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<Original Article>

Characterization of antimicrobial resistance and application of RFLP for epidemiological monitoring of thermophilic *Campylobacter* spp. isolated from dogs and humans in Korea

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Abstract : An antimicrobial susceptibility test was conducted to compare the resistance rates among *Campylobacter* spp. isolates from dogs (n = 50) raised under diverse conditions and humans (n = 50). More than 60% of *Campylobacter* (*C.*) *jejuni* from dogs and humans showed resistance to nalidixic acid, enrofloxacin and ciprofloxacin. *C. jejuni* isolates from humans showed higher resistance to tetracycline (83.3%) and ampicillin (91.3%) than those from dogs. None of the *C. jejuni* or *Campylobacter coli* isolates from humans or dogs were resistant to erythromycin. Overall, 85% of *Campylobacter* spp. isolates showed a multidrug resistant phenotype. Nucleotide sequencing analysis of the *gryA* gene showed that 100% of NA^R/CIP^R *C. jejuni* isolates from dogs and humans had the Thr-86^{th}-Ile mutation, which is associated with fluoroquinolone resistance. *fla*A PCR restriction fragment length polymorphism (RFLP) typing to differentiate the isolates below the species level revealed 12 different clusters out of 73 strains. The human isolates belonged to eight different RFLP clusters, while five clusters contained dog and human isolates.

Keywords : antibiotic resistance, *Campylobacter* spp., DNA gyrase, point mutation, RFLP

Introduction

Campylobacter spp. are one of the leading causes of acute bacterial diarrhea worldwide. Infection with *Campylobacter* (*C.*) *jejuni* or *Campylobacter* (*C.*) *coli* is characterized by the sudden onset of fever, abdominal pains, and diarrhea with blood and leukocytes [4, 20]. Occasionally, postinfectious sequale following *C. jejuni* infection includes reactive arthritis and Guillian-barre syndrome [8]. There are many possible sources of infection with *C. jejuni* and *C. coli*, as they constitute the normal intestinal flora in a wide range of birds and mammals [4, 31]. Large-scale outbreaks of human campylobacteriosis are rare and usually linked to the consumption of contaminated water or raw milk. Sporadic cases of campylobacteriosis are frequently associated with the consumption of undercooked chicken, thus contaminated poultry meat is a major risk factor contributing to human infections [14, 17].

The household dog has previously been identified as a risk factor for human campylobacteriosis [1, 3, 29]. Most of dogs carrying the bacteria are asymptomatic, serving as reservoirs capable of shedding *Campylobacter* spp. in the feces. The fecal shedding of bacteria may increase the chance of infection in other animals by contaminating the environment [12, 23].

Most human infection with *C. jejuni* and *C. coli* is self-limiting and antimicrobials are usually not recommended for treatment. Occasionally, treatment with erythromycin or fluoroquinolone is used for individuals with invasive or severe infection [23]. Recently, resistance to fluoroquinolone is increasing worldwide. The increase of antimicrobial resistance in *Campylobacter* spp. is recognized as a major public health concern, and fluoroquinolone resistance is the most common among the human-origin isolates of both *C. jejuni* and *C. coli* [22, 27].

The primary target of the fluoroquinolones to *C. jejuni* has been shown to be DNA gyrase, a type II topoisomerase that is an essential enzyme for DNA replication [9, 26]. DNA gyrase is composed of two A and B subunits encoded by the *gyr*A and *gyr*B genes, respectively. A point mutation at the codon 86 (ACA to ATA) in the quinolone resistance-deter-

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mining region (QRDR) of the *gyr*A gene, leading to substitution of isoleucine (Ile) for threonine (Thr), is the most common cause of fluoroquinolone resistance among *C. jejuni* isolates [25, 28]. There are anecdotal reports of mutations leading to additional amino acid changes as well as silent nucleotide mutations [19, 21].

There are several methods available for epidemiological strain typing to determine the source of *Campylobacter* spp. infection. These methods include PCR restriction fragment length polymorphism (PCR-RFLP) analysis based on the flagellin gene (*fla*A typing), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) analysis. Assessment of the strain typing methods based on the genotypes of *Campylobacter* spp. has been described in a recent review paper [30]. The RFLP method is simple and reasonable, and has sufficient discriminatory power useful for the typing of *C. jejuni* and *C. coli* [17].

Accordingly, the aim of this study was to investigate the prevalence of antimicrobial resistant *Campylobacter* spp. isolated from dogs and humans and to confirm the relationship between fluoroquinolone-resistance and the *gyr*A mutation by DNA sequencing of the *gyr*A gene. In order to investigate epidemiological relatedness of *Campylobacter* spp. isolated from dogs and humans, a PCR-RFLP method for the *fla*A typing was also employed [19].

Materials and Methods

Bacterial strains and growth condition

A total of 41 *C. jejuni* was isolated from dogs in Gyeongnam and Busan area, in the period between August 2009 and April 2010. Nine strains of *C. coli* were isolated from dogs in Gyeongnam and Busan area, in the same period. Strains of 46 *C. jejuni* and 4 *C. coli* isolated from humans with diarrhea in Seoul area during the year 2008-2009 were kindly provided by Korea National Institute of Health. All isolates were stored in liquid nitrogen at −150°C. Strains were cultured at 42°C for 48 h on Mueller-Hinton agar supplemented with 5% sheep blood in a microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2).

Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed by agar dilution method with Mueller-Hinton agar supplement with 5% sheep blood, according to the protocols of Clinical and Laboratory Standards Institute (CLSI) [5]. *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were used as the quality control organisms for the test. Erythromycin (EM 0.03-64 μg/mL), tetracycline (TC 0.125-512 μg/mL), nalidixic acid (NA 0.25-512 μg/mL), enrofloxacin (ENO 0.03-64 μg/mL), ciprofloxacin (CIP 0.03-128 μg/mL), gentamicin (GM 0.25-512 μg/mL) and ampicillin (AM 0.25-512 μg/mL) were added to the agar plate by a ten-fold serial dilution. Bacterial turbidity for plating was adjusted by using a 0.5 Macfarland standard, according to the guideline of CLSI [5].

Minimal inhibitory concentration (MIC) values for resistance criteria were set towards EM (≥ 32 μg/mL), TC (≥ 16 μg/mL), NA (≥ 64 μg/mL), ENO (≥ 2 μg/mL), CIP (≥ 4 μg/mL), GM (≥ 8 μg/mL), and AM (≥ 32 μg/mL) in accordance with a previous report [2]. Multidrug resistance (MDR) was defined as the case exhibiting resistance to two or more antimicrobials at the same time. The bacterial inoculum was applied to the plate with multipoint inoculator. Then, the agar plates were incubated at 42°C for 48 h in a microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2).

