isolates was analyzed by linear regression using SPSS (SPSS Inc., USA).

RESULT

Isolation and Identification

All *Campylobacter* isolates were identified using several biochemical test and multiplex PCR kit. Templates DNAs were amplified 800 bp common band for *Campylobacter* spp., 502 bp band for *C. coli*, 161 bp band for *C. jejuni* (Fig. 1). Total 99 of *Campylobacter* were isolated from 117 duck's fecal samples. Ninety three (93.9%) strains of *C. jejuni* and 6 (6.1%) of *C. coli* were isolated (Table 2). *C. jejuni* was isolated more than 82% in all farm tested. *C. coli* strains were isolated from only three farms, the range of 6.25-17.65%

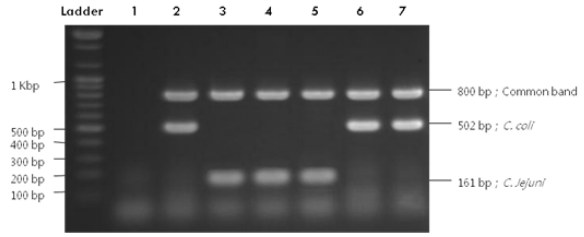

Fig. 1. Identification of *Campylobacter* spp. using Multiperfect™ *Campylobacter* strain PCR Kit. Ladder: 100 bp maker, lane1: negative control: *E. coli* DH5α, lane2: *C. coli* ATCC 33559, lane3: *C. jejuni* ATCC 33560, lane 4-5: *C. jejuni* isolates, lane 6-7: *C. coli* isolates.

Table 2. Isolation rate of *Campylobacter* spp. from ducks

Farms	*Campylobacter* spp. (%)	*C. jejuni* (%)	*C. coli* (%)
A (n=20)	17 (85%)	14 (82.35%)	3 (17.65%)
B (n=19)	18 (94.7%)	18 (100%)	0 (0%)
C (n=18)	15 (83.3%)	13 (86.67)	2 (13.33%)
D (n=16)	16 (80%)	15 (93.75)	1 (6.25%)
E (n=17)	17 (85%)	17 (100%)	0 (0%)
F (n=16)	16 (80%)	16 (100%)	0 (0%)
Total (n=117)	99 (84.6%)	93 (93.9%)	6 (6.1%)

PCR detection of virulence and GBS associated genes

The prevalence of virulence associated genes of *C. jejuni* isolates from ducks was determined by PCR methods. Among the 10 virulence associated genes, *cad*F, *dna*J, *fla*A and *ceu*E genes were found in all of the *C. jejuni* isolates (72 strains) from ducks. *rac*R, *pld*A, *iam*A, *cia*B, *vir*B11, and *doc*C genes were detected 87.5% (63/72), 84.7% (61/72), 77.8% (56/72), 48.6% (35/72), 13.9% (10/72) and 11.1% (8/72), respectively. PCR detection was performed of GBS associated genes for *C. jejuni* from fecal samples. The *gal*E genes were detected 97.2% (70/72) of *C. jejuni* isolates. *cgt*B and *wla*N genes were detected 62.5% and 13.9% of *C. jejuni* isolates, respectively (Table 3).

Patterns of virulence and GBS associated genes

Patterns of virulence associated genes of *C. jejuni* isolates were analyzed. A total of 7 patterns were observed

Table 3. Detection rates of virulence and GBS associated genes of *C. jejuni* isolates from ducks

	Genes	No. of strains	%
Adherence and invasion associated genes	*cad*F	72/72	100%
	*dna*J	72/72	100%
	*fla*A	72/72	100%
	*ceu*E	72/72	100%
	*rac*R	63/72	87.5%
	*pld*A	61/72	84.7%
	*iam*A	56/72	77.8%
	*cia*B	35/72	48.6%
	*vir*B11	10/72	13.9%
	*doc*C	8/72	11.1%
GBS associated genes	*gal*E	70/72	97.2%
	*cgt*B	45/72	62.5%
	*wla*N	10/72	13.9%

in PCR products. When compared the gene harboring patterns of 72 *C. jejuni* isolates from ducks, 10 kinds of gene containing harboring patterns were 24 strains, 9 kinds of gene harboring patterns were 18 strains, 8 kinds of gene harboring pattern were 11 strains, 6, 7, 11 kinds of gene harboring pattern were 6 strains, and 6 kinds of gene harboring pattern were 6 strains in the order (Table 4).

