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Macrolide resistance mechanisms and virulence factors in erythromycin-resistant *Campylobacter* species isolated from chicken and swine feces and carcasses

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ABSTRACT. Resistance to antimicrobials was measured in 73 isolates of *Campylobacter jejuni* (*C. jejuni*) and 121 isolates of *Campylobacter coli* (*C. coli*) from chicken and swine feces and carcasses in Korea. Both bacterial species showed the highest resistance to (fluoro) quinolones (ciprofloxacin and nalidixic acid) out of the nine antimicrobials tested. Erythromycin resistance was much higher in *C. coli* (19.0%, 23/121) than in *C. jejuni* (6.8%, 5/73). The mutation in the 23S rRNA gene was primarily responsible for macrolide resistance in *Campylobacter* isolates. Several amino acid substitutions in the L4 and L22 ribosomal proteins may play a role in the mechanism of resistance, but the role requires further evaluation. A total of eight virulence genes were detected in 28 erythromycin-resistant *Campylobacter* isolates. All *C. jejuni* isolates carried more than four such genes, while *C. coli* isolates carried fewer than three such genes. The high rate of resistance highlights the need to employ more prudent use of critically important antimicrobials, such as fluoroquinolones and macrolides, in swine and poultry production, and to more carefully monitor antimicrobial resistance in *Campylobacter* isolates in food animals.
KEY WORDS: *Campylobacter*, macrolide resistance, virulence factor

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Campylobacteriosis is one of the most commonly reported gastrointestinal diseases worldwide [7]. *Campylobacter* spp., such as *Campylobacter jejuni* and *Campylobacter coli*, are usually normal intestinal flora in animals. Contamination of food products during processing is the main source of food poisoning in humans. Although campylobacteriosis is generally a self-limiting disease, antimicrobial treatment may be required for systemic *Campylobacter* infections, such as severe or long-lasting infections, in immune-deficient people or immunosuppressed patients [20].

Macrolides are one of only a few antimicrobials available to treat *Campylobacter* infection [21]. Macrolides, such as erythromycin and tylosin, are also widely used in animal industry [12]. The potential risk that macrolide-resistant *Campylobacter* spp. will be transmitted from animal products to humans has raised concerns that using macrolides in animals will compromise the treatment of human infections.

Two main mechanisms of macrolide resistance, ribosomal target modifications and active efflux, may be involved. High-level resistance is mainly caused by mutations at positions 2,058 and 2,059 (*Escherichia coli* numbering) of the 23S rRNA gene [2, 3, 11, 18]. In addition, several modifications in the ribosomal proteins L4 and L22, which are associated with macrolide resistance, have been reported in *Campylobacter* [2, 3, 11, 18]. The other resistance mechanism is mediated by the CmeABC efflux pump, which protects *Campylobacter* against erythromycin, tetracyclines, bile salts, detergents and dyes [2, 13].

Studies conducted in Korea [8, 9, 16] demonstrated a relatively high level of antimicrobial resistance in *Campylobacter* from animals and meats, compared with that in the European Union, Canada and United States [9]. Furthermore, outbreaks of food poisoning caused by *Campylobacter* have increased in Korea recently [16]. The choices for treating *Campylobacter* infections are limited, because there is a high level of resistance to (fluoro) quinolones among *Campylobacter* found in food animals and meats in Korea [8, 9]. Thus, macrolides are very important antimicrobials for treatment of *Campylobacter* infection in human in Korea. The aims of the present study are to examine antimicrobial resistance and to investigate the molecular mechanisms involved in macrolide resistance, focusing on region V of the 23S rRNA gene, the *rplD* (L4) and *rplV* (L22) genes, and to detect the presence of virulence factors in erythromycin-resistant *C. jejuni* and *C. coli* strains isolated from animals and carcasses in Korea.