DNA extraction

Genomic DNA was extracted from the fresh culture by using QIA amp DNA Mini kit (Qiagen, Germany) according to the recommendations of the manufacturer. The DNA concentration was measured spectrophotometrically at A_{260}.

DNA sequencing of the *gyr*A gene

DNA sequencing of the *gyr*A genes was performed to confirm the relationship between the fluoroquinolone resistance and the *gyr*A mutation. The DNA amplification was carried out in a thermal cycler using a forward primer (5'-GCTATGCAAATGATGAGGC-3') and a reverse primer (5'-CAGTATAACGCATCGCAGCGG-3'). The reaction mixture contained 5 μL of PCR buffer [10 mM Tris-HCl (pH8.5), 50 mM KCl, 1.5 m M $MgCl_2$] , 1 μL of 10 mM dNTP mixture, 1 μL of *Taq* polymerase (2.5 U), 1 μL of bacterial DNA extract as template, and 2 μM of primer mixture in a PCR tube with a final 50 μL volume. Each PCR product was purified and subjected to (ABI 3730 DNA Analyzer; Applied Biosystems, USA).

The *fla*A-RFLP

The flagellin gene typing was performed according to the method of Nachamkin *et al.* [19] in order for comparison of virulence, antimicrobial resistance, and the genotypic profile of the *C. jejuni* and *C. coli* isolates from the dog and human stools.

The amplification reaction was carried out in a DNA thermal cycler using forward primer (5'-GGATTTCGTATTAACACAAATGGTGC-3') and reverse primer (5'-CTGTAGTA ATCTTAAACATTTG-3'). PCR was performed in a 50 μL mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs, 2.5 μ *Taq* DNA polymer (Takara Bio, Japan), 1 mM of the two primers, and 1 μL template DNA. Samples were preincubated at 95°C 5 min and cycled 30 times at 95°C for 20 sec, 52°C for 45 sec, and 72°C for 60 sec. The samples were then incubated at 72°C for 5 min and maintained at 4°C. A reagent blank contained all components of the reaction mixture except template DNA, for which sterile distilled water was substituted. This step was included in every PCR procedure.

After amplification, 5 μL of the PCR product was electrophoresed on a 12.5% acrylamide gel (Bio-rad Laboratories, USA) in Tris borate-EDTA (TBE) buffer. The gels were stained with 0.5 μg/mL ethidium bromide to determine the

presence or absence of the PCR products. The amplicon of *fla*A gene was digested with the restriction enzyme *Dde*I for 2 h at 37°C. The digested fragments were separated and analyzed by electrophoresis. Acrylamide gels were runned at 75 V for 2 h in 1.0 × TBE buffer. Photographs of gels stained with 0.5 μg/mL ethidium bromide were analysed by Fingerprinting Plus software package (ver. 1.2; Bio-Rad Laboratories). A 1.0 kb DNA ladder was used as a molecular marker.

Table 1. Comparison of antimicrobial resistance phenotypes of *C. jejuni* isolated from humans and dogs raised in different raising conditions and areas

	Household dogs (n = 10)				Stray dogs (n = 23)				Breading dogs (n = 8)				Humans (n = 46)			
Antimicrobial	MIC_{50}	MIC_{90}	MIC range	% R	MIC_{50}	MIC_{90}	MIC range	% R	MIC_{50}	MIC_{90}	MIC range	% R	MIC_{50}	MIC_{90}	MIC range	% R
Erythromycin (≥ 32 μg)	0.5	1	0.5~2	**0***	0.5	2	0.5~4	**0***	0.5	4	0.5~4	**0***	2	4	0.125~16	**0**
Tetracycline (≥ 16 μg)	0.5	64	0.5~256	**40**	0.5	64	0.5~256	**21.7**	0.5	512	0.5~512	**25**	64	128	0.125~256	**82.6**
Nalidixic acid (≥ 64 μg)	128	256	8~256	**90**	128	256	8~256	**78.3***	128	256	4~256	**75**	128	256	0.125~512	**87**
Enrofloxacin (≥ 2 μg)	4	8	0.25~8	**80**	4	4	0.25~4	**56.5**	4	8	2~8	**100**	4	16	0.03~64	**80.4**
Ciprofloxacin (≥ 4 μg)	8	16	0.125~64	**60**	8	16	0.125~16	**78.3***	8	64	8~64	**100**	8	32	0.03~128	**80.4**
Gentamicin (≤ 8 μg)	1	128	0.5~128	**20*****	1	128	0.5~256	**43.5*****	0.5	0.5	0.5	**0*****	1	2	0.25~64	**6.52**
Ampicillin (≤ 32 μg)	0.5	16	0.5~128	**10**	2	8	0.5~128	**8.7**	0.5	32	0.5~32	**12.5**	512	>512	8~512	**91.3**

MIC_{50} and MIC_{90} indicate the concentration (μg/mL) at which 50% and 90% of isolates tested were susceptible to the antimicrobial, respectively. MIC range indicates the MIC level for *C. jejuni* isolates at a particular antimicrobial concentration. %R indicates the ratio of resistance strains for the antimicrobial agent. (≥): resistance breaks point (μg/mL) for the antimicrobials. *≤ 0.5, **≤ 0.01, ***≤ 0.001 level of significant statistical difference.

Table 2. Comparison of antimicrobial resistance phenotypes of *C. coli* isolated from humans and dogs raised in different raising condition and areas

	Household dogs (n = 4)				Stray dogs (n = 3)				Breading dogs (n = 2)				Humans (n = 4)			
Antimicrobial	MIC_{50}	MIC_{90}	MIC range	% R	MIC_{50}	MIC_{90}	MIC range	% R	MIC_{50}	MIC_{90}	MIC range	% R	MIC_{50}	MIC_{90}	MIC range	% R
Erythromycin (≥ 32 μg)	0.5	2	0.5~2	**0**	0.5	4	0.5~4	**0**	0.5	0.5	0.5	**0**	4	4	0.5~4	**0**
Tetracycline (≥ 16 μg)	0.5	0.5	0.5	**0**	32	64	4~64	**66.7**	0.5	2	0.5~2	**0**	64	256	0.5~256	**75**
Nalidixic acid (≥ 64 μg)	32	128	2~128	**50**	128	256	4~256	**66.7**	32	64	32~64	**50**	8	256	4~256	**50**
Enrofloxacin (≥ 2 μg)	16	64	16~64	**100**	16	64	8~64	**100**	8	16	8~16	**100**	0.125	32	0.06~32	**50**
Ciprofloxacin (≤ 4 μg)	0.125	0.125	0.125	**0**	0.125	0.125	0.125	**0**	0.125	0.125	0.125	**0**	0.125	64	0.06~64	**50**
Gentamicin (≤ 8 μg)	4	4	4	**0****	1	1	1	**0**	1	1	1	**0****	1	32	1~32	**25**
Ampicillin (≥ 32 μg)	0.5	1	0.5~1	**0**	16	32	1~32	**33.3**	0.5	1	0.5~1	**0**	128	512	64~512	**100**