Antimicrobial susceptibility of *C. jejuni* isolates from ducks

To determine antimicrobial susceptibility of *C. jejuni* isolated from duck's fecal samples, agar dilution method

Table 4. Virulence and GBS associated gene patterns of of *C. jejuni* isolates from ducks

No.	PCR patterns	No. of strains	Strains
6 (n=6)	*gal*E, *cad*F, *dna*J, *fla*A, *vir*B11, *ceu*E	6	cj-D56, cj-D57, cj-D58, cj-D60, cj-D63, cj-D64
7 (n=6)	*cgt*B, *gal*E, *cad*F, *dna*J, *fla*A, *vir*B11, *ceu*E	3	cj-D54, cj-D55, cj-D62
	*gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *iam*A, *ceu*E	2	cj-D12, cj-D39
	*gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *pld*A, *ceu*E	1	cj-D8
8 (n=11)	*cgt*B, *gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *pld*A, *ceu*E	1	cj-D6
	*gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *iam*A, *pld*A, *ceu*E	8	cj-D11, cj-D17, cj-D18, cj-D20, cj-D24, cj-D25, cj-D28, cj-D31
	*wla*N, *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *pld*A, *ceu*E	2	cj-D13, cj-D14
9 (n=18)	*cgt*B, *gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *pld*A, *ceu*E	1	cj-D9
	*cgt*B, *gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *iam*A, *pld*A, *ceu*E	11	cj-D4, cj-D16, cj-D19, cj-D22, cj-D26, cj-D27, cj-D29, cj-D30, cj-D35, cj-D36, cj-D42
	*cgt*B, *gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *pld*A, *ceu*E. *doc*C	1	cj-D7
	*gal*E, *wla*N, *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *pld*A, *ceu*E	1	cj-D2
	*gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *iam*A, *pld*A, *ceu*E	4	cj-D5, cj-D40, cj-D41, cj-D59
10 (n=24)	*cgt*B, *gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *iam*A, *pld*A, *ceu*E	18	cj-D1, cj-D10, cj-D32, cj-D33, cj-D34, cj-D37, cj-D43, cj-D44, cj-D45, cj-D46, cj-D48, cj-D53, cj-D65, cj-D66, cj-D67, cj-D68, cj-D69, cj-D70
	*cgt*B, *gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *iam*A, *pld*A, *vir*B11, *ceu*E	1	cj-D38
	*cgt*B, *gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *iam*A, *pld*A, *ceu*E, *doc*C	2	cj-D71, cj-D72
	*gal*E, *wla*N, *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *iam*A, *pld*A, *ceu*E	2	cj-D3, cj-D15
	*gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *iam*A, *pld*A, *ceu*E, *doc*C	1	cj-D47
11 (n=6)	*cgt*B, *gal*E, *wla*N, *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *iam*A, *pld*A, *ceu*E	3	cj-D21, cj-D23, cj-D50
	*cgt*B, *gal*E, *wla*N, *cad*F, *dna*J, *fla*A, *rac*R, *iam*A, *pld*A, *ceu*E, *doc*C	1	cj-D51
	*cgt*B, *gal*E, *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *iam*A, *pld*A, *ceu*E, *doc*C	2	cj-D52, cj-D62
12 (n=1)	*cgt*B, *gal*E, *wla*N, *cad*F, *dna*J, *fla*A, *rac*R, *cia*B, *iam*A, *pld*A, *ceu*E, *doc*C	1	cj-D49
Total 21 patterns			Total of 72 strains