MIC_{50} and MIC_{90} indicate the concentration (μg/mL) at which 50% and 90% of isolates tested were susceptible to the antimicrobial, respectively. MIC range indicates the MIC level for *C. coli* isolates at a particular antimicrobial concentration. %R indicates the ratio of resistance strains for the antimicrobial agent. (≥); resistance breaks point (μg/mL) for the antimicrobials. *≤ 0.5, **≤ 0.01, ***≤ 0.001 level of significant statistical difference.

The *fla*A RFLP pattern was analyzed by BioNumeric software (ver. 4.01; Applied Maths, Belgium). Images of gel were normalized by alingment with 1.0 Kb DNA ladder (Takara Bio). Matching and the dendrogram of the fingerprints determined by the unweighted pair group method with averages were performed using the Dice coefficient with 1% tolerance windows.

Stastical analysis

Data were analyzed by Student's *t* tests. Differences were considered significant at $p < 0.05$. Data are expressed as means ± standard error.

Results

Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed for comparison of resistance rate in *Campylobacter* isolates from dogs raised in diverse condition and humans. Antimicrobial resistance measured against 7 antimicrobial agents is shown in Tables 1 and 2. None of the *C. jejuni* and *C. coli* isolates from humans and dogs was resistant to erythromycin. Antimicrobial resistance to tetracycline was observed in 45%, 21.7%, 25%, and 82.6% of the *C. jejuni* isolates from household dogs, stray dogs, breeding dogs, and humans, respectively. *C. jejuni* isolates from humans showed higher resistance to tetracycline than those isolated from dogs ($p \leq 0.01$). Tetracycline resistance of *C. jejuni* from humans showed higher MIC 50 (64 μg/mL) than those isolated from dogs (0.5 μg/mL). More than 60% of *C. jejuni* from dogs and humans showed resistance to nalidixic acid, enrofloxacin, and ciprofloxacin. Especially, all of the *C. jejuni* from breeding dogs were resistant to enrofloxacin and ciprofloxacin. Nalidixic acid resistance of *C. jejuni* from humans and three different raising groups of dogs revealed equal level of MIC_{50} (128 μg/mL) and MIC_{90} (256 μg/mL). Antimicrobial resistance to gentamicin was observed in 20%, 43.5%, and 6.25% of *C. jejuni* from household dogs, stray dogs and humans, respectively. However, none of the *C. jejuni* from breading dogs was resistant to gentamicin. Antimicrobial resistance to ampicillin was observed in 10%, 8.7%, 12.5%, and 91.3% of *C. jejuni* from household dogs, stray dogs, breading dogs, and humans, respectively. *C. jejuni* from humans showed higher resistance to ampicillin than those isolated from dogs ($p \leq 0.05$). Antimicrobial resistance to tetracycline was observed in 66.7% and 75% of *C. coli* isolates from stray dogs and humans, respectively. However, none of the *C. coli* from household dogs and breeding dogs was resistant to tetracycline. More than 50% of *C. coli* from dogs and humans showed resistance to nalidixic acid and enrofloxacin. Enrofloxacin resistant *C. coli* from dogs had higher MIC_{50} (16 μg/mL) than those isolated from humans (0.125 μg/mL). Antimicrobial resistance to ciprofloxacin and gentamicin was observed in 50% and 25% of *C. coli* from humans, respectively. However, none of *C. coli* from dogs was resistant to ciprofloxacin and gentamicin. Antimicrobial resistance to ampicillin was observed in 33.5% and all of *C. coli* from stray dogs and humans, respectively. However, none of *C. coli* from household dogs and breeding dogs was resistant to ampicillin.

Table 3. Drug resistance patterns of *C. jejuni* and *C. coli* isolates from dogs and humans

Resistance pattern	Number of strains				Total
	Dogs		Humans		
	C. jejuni	*C. coli*	*C. jejuni*	*C. coli*	
EM	–	–	–	–	–
NA	1	–	–	–	1
CIP	1	–	–	–	1
ENO	–	3	–	–	3
AM	–	–	5	1	6
TE, AM	–	1	–	–	1
NA, ENO	2	4	–	–	6
NA, CIP	3	–	–	–	3
NA, AM	–	–	1	–	1
ENO, CIP	2	–	–	–	2
TE, NA, ENO	1	–	–	–	1
TE, NA, CIP	1	–	–	–	1
TE, NA, AM	–	–	1	–	1
TE, ENO, GM	1	–	–	–	1
TE, ENO, AM	1	–	–	–	1
TE, GM, AM	–	–	–	1	1
NA, ENO, CIP	8	–	–	–	8
NA, CIP, GM	3	–	–	–	3
TE, NA, ENO, CIP	4	–	3	–	7
TE, NA, ENO, AM	–	1	–	–	1
TE, NA, CIP, AM	–	–	1	–	1
NA, ENO, CIP, GM	6	–	-	–	6
NA, ENO, CIP, AM	1	–	4	–	5
TE, NA, ENO, CIP, GM	1	–	–	–	1
TE, NA, ENO, CIP, AM	2	–	27	2	31
TE, NA, ENO, CIP, GM, AM	–	–	3	–	3
Total	**41**	**9**	**46**	**4**	**100**

EM: erythromycin, NA: Nalidixic acid, CIP: ciprofloxacin, ENO: enrofloxacin, AM: ampicillin, TE: tetracycline, GM: gentamicin.

According to the antimicrobial susceptibility test, a large number of *Campylobacter* spp. isolates used in this study showed a multidrug resistance phenotype. A total of 26 antimicrobial resistance pattern was determined (Table 3). Of 100 *Campylobacter* spp. isolates, 32 strains (32.0%) were resistant to 5 drugs, 20 strains (20.0%) were resistant to 4 drugs, 17 strains (17.0%) were resistant to 3 drugs and 13 strains (13.0%) were resistant to 2 drugs. Eighty-five percent (85.0%) of the isolates were resistant to two or more kinds of antibiotics (Table 3).

Table 4. Mutation rates in each codon on *gyr*A gene of *C. jejuni* isolated from dogs and humans

Species	Origin	78	81	86	90	110	117	118	119
		GGC (Gly)	CAT (His)	ATA (Ile)	AAT (Asn)	GGT (Gly)	GGC (gly)	GAC (Asp)	AGC (Ser)
C. jejuni	Dogs	0/41	11/41	34/41	0/41	0/41	0/41	0/41	8/41
	%	0	26.8	82.9	0	0	0	0	19.5
C. jejuni	Human	0/46	11/46	35/46	0/46	1/46	1/46	0/46	2/46
	%	0	24.0	76.0	0	2.2	2.2	0	4.3

Table 5. Summary of silent and missense mutation in the QRDR of topoisomerase II, *gyr*A gene *of C. jejuni* isolated from dogs and humans

Type of mutation	Nucleotide change	Amino acid change	Number of isolates from NA^R·CIP^R		Number of isolates from NA^S·CIP^S	
			Dogs (n = 29)	Humans (n = 34)	Dogs (n = 5)	Humans (n = 7)
Silent mutation	GGT→GGC	Gly-78→Gly	0	0	0	0
	CAC→CAT	His-81→His	7	9	0	2
	GGC→GGT	Gly-110→Gly	0	1	0	0
	GGT→GGC	Gly-117→Gly	0	0	0	1
	GAT→GAC	Asp-118→Asp	0	0	0	0
	AGT→AGC	Ser-119→Ser	6	1	0	1
Missense mutation	ACA→ATA	Thr-86→Ile	29	34	0	0
	GAT→AAT	Asp-90→Asn	0	0	0	0

NA^R: nalidixic acid resistance, CIP^R: ciprofloxacin resistance, NA^S: nalidixic acid susceptibility, CIP^S: ciprofloxacin susceptibility.

Mutations in QRDR of *gyr*A gene

The relationship between quinolone resistance and *gyr*A mutation was confirmed by DNA sequencing of the *gyr*A gene in *Campylobacter*. A total of 87 strains of *C. jejuni* were selected for analysis of DNA sequence mutations in the QRDR in the *gyr*A gene. The missense mutation of the 86th codon (ACA→ATA; Thr→IIe) was observed in 34 strains (82.9%) of *C. jejuni* isolated from dogs, and 35 strains (76%) of *C. jejuni* isolated from humans, respectively. However, amino-acid substitution (Asp→Asn) in the 90th codon was not found in the *gyr*A sequence. The silent mutations at the codons 78, 81, 110, 117, 118, and 119 were assessed (Tables 4 and 5). The results showed that the silent mutation at the codons 81 (26.8%), 119 (19.5%) and 81 (24%), 110 (2.2%), 117 (2.2%), 119 (4.3%) in the QRDR of the *gyr*A genes in *C. jejuni* were isolated from dogs and humans respectively. Also sequencing analysis showed that 100% of NA^R and CIP^R *C. jejuni* isolates (n = 63) from dogs and humans have the mutation Thr-86^{th}-Ile, which is known to be associated with quinolone resistance, and the mutations were not exhibited in all fluorquinolone-susceptible *C. jejuni* at the 86th codon (Table 5).

*fla*A - RFLP analysis

Ninety-four strains of *Campylobacter* spp. were selected at random for the analysis of *fla*A-RFLP after digestion with *Dde*I restriction enzyme to compare phenotype and genotype of the *Campylobacter* isolates from dogs and humans (Fig. 1). The *fla*-RFLP typing distributed 73 strains of *C. jejuni* and *C. coli* into 12 major *fla*-RFLP clusters. Twenty one strains of *C. jejuni* and *coli* were not included in major cluster (Fig. 2), because they had low similarity (lower than 90% similarity). The frequently detected patterns of the *fla*-RFLP cluster were cluster 1 (n = 12) of *C. jejuni* and cluster 5, 6, 7

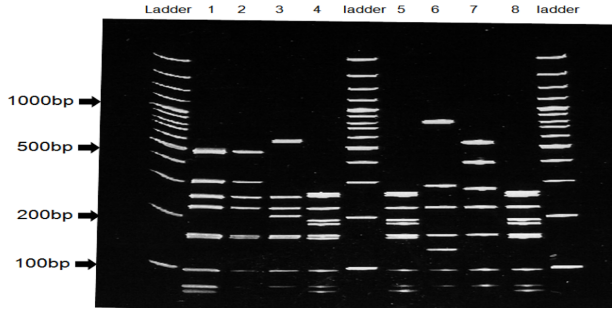


Fig. 1. Representative patterns of gel elctrophoresis of *fla*-RFLP profile for *Campylobacter* (*C.*) *jejuni* and *Campylobacter* (*C.*) *coli* isolates from dogs. Restriction fragments generated by digestion with *Dde*I. Lane 1, *C. jejuni* D37; Lane 2, *C. jejuni* D38; Lane 3, *C. jejuni* D39; Lane 4, *C. jejuni* D40; Lane 5, *C. jejuni* D41; Lane 6, *C. coli* D42; Lane 7, *C. coli* D43; Lane 8, *C. coli* D44.

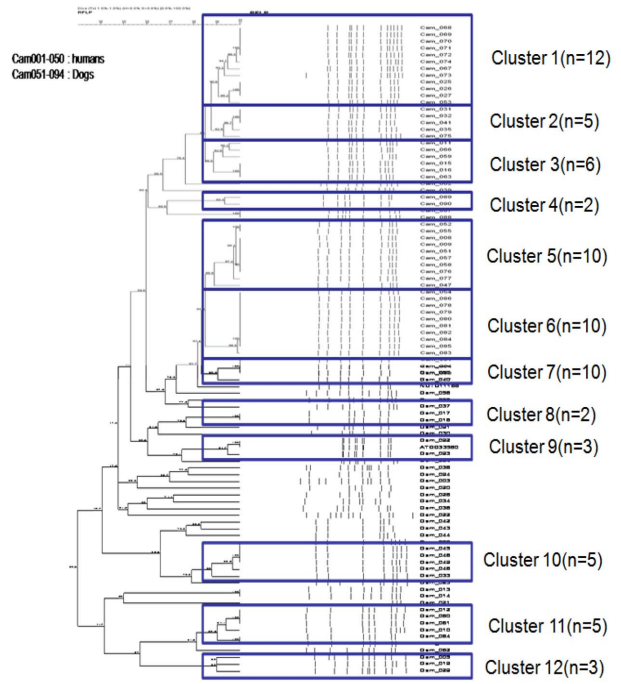


Fig. 2. Dendrogram of *fla*A-RFLP patterns of *C. jejuni* and *C. coli* isolated from dogs and humans in Korea. Dendrogram of RFLP banding profiles obtained with 94 *Campylobacter* spp. strains isolated from dogs and humans. The dendrogram shows the results of a phylogenetic comparison. The box indicates the 90% similarity threshold. Cam001-050, humans; Cam051-094, dogs.