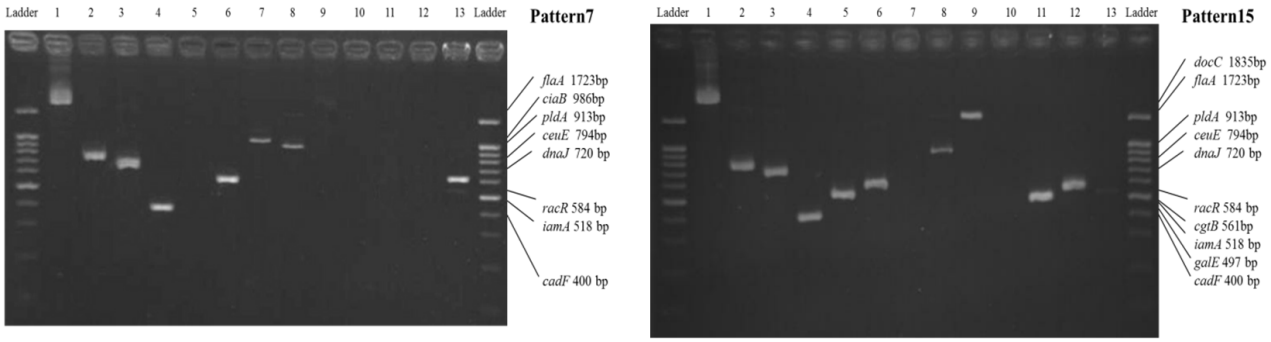

Fig. 2. Representative PCR amplification patterns for virulence and GBS associated genes of *Campylobacter* isolates. Ladder: 100 bp maker, lane1: *fla*A, lane2: *ceu*E, lane3: *dna*J, lane4: *cad*F, lane5: *iam*A, lane6: *rac*R, lane7: *cia*B, lane8: *pld*A, lane9: *doc*C, lane10: *vir*B11, lane11: *gal*E, lane12: *cgt*B, lane13: *wla*N.

Table 5. Distributions of MIC of 11 antimicrobial agents for 72 *C. jejuni* isolates from ducks

	Antimicrobial Agent	Number of isolates with MIC (mg/L) of															Mode	MIC_{50}	MIC_{90}	R (%)
		0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512				
β-lactams	Ampicillin (≥ 32)					31	1	4	9	5	9	6	1	2	2	2		0.5	2	32	(18.1)
Aminoglycosides	Streptomycin (≥ 64)				39	2	12	7	0	0	1	0	1	1	9			0.25	0.25	256	(15.3)
	Kanamycin (≥ 64)					5	2	0	3	6	2	14	25	0	2	13		64	64	512	(55.6)
Tetracyclines	Tetracycline (≥ 16)						36	4	3	2	3	3	8	8	4	1		1	1	128	(37.5)
	Oxytetracycline (≥ 8)					16	2	1	8	5	5	12	11	8	1	3		0.5	16	128	(62.5)
Quinolones	Nalidixic acid (≥ 64)							29	3	4	3	6	9	14	4			2	8	128	(37.5)
	Ofloxacin (≥ 8)	55	0	0	2	3	3	1	3	2	3							0.03	0.03	4	(6.9)
	Norfloxacin (≥ 16)			42	0	2	0	2	3	2	3	4	9	5				0.125	0.125	64	(51.2)
	Enrofloxacin (≥ 2)				30	0	3	3	10	21	5							0.25	2	8	(54.2)
	Ciprofloxacin (≥ 4)			29	4	1	2	0	1	2	21	12						0.125	1	32	(50.0)
Macrolides	Erythromycin (≥ 16)	24	35	1	3	5	4											0.06	0.06	0.5	(0)