of *C. coli* isolates (Fig. 2). Eight *fla*-RFLP clusters of 1, 2, 3, 5, 8, 10, 11, and 12 included *C. jejuni* and *C. coli* from human. Other 8 *fla*-RFLP clusters of 1, 2, 3, 4, 5, 6, 7, and 9 included *C. jejuni* and *C. coli* isolated from dogs. The clusters of 8, 10, and 12 have only human isolates and clusters of 4, 6, 7, and 9 were assigned only to dog isolates (Fig. 2). Clusters of 1, 2, 3, 5, and 11 included *C. jejuni* and *C. coli* isolated from dogs and humans simultaneously and the genotype and the phenotype were compared in these clusters (Fig. 3). No strain with a complete homogenous genotype and phenotype was detected.

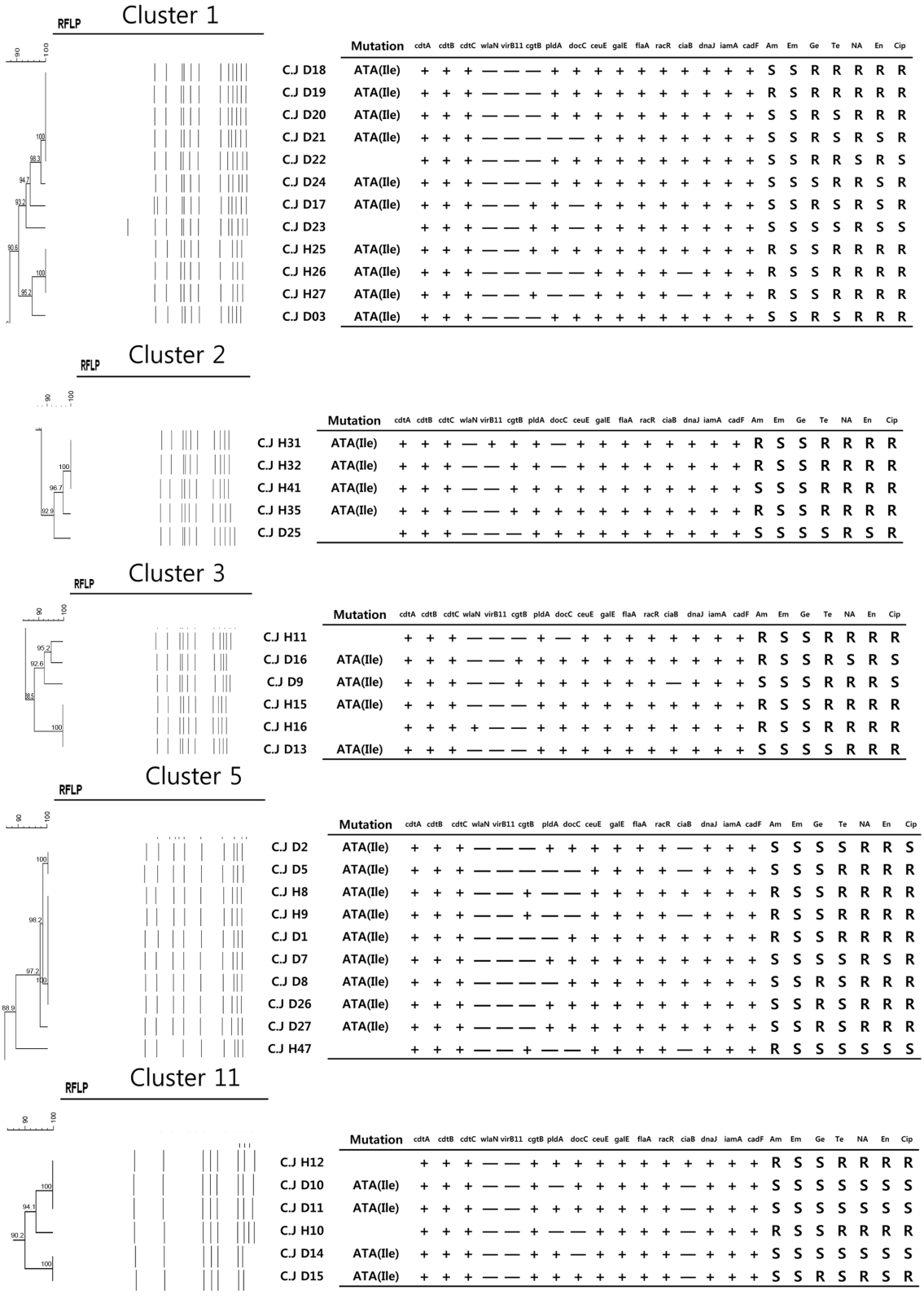
Discussion

A tendency of increase of antimicrobial resistance in *Campylobacter* spp. can be recognized as a major public health concern [7, 23]. In this study, the prevalence of antimicrobial resistance in *Campylobacter* spp. isolated from dogs raised in diverse conditions and humans was investigated, and the relationship between fluoroquinolon resistance and the *gyr*A mutation was revealed. In this study, no erythromycin resistant *Campylobacter* spp. isolates from dogs and humans was identified. This result agrees with the previous studies which suggested that none of *C. jejuni* isolates from humans and companion animals was resistant [16, 28]. However, other studies reported 3-6.8% of erythromycin resistance in *C. jejuni* isolated from humans [7, 18].

Resistance to ampicillin and other β-lactam antimicrobials has been broadly studied by previous reports [20, 28]. *C. jejuni* resistance to ampicillin is known to be correlated with production of ß-lactamase. The β-lactamase is produced by cj0299 open reading frame which is located on a chromosomal DNA in *Campylobacter* spp. [21]. The resistance to ampicillin we observed in *C. jejuni* was 8.7-12.5% from dog isolates and 91.3% from human isolates. In *C. coli*, a lower level of resistance was observed in the isolates from dogs. Frequency of ampicillin resistance of *C. jejuni* from dogs and humans in this study are similar with the previous reports [1, 32]. *C. jejuni* isolates from humans showed higher resistance to ampicillin than those isolated from dogs ($p \leq 0.05$). However, *C. jejuni* isolates from three different raising groups of dogs were not statistically significant.

The rates of tetracycline resistance were previously reported, ranging from 13 to 78.8% among the *Campylobacter* spp. isolates from humans and companion animals [16, 18, 30]. A higher rate of resistance to tetracycline was also observed, mainly in *C. coli* isolated from pigs and broilers (62 and 52%, respectively) [30]. In this study, the resistance of *C. jejuni* to tetracycline ranged from 21.7~40% in dog isolates and 83.3% in human isolates. *C. jejuni* isolates from humans showed a higher resistance to tetracycline than those isolated from dogs ($p \leq 0.01$). However, *C. jejuni* isolates from three different raising groups of dogs were not statistically significant. Since tetracycline has been used as an alternative treatment for *Campylobacter* gastroenteritis, the tetracycline resistance in the isolates of humans has been increasing [18, 20], moreover, the usage of tetracyclines is increasing due to inexpensive cost and their broad spectrum of activity.

Fluoroquinolone resistance is mostly shown in the human isolates of both *C. jejuni* and *C. coli* [25]. Among *C. jejuni* isolates from dogs, the rates of resistance were 75~90% for nalidixic acid, 56.5~100% for enrofloxacin and 60~100% for ciprofloxacin. In the results of antimicrobial susceptibility test for *Campylobacter* spp. isolates from humans for quinolones, the rates of resistance were 87% for nalidixic acid, 80.4% for enrofloxacin, and 80% for ciprofloxacin. The fluoroquinolone resistance of *C. jejuni* from humans and dogs in this study was similar with previous reports [3, 15, 29]. The results of *C. coli* isolates from humans were 50% resistant to three fluoroquinolones. Among *C. coli* isolates from dogs, the rates of resistance were 50~66.7% for nalidixic acid and 100% for enrofloxacin. However, all strains of *C. coli* isolated from dogs were susceptible to ciprofloxacin. Globally, antimicrobial resistance rate of *C. coli* isolates from humans and dogs raised in diverse condition did not show significant stastical difference because of a small number of isolates used in this study. Many previous studies showed that the Thr-86th-Ile (ACA→ATA) mutation in the QRDR of *gyr*A is the most common mechanism causing nalidixic acid or ciprofloxacin resistance in *C. jejuni* [11]. Piddock *et al.* [22] had demonstrated that a Thr-86th to Ile change is present



	Mutation	cdtA	cdtB	cdtC	wlaN	virB11	cgtB	pldA	docC	ceuE	galE	flaA	racR	ciaB	dnaJ	iamA	cadF	Am	Em	Ge	Te	NA	En	Cip
C.J D18	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	S	S	R	R	R	R	R
C.J D19	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	R	S	R	R	R	R	R
C.J D20	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	S	S	R	S	R	R	R
C.J D21	ATA(Ile)	+	+	+	—	—	—	—	—	+	+	+	+	+	+	+	+	S	S	R	S	R	S	R
C.J D22		+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	S	S	R	R	S	R	S
C.J D24	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	S	S	S	R	R	S	R
C.J D17	ATA(Ile)	+	+	+	—	—	+	+	—	+	+	+	+	+	+	+	+	S	S	R	S	R	S	R
C.J D23		+	+	+	—	—	+	+	—	+	+	+	+	+	+	+	+	S	S	S	S	R	S	S
C.J H25	ATA(Ile)	+	+	+	—	—	+	+	+	+	+	+	+	+	+	+	+	R	S	S	R	R	R	R
C.J H26	ATA(Ile)	+	+	+	—	—	—	—	—	+	+	+	+	—	+	+	+	R	S	S	R	R	R	R
C.J H27	ATA(Ile)	+	+	+	—	—	+	—	—	+	+	+	+	—	+	+	+	R	S	S	R	R	R	R
C.J D03	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	S	S	R	S	R	R	R

	Mutation	cdtA	cdtB	cdtC	wlaN	virB11	cgtB	pldA	docC	ceuE	galE	flaA	racR	ciaB	dnaJ	iamA	cadF	Am	Em	Ge	Te	NA	En	Cip
C.J H31	ATA(Ile)	+	+	+	—	+	+	+	—	+	+	+	+	+	+	+	+	R	S	S	R	R	R	R
C.J H32	ATA(Ile)	+	+	+	—	—	+	+	—	+	+	+	+	+	+	+	+	R	S	S	R	R	R	R
C.J H41	ATA(Ile)	+	+	+	—	—	+	+	+	+	+	+	+	+	+	+	+	S	S	S	R	R	R	R
C.J H35	ATA(Ile)	+	+	+	—	—	+	+	+	+	+	+	+	+	+	+	+	R	S	S	R	R	R	R
C.J D25		+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	S	S	S	S	R	S	R

	Mutation	cdtA	cdtB	cdtC	wlaN	virB11	cgtB	pldA	docC	ceuE	galE	flaA	racR	ciaB	dnaJ	iamA	cadF	Am	Em	Ge	Te	NA	En	Cip
C.J H11		+	+	+	—	—	—	+	—	+	+	+	+	+	+	+	+	R	S	S	R	R	R	R
C.J D16	ATA(Ile)	+	+	+	—	—	+	+	+	+	+	+	+	+	+	+	+	R	S	S	R	S	R	S
C.J D9	ATA(Ile)	+	+	+	—	—	+	+	+	+	+	+	+	—	+	+	+	S	S	S	R	R	R	S
C.J H15	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	R	S	S	R	R	R	R
C.J H16		+	+	+	+	—	—	+	+	+	+	+	+	+	+	+	+	R	S	S	R	R	R	R
C.J D13	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	S	S	S	S	R	R	R