Dark solid bar represent MIC for each drug according to CLSI (2009)
MIC_{50} and MIC_{90} indicate the concentration (μg/mL) at which 50% and 90% of isolates tested were susceptible to the antimicrobial, respectively.
(≥) indicates breakpoint of antibicrobial resistance.
R (%) indicates the rate of resistance strains for the antimicrobial agents.

was used. Antimicrobial susceptibility test was performed on with 72 *C. jejuni* isolates. The MICs (minimum inhibitory concentrations) of all 11 antimicrobial agents were summarized in Table 5. The proportions of resistant isolates for the different antimicrobial agents were as following; the rates of resistance were 62.5% for oxytetracycline, 55.6% for kanamycin, 54.2% for enrofloxacin, 52.1% for norfloxacin, 50% for ciprofloxacin, 37.5% for tetracycline and nalidixic acid, respectively, 18.1% for ampicillin, 15.3% for streptomycin and 6.9% for ofloxacin. All the isolates were susceptible to erythromycin.

Total 10 multidrug resistant patterns were determined by antimicrobial susceptibility test for 72 *C. jejuni* isolates to 11 antimicrobials (Table 6). Most common multiple drug resistant pattern in *C. jejuni* was the resistance to three drugs resistance (19.4%), followed by five and six drug resistant *C. jejuni* isolates were twelve (16.7%), four drug resistant *C. jejuni* isolates were eleven (15.3%). Four *C. jejuni* isolates (5.6%) were susceptible to all 11 drugs. Also, more than nine drug resistant *C. jejuni* isolates were two of seventy-two (2.8%). Sixty-six *C. jejuni* isolates (91.6%) were resistant to multiple drugs (two or more).

Table 6. The multiple drug resistance patterns of *C. jejuni* isolates from ducks

Resistance patterns	No. of isolates	%
Sensitive to all 11 drugs	4	5.6%
1 drug resistance	2	2.8%
2 drug resistance	7	9.7%
3 drug resistance	14	19.4%
4 drug resistance	11	15.3%
5 drug resistance	12	16.7%
6 drug resistance	12	16.7%
7 drug resistance	6	8.3%
8 drug resistance	2	2.8%
9 drug resistance	2	2.8%
Total (n=72)	72	100%

Table 7. Adherence and invasion numbers of *C. jejuni* isolates from ducks (*; $p < 0.05$)

Strains	Adherence (CFU/well)	Invasion (CFU/well)
Cj_47	$4.772\pm2.558\times10^{5}$*	$8.47\pm5.16\times10^{4}$*
Cj_52	$1.053\pm0.451\times10^{6}$*	$7.99\pm4.69\times10^{4}$*
Cj_35	$2.998\pm1.210\times10^{5}$*	$6.27\pm4.57\times10^{4}$
Cj_51	$5.099\pm5.646\times10^{5}$	$5.80\pm5.90\times10^{4}$
Cj_38	$3.458\pm1.065\times10^{5}$*	$5.60\pm3.09\times10^{4}$*
Cj_25	$3.111\pm0.859\times10^{5}$*	$4.44\pm3.76\times10^{4}$
Cj_38	$2.560\pm1.804\times10^{5}$	$4.34\pm2.14\times10^{4}$*
Cj_42	$2.744\pm1.499\times10^{5}$*	$3.30\pm2.51\times10^{4}$
Cj_37	$3.517\pm1.069\times10^{5}$*	$3.01\pm3.07\times10^{4}$
Cj_23	$8.95\pm6.24\times10^{4}$	$2.72\pm1.47\times10^{4}$*
Cj_15	$1.505\pm1.209\times10^{5}$	$2.59\pm1.37\times10^{4}$*
Cj_66	$4.744\pm1.644\times10^{5}$*	$2.58\pm1.61\times10^{4}$*
Cj_2	$2.599\pm1.440\times10^{5}$*	$1.39\pm0.15\times10^{4}$*
Cj_39	$5.53\pm5.40\times10^{4}$	$6.47\pm1.58\times10^{3}$*
Cj_36	$9.69\pm11.88\times10^{4}$	$3.43\pm2.97\times10^{3}$
Cj_55	$4.21\pm1.27\times10^{4}$	$3.17\pm2.96\times10^{3}$
Cj_54	$6.49\pm6.29\times10^{4}$	$3.10\pm1.87\times10^{3}$
Cj_6	$7.15\pm4.46\times10^{4}$	$2.33\pm2.01\times10^{3}$
Cj_12	$5.84\pm2.44\times10^{4}$	$1.50\pm2.33\times10^{3}$
Cj_13	$4.96\pm1.56\times10^{4}$	$1.00\pm1.73\times10^{3}$
E. coli DH5α	$5.25\pm1.88\times10^{4}$	$7.33\pm2.52\times10^{2}$
Means	$2.646\pm2.486\times10^{5}$	$3.03\pm2.7\times10^{4}$