	Mutation	cdtA	cdtB	cdtC	wlaN	virB11	cgtB	pldA	docC	ceuE	galE	flaA	racR	ciaB	dnaJ	iamA	cadF	Am	Em	Ge	Te	NA	En	Cip
C.J D2	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	—	+	+	+	S	S	S	S	R	R	S
C.J D5	ATA(Ile)	+	+	+	—	—	—	—	—	+	+	+	+	—	+	+	+	S	S	S	R	R	R	R
C.J H8	ATA(Ile)	+	+	+	—	—	+	—	—	+	+	+	+	+	+	+	+	R	S	S	R	R	R	R
C.J H9	ATA(Ile)	+	+	+	—	—	+	—	—	+	+	+	+	—	+	+	+	R	S	S	R	R	R	R
C.J D1	ATA(Ile)	+	+	+	—	—	—	—	+	+	+	+	+	+	+	+	+	R	S	S	R	R	R	R
C.J D7	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	S	S	S	S	R	S	R
C.J D8	ATA(Ile)	+	+	+	—	—	—	—	+	+	+	+	+	+	+	+	+	S	S	R	S	R	R	R
C.J D26	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	S	S	R	S	R	R	R
C.J D27	ATA(Ile)	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	S	S	R	S	R	R	R
C.J H47		+	+	+	—	—	+	—	—	+	+	+	+	—	+	+	+	R	S	S	S	S	S	S

	Mutation	cdtA	cdtB	cdtC	wlaN	virB11	cgtB	pldA	docC	ceuE	galE	flaA	racR	ciaB	dnaJ	iamA	cadF	Am	Em	Ge	Te	NA	En	Cip
C.J H12		+	+	+	—	—	+	+	+	+	+	+	+	+	+	+	+	R	S	S	R	R	R	R
C.J D10	ATA(Ile)	+	+	+	—	—	+	—	+	+	+	+	+	—	+	+	+	S	S	S	S	S	S	S
C.J D11	ATA(Ile)	+	+	+	—	—	+	+	+	+	+	+	+	—	+	+	+	S	S	S	S	S	S	S
C.J H10		+	+	+	—	—	+	—	—	+	+	+	+	—	+	+	+	R	S	S	R	R	R	R
C.J D14	ATA(Ile)	+	+	+	—	—	+	+	—	+	+	+	+	—	+	+	+	S	S	S	S	S	S	S
C.J D15	ATA(Ile)	+	+	+	—	—	+	+	+	+	+	+	+	—	+	+	+	S	S	R	S	R	S	R

Fig. 3. Dendrogram highlighting the *fla*A-RFLP patterns. Virulence genes and antimicrobial resistance profile of Cluster 1, 2, 3, 5 and 11 included *C. jejuni* and *C. coli* isolated from dogs and humans, simultaneously. Restriction fragments generated by digestion with *Dde*I. Virulence gene, data not suggested; +, detected; –, not detected. CJD: *C.jejuni* isolated from dogs, CJH: *C. jejuni* isolated from humans, Am: ampicillin, Em: erythromycin, Ge: gentamicin, Te: tetracycline, Na: nalidixic acid, En: Enrofloxacin, Cip: ciproxacin, R: resistance, S: sensitive.

in the huge majority of fluoroquinolone resistant *Campylobacter* spp., whether isolated from dogs or humans. The association of this substitution with quinolone resistance in *Campylobacter* spp. has been reported previously [6].

Regarding the *gyr*A mutation in the QRDR, it was found that all the fluoroquinolone resistant isolates (n = 63) possessed the mutation at the 86th codon (threonine to isoleucine) of the *gyr*A gene originated from *Campylobacter* spp. isolates used in this study. In the case of silent mutation, the mutations were found at the codons 81 (CAC→CAT), 110 (GGC→GGT), 117 (GGT→GGC), 119 (AGC→AGT), which is in line with the previous reports [33] and sequencing analysis showed that 100% of NA^R / CIP^R *C. jejuni* isolates from dogs and humans had the mutation Thr-86^{th}-Ile, which is known to be associated with quinolone resistance.

The *fla*A-PCR-RFLP has been applied as one of the tools of epidemiological study to differentiate isolates below the species level [10, 13]. Accordingly, *fla*A-RFLP typing method was used to analyze the genotype. Ninety four strains of *Campylobacter* isolates were subtyped by *fla*A-PCR-RFLP into 12 major clusters. Interestingly, clusters of 1, 2, 3, 5, and 11 included dog and human isolates simultaneously. These results suggest epidemiological co-relationship between *C. jejuni* isolated from dogs and humans. However, these results may not simply imply that there is no relationship between the *Campylobacter* spp. isolated from dogs and humans, because these strains were not isolated from homogenous environment at the same time. For further study, molecular epidemiological comparison of *Campylobacter* spp. isolated from pet owners and their dogs would be necessary.