There was considerable correlation (R^2=0.627) between the adherence and invasion ability of *C. jejuni* isolates for INT-407 cell.

Adhesion and invasion ability of *C. jejuni* isolates into INT-407 cells

Gentamicin protection assays were performed to determinate the adherence and invasion ability of *C. jejuni*. Twenty isolates were randomly selected considering virulence associated gene patterns. For comparison, *E. coli* DH5α were included in the gentamicin protection assays as control, respectively. After 3 hr incubation, the adherence (intracellular and extracellular bacteria) abilities of the 20 isolates to INT-407 cells were between $4.21\pm1.27\times10^{4}$ CFU/well and $1.053\pm0.451\times10^{6}$ CFU/well from the isolates of cj-55 and cj-52, respectively, and that can be expressed as 0.1033% to 5.2655% to the infecting inoculum. The invasion (intracellular bacteria) abilities of the 20 isolates to INT-407 were between $1.00\pm1.73\times10^{3}$ CFU/well and $8.47\pm5.16\times10^{4}$ CFU/well from the isolates of cj-13 and cj-47, respectively, and that can be expressed as 0.0050% to 0.4235% to the infecting inoculums (Table 7). The average CFU/well of 20 *C. jejuni* isolates from ducks for adherence to and invasion were $2.646\pm2.886\times10^{5}$ and $3.03\pm2.7\times10^{4}$ respectively, and that was 1.3230±1.2139% and 0.1516± 0.1343% of the starting viable inoculum. There was a considerable correlation (adjusted R^2=0.627) between the adherence ability and invasion ability of the *C. jejuni* (Table 7).

DISCUSSION

Birds, including ducks, have been implicated as vectors of transmission of *C. jejuni* (Waldenström et al, 2002). In this study, total of 99 (85.4%) strains of campylobacters were isolated from 117 duck's fecal samples. It has confirmed the importance of ducks as reservoirs in the distribution of thermophilic *Campylobacter* spp. in particular *C. jejuni* in Korea. High prevalence of Campylobacter spp. in ducks was agreement with other findings (Nonga and Muhairwa, 2010).

Among the 99 strains of *Campylobacter* spp., 93 (93.9%) strains were *C. jejuni* and 6 (6.1%) strains were *C. coli*. By previous studies, *C. jejuni* has been isolated from 29.4% to 88.3% from ducks (Prescott and Bruin-Mosch 1981; Luechtefeld et al, 1980). In this study, the most of *Campylobacter* spp. isolates were *C. jejuni* (93.9%). This result agreed with previous study by Nonga and Muhairwa (2010) (81.9%), Prescott and Bruin-Mosch (1981) (88.3%). However, other researchers reported higher prevalance of *C. coli* than *C. jejuni* in ducks. However, the other studies showed no difference between prevalence of *C. coli* and *C. jejuni* in ducks (Aquino et al, 2002). The difference in species of isolates may depend on the local environmental conditions, sampling techniques and methods, seasonal conditions and laboratory methodologies.

Globally, the occurrences of resistance to several major antibiotics useful in the treatment of campylobacteriosis are increasing and multiple drug resistance patterns to several classes of antimicrobials are emerging (Lehtopolku et al, 2010; Steinbrückner et al, 2010). These studies warn the potential risk of drug resistance in *C. jejuni*, which could resultingly be transferred to humans through food chain. In this study, the antimicrobial susceptibility test was performed on 72 *C. jejuni* isolates

from duck's fecal samples. The rates of drug resistance were 62.5% for oxytetracycline, 55.6% for kanamycin, 54.2% for enrofloxacin, 50% for ciprofloxacin, 37.5% for tetracycline and nalidixic acid, respectively, 18.1% for ampicillin, 15.3% for streptomycin and 6.9% for ofloxacin. All the *C. jejuni* isolates were susceptible to erythromycin. Erythromycin has been used as primary treatment drug for campylobacteriosis. Although the rate of erythromycin resistant *C. jejuni* isolates has been reported to be 3.2-42% in humans and animals in other countries (Gibreel and Taylor, 2006; Boonmar et al, 2007), none of erythromycin resistant *C. jejuni* isolates were in this study. This result was consistent with the report in Japan (Bakeli et al, 2008). The prevalence of quinolone-resistant *C. jejuni* isolates has continued to increase in other countries, although the rate varies from country to country. Therefore, increasing attention has been paid to the acquisition of resistance to quinolone drugs by *C. jejuni* (Smith et al, 1999). Contrary to the present study where drug resistance was 54.2%, many studies reported a high level of ciprofloxacin resistance (more than 80%) (Prats et al, 2000). The low resistance rate observed in this study may be due to the fact that fluoroquinolones have not been extensively used in veterinary practice, especially, duck industry in Korea. In this study, the resistance rates of *C. jejuni* to tetracycline were revealed as 37.5%. Previously, it has been shown that 38.5% of *C. jejuni* isolates from human in Germany are resistant to tetracycline. However, high resistance rates to tetracyclines has previously been researched by other researcher (Prats et al, 2000). The tetO genes, associated to the tetracycline resistance in *C. jejuni*, and that mediated by conjugative plasmids or chromosomal DNA elements. So, there is possibility of gene transfer among *Campylobacter* spp. and other bacteria in the animals. Multiple drug resistant patterns were determined (Table 7). The Sixty-six *C. jejuni* isolates(91.6%) were resistant to multiple drugs. These results indicating that it could be a serious public health-risk factor in human. And these data emphasize the need of drug resistance surveillance and effective national monitoring program in Korea.

Several studies have reported that both adherence and invasion are multifactorial processes in *C. jejuni*. Several virulence factors have been identified in previous studies (Datta et al, 2003; Bacon et al, 2000; Nawaz et al, 2003).

In this study, the prevalence of virulence associated genes was determined among the population of *C. jejuni* isolates from ducks by PCR detection. *cad*F, *dna*J, *fla*A and *ceu*E genes were found in all of isolates from ducks. *rac*R, *pld*A, *iam*A, *cia*B, *vir*B11, and *doc*C genes were detected 87.5%, 84.7%, 77.8%, 48.6%, 13.9% and 11.1%, respectively.

The *cad*F gene was detected in all *Campylobacter* isolates of *C. jejuni* from duck's fecal samples. Similar observations were reported in *Campylobacter* spp. isolates from human specimens as well as from chicken carcasses (Datta et al, 2003).

The *dna*J gene was detected in all isolates, racR gene was detected in 87.5% of isolates. In the previous study, dnaJ gene were detected in 100% of isolates in many studies, however racR gene was detected in the range of 85.7-100% (Datta et al, 2003; Talukder et al, 2008).