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References

1. **Acke E, McGill K, Quinn J, Jones BR, Fanning S, Whyte P.** Antimicrobial resistance profile and mechanisms of resistance in *Campylobacter jejuni* isolates from pets. Foodborne Pathog Dis 2009, **6**, 705-710.
2. **Andersen SR, Saadbye P, Shukri NM, Rosenguist H, Nielsen NL, Boel J.** Antimicrobial resistance among *Campylobacter jejuni* isolated from raw poultry meat at retail level in Denmark. Int J Food Microbial 2006, **107**, 250-255.
3. **Andzejewska M, Szczepańska B, Klawe JJ, Spica D, Chudzińska M.** Prevalence of *Campylobacter jejuni and Campylobacter coli* species in cats and dogs from Bydgoszcz (Poland) region. Pol J Vet sci 2013, **16**, 115-120.
4. **Butzler JP.** *Campylobacter*, from obscurity to celebrity. Clin Microbial Infect 2004, **10**, 868-876.
5. **CLSI.** Method for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline. CLSI document M45-A. Clinical and Laboratory Standard Institute, Wayne, 2006.
6. **Cooper R, Segal H, Lastovica AJ, Elisha BG.** Genetic basis of quinolone resistance and epidemiology of resistant and susceptible isolates of porcine *Campylobacter coli* strains. J Appl Microbiol 2002, **93**, 241-249.
7. **Corcoran D, Quinn T, Cotter L, Whyte P, Fanning S.** Antimicrobial resistance profiling and fla-typing of Irish thermophillic *Campylobacter* spp. of human and poultry origin. Lett Appl Microbiol 2006, **43**, 560-565.
8. **Dingle KE, Van Den Braak N, Colles FM, Price LJ, Woodward DL, Rodgers FG, Endtz HP, Van Belkum A, Maiden MCJ.** Sequence typing confirms that *Campylobacter jejuni* strains associated with Gullian-Barré and Miller-Fisher syndromes are of diverse genetic lineage, serotype, and flagella type. J Clin Microbial 2001, **39**, 3346-3349.
9. **Drlica K, Zhao X.** DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol Mol Biol Rev 1997, **61**, 377-392.
10. **Ertaş HB, Çentinkya B, Muz A, Öngör H.** Genotyping of broiler-originated *Campylobacter jejuni* and *Campylobacter coli* isolates using *fla* typing and random amplified polymorphic DNA methods. Int J Food Microbiol 2004, **94**, 203-209.
11. **Hakanen A, Jalava J, Kotilainen P, Jousimies-Somer H, Siitonen A, Huovinen P.** *gyr*A polymorphism in *Campylobacter jejuni*: detection of *gyr*A mutations in 162 *C. jejuni* isolates by single-strand conformation polymorphism and DNA sequencing. Antimicrob Agents Chemother 2002, **46**, 2644-2647.
12. **Hald B, Madsen M.** Healthy puppies and kittens as carriers of *Campylobacter spp.*, with special reference to *Campylobacter upsaliensis*. J Clin Microbiol 1997, **35**, 3351-3352.
13. **Harrington CS, Moran L, Ridley AM, Newell DG, Madden RH.** Inter-laboratory evaluation of three flagellin PCR/RFLP methods for typing *Campylobacter jejuni* and *C. coli*: the CAMPYNET experience. J Appl Microbiol 2003, **95**, 1321-1333.
14. **Kapperud G, Skjerve E, Bean NH, Ostroff SM, Lassen J.** Risk factors for sporadic *Campylobacter* infections: results of a case-control study in southeastern Norway. J Clin Microbiol 1992, **30**, 3117-3121.
15. **Kayman T, Abay S, Hizlisoy H.** Identification of *Campylobacter* spp. isolates with phenotypic methods and multiplex polymerase chain reaction and their antibiotic susceptibilities. Mikrobiyol Bul 2013, **47**, 230-239.
16. **Lee MK, Billington SJ, Joens LA.** Potential virulence and antimicrobial susceptibility of *Campylobacter jejuni* isolates from food and companion animals. Foodborne Pathog Dis 2004, **1**, 223-230.
17. **Lienau JA, Ellerbroek L, Klein G.** Tracing flock-related *Campylobacter* clones from broiler farms through slaughter to retail products by pulsed-field gel electrophoresis. J Food Prot 2007, **70**, 536-542.
18. **McGill K, Cowley D, Moran L, Scates P, O'Leary A, Madden RH, Carroll C, McNamara E, Moore JE, Fanning S, Collins JD, Whyte P.** Antibiotic resistance of retail food and human *Campylobacter* isolates on the island of Ireland from 2001-2002. Epidemiol Infect 2006, **134**, 1282-1291.
19. **Nachamkin I, Bohachick K, Patton CM.** Flagellin gene typing of *Campylobacter jejuni* by restriction fragment

length polymorphism analysis. J Clin Microbiol 1993, **31**, 1531-1536.

20. **Nachamkin I, Engberg J, Aerestrup FM.** Diagnosis and antimicrobial susceptibility of *Campylobacter* species. In: Nachamkin I, Blaser MJ (eds.). *Campylobacter* spp. 2nd ed. pp. 45-66, ASM press, Washington DC, 2000.
21. **Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S, Jagels K, Karlyshev AV, Moule S, Pallen MJ, Penn CW, Quail MA, Rajandream MA, Rutherford KM, van Vliet AHM, Whitehead S, Barrell BG.** The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 2000, **403**, 665-668.
22. **Piddock LJV, Ricci V, Pumbwe L, Everett MJ, Griggs DJ.** Fluoroquinolone resistance in *Campylobacter* species from man and animals: detection of mutations in topoisomerase genes. J Antimicrob Chemother 2003, **51**, 19-26.
23. **Quinn JP.** Clinical strategies for serious infection: a North American perspective. Diagn Microbiol Infect Dis 1998, **31**, 389-395.
24. **Robinson RA, Pugh RN.** Dogs, zoonoses and immunosuppression. J R Soc Promot Health 2002, **122**, 95-98.
25. **Ruiz J, Goñi P, Marco F, Gallardo F, Mirelis B, Jimenez De Anta T, Vila J.** Increased resistance to quinolones in *Campylobacter jejuni*: a genetic analysis of *gyr*A gene mutations in quinolone-resistant clinical isolates. Microbiol Immunol 1998, **42**, 223-226.
26. **Segreti J, Gootz TD, Goodman LJ, Parkhurst GW, Quinn JP, Martin BA, Trenholme GM.** High-level quinolone resistance in clinical isolates of *Campylobacter jejuni.* J Infect Dis 1992, **165**, 667-670.
27. **Tauxe RJ.** Epidemiology of *Capylobacter jejuni* infections in the United States and other industrialized nations. In: Nachamkin I, Blaser MJ, Tomkins LS (eds.). *Campylobacter Jejuni*: Current Status and Future Trends. pp. 9-19, American Society for Microbiology, Washington DC, 1992.
28. **Thakur S, Zhao S, McDermott PF, Harbottle H, Abbott J, English L, Gebreyes WA, White DG.** Antimicrobial resistance, virulence, and genotypic profile comparison of *Campylobacter jejuni* and *Campylobacter coli* isolated from humans and retail meats. Foodborne Pathog Dis 2010, **7**, 835-844.
29. **Tsai HJ, Huang HC, Lin CM, Lien YY, Chou CH.** Salmonellae and Campylobacters in household and stray dogs in northern Taiwan. Vet Res Commun 2007, **31**, 931-939.
30. **Van Looveren M, Daube G, De Zutter L, Dumont JM, Lammens C, Wijdooghe M, Vandamme P, Jouret M, Cornelis M, Goossens H.** Antimicrobial susceptibilities of *Campylobacter* strains isolated from food animals in Belgium. J Antimicrob Chemother 2001, **48**, 235-240.
31. **Wassenaar TM, Newell DG.** Genotyping of *Campylobacter* spp. Appl Environ Microbiol 2000, **66**, 1-9.
32. **Yong JR, Wu HS, Chiang CS, Mu JJ.** Pediatric campylobacteriosis in northern Taiwan from 2003-2005. BMC Infect Dis 2008, **8**, 151.
33. **Zirnstein G, Li Y, Swaminathan B, Angulo F.** Ciprofloxacin resistance in *Campylobacter jejuni* isolates: detection of *gyr*A resistance mutations by mismatch amplification mutation assay PCR and DNA sequence analysis. J Clin Microbiol 1999, **37**, 3276-3280.