The *fla*A gene was detected in all of *C. jejuni* isolates and *pld*A gene was detected in 84.7%. In the previous studies, *fla*A and *pld*A were detected in all of isolates (Wassenaar et al, 1991). The *cia*B gene was detected in 48.6% of *C. jejuni isolates*. In the previous study, *cia*B gene was detected in the range of 41-100% (Datta et al, 2003; Talukder et al, 2008). The *iam* gene was found in 77.8% of isolates. Similar observation were reported 88.11% of aim gene in *C. jejuni* and *C. coli* isolates from broiler feces (Khoshbakht et al, 2013).

The *ceu*E genes was detected in all isolates. This data was higher than that of Talukder et al. (2008) and Ripabelli et al. (2010) which found a prevalence of 82.5% and 86.3%, respectively., However, other authors in Denmark were agreed with present results (Bang et al, 2003). The product of *ceu*E gene is important for pathogenicity because of its involvement in iron acquisition and bacterial infectivity (Gonzalez et al, 1997).

The *vir*V11 genes were detected in the range of 0-35.7% (Talukder et al, 2008; Nielsen et al, 2010). In this study, the 13.9 % prevalence of the *vir*B11 gene in duck's fecal isolates of *C. jejuni* is in agreement with 10.3% in humans reported in Bacon et al.(2000) and 16.7% in Krutkiewicz et al.(2010). This result suggest-

ing that some poultry isolates are capable of invading the human intestine.

Methyl-accepting chemotaxis proteins (MCPs) were postulated to form complexes with each other and to be involved in chick colonization (Hendrixon and DiRita, 2004). The *doc*C were suggested to be candidates of such a complex (Hendrixon and DiRita, 2004). In this study, the *doc*C gene was detected in 8 of 72 strains (11.1%). However, Muller et al.(2006) reported 54.5% of docC gene in *C. jejuni* from broilers than higher prevalence in this study.

In the comparison of detection rates to 3 kinds of GBS associated genes for *C. jejuni* from ducks. *gal*E gene were detected 97.2% of *C. jejuni*. *cgt*B and *wla*N genes were detected 62.5% and 13.9%, repectively. The *cgt*B gene and *wla*N gene were detected in other studies, in the range of 4.7% to 100% (Datta et al, 2003; Nawaz et al, 2003), respectively. Further studies are necessary to discover the importance of LOS structures in connection with GBS. However, the presence of *cgt*B, *wla*N and *gal*E gene suggest that some duck's fecal isolates are capable cause of GBS.

However, it is important to refer that the prevalence of a few genes cannot be used to confirm whether *C. jejuni* is more virulent or not. In addition, specific sequence variation at the primer binding site cause a negative result by PCR, so negative results does not indicate the absolute absence of a gene. This is a limitation of the PCR method for detecting target regions. In order to obtain accurate results, more than researches are going to need.

Adherence and invasion, known as an independent process, but colonization or adherence of microbial pathogens to mucosal surfaces is the primary step of infection and appears to be a prerequisite for invasion in most cases (Monteville et al, 2003). In this study, I examined the ability of *C. jejuni* strains isolated from the fecal contents of ducks to adhere and invade to the monolayer cells of the INT-407 human intestinal cell line. Since there is no perfect animal model, the cell culture model intestinal epithelial cells is an effective tool for assessing the abilities of *C. jejuni* isolates to adhere to and invade the human intestinal epithelium. In present study, the range of adherence and invasion were 0.1033% to 5.2655% and 0.0050% to 0.4235%, respectively. Biswas et al (2000). reported that 0.7416～2.1714% and 0.0012～0.4226% of the range of adherence and invasion, respectively and 0.03～0.73% and 0.007～0.28% in Thlailand (Chansiripornchai and Sasipreeyajan, 2009). Also, I analyzed the relationship between adherence and invasion efficiency of *C. jejuni* using linear regression analysis (SPSS). There was a considerable correlation (adjusted R^2=0.627) between the adherence and the invasion ability of the *Campylobacter* isolates. Thus, the adherence of *C. jejuni* may facilitate invasion into host cells. However, in order to understand clearly the relationship of adherence and invasion, furthermore experiments will be conducted, *in vivo*.

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