MATERIALS AND METHODS

Bacteria collection: *Campylobacter* isolates were recovered from laboratories and centers participating in the Korean Veterinary Antimicrobial Resistance Monitoring System (KVARMS). We collected 194 *Campylobacter* spp. isolated from chicken and swine animal feces and carcasses in 2010: 73 *C. jejuni* from chicken feces (n=43) and chicken carcasses (n=30), and 121 *C. coli* from pig feces (n=46), pig carcasses (n=12), chicken feces (n=38) and chicken carcasses (n=25). Animal feces and carcass samples were

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collected from slaughterhouses in nine provinces. *Campylobacter* were isolated using Bolton broth (Thermo Scientific, Basingstoke, U.K.) and *Campylobacter* blood-free selective agar (Thermo Scientific) and confirmed by polymerase chain reaction (PCR) [5].

Antimicrobial resistance: Minimum inhibitory concentrations (MICs) for *Campylobacter* were determined by the broth dilution method using commercially available Sensititre® panel Campy (TREK Diagnostic Systems, West Sussex, U.K.) according to the manufacturer's instructions. Briefly, the antimicrobials, azithromycin, ciprofloxacin, clindamycin, erythromycin, florfenicol, gentamicin, nalidixic acid, telithromycin and tetracycline, were tested. The interpretation of MICs was carried out according to the National Antimicrobial Resistance Monitoring System (NARMS, 2011) [17]. *C. jejuni* ATCC 33560 was used as a quality control strain.

Analysis of the molecular mechanisms of macrolide resistance: Domain V of the 23S rRNA [3], L4 protein [2] and L22 protein [3] were amplified by the PCR. Amplified PCR products were purified, and the products were then directly sequenced at Macrogen (Seoul, Korea). DNA sequences of resistant and susceptible strains were compared with the sequence of the *C. coli* JV20 genome (GeneBank accession number NZ_ AEER01000024).

Detection of virulence genes: The presence of 12 *Campylobacter* virulence genes, *flaA, flhA, cadF, docA, cdtA, cdtB, cdtC, ciaB, iam, wlaN, virB11* [4] and *ceuE* [1], in 194 *Campylobacter* spp. was detected by PCR as in previously described work [1, 4].

RESULTS

Antimicrobial resistance: The MICs at which 50% and 90% of 194 *Campylobacter* isolates were inhibited (MIC_{50} and MIC_{90}, respectively), and the proportions of resistant isolates for the different antimicrobial agents are summarized in Table 1. No differences were identified in the frequency of the same antimicrobial profiles between *C. jejuni* and *C. coli* or between strains of the same species that originated either from feces or carcasses. Resistance to (fluoro) quinolone antimicrobials was highest (ranging from 67% to 100%) in both *C. jejuni* and *C. coli* from all samples except pig carcasses. Resistance rates to tetracycline were the second highest, ranging from 42% to 87% among the *Campylobacter* isolates. A total of 30 (15.5%)*Campylobacter* isolates showed phenotypic resistance to erythromycin. Macrolide resistance was observed in 23 of 121 (19.0%)*C. coli*, compared with only 5 of 73 (6.8%)*C. jejuni*. Erythromycin resistance varied among bacterial species and source animals. *C. coli* isolates from pig feces (23.9%) and pig carcasses (25.0%) showed higher macrolide resistance than did isolates from chicken feces (18.4%) and chicken carcasses (8.0%).

Erythromycin-resistant mechanisms of Campylobacter spp.: Sequence analysis of the internal 316-bp amplicon of the 23S rRNA gene revealed an A2075G transition in all high-level erythromycin-resistant isolates (Table 2). No mutation was identified in this region in any of the intermediate-level

Table 1. Antimicrobial resistance among *Campylobacter jejuni* and *Campylobacter coli* isolates from chicken and swine feces and carcasses

Antimicrobials	*C. jejuni* (n=73)						*C. coli* (n=121)									
	Chicken feces (n=43)			Chicken carcasses (n=30)			Pig feces (n=46)			Pig carcasses (n=12)			Chicken feces (n=38)			Chicken carcasses (n = 25)		
	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)	MIC_{50}	MIC_{90}	R (%)
Azithromycin	0.03	0.06	0 (0)	0.03	2	0 (0)	0.03	64	11 (23.9)	0.06	4	4 (33.3)	0.03	64	8 (21.1)	0.06	1	1 (4.0)
Ciprofloxacin	8	32	35 (81.4)	16	64	29 (96.7)	16	32	39 (84.8)	8	16	8 (66.7)	8	32	38 (100)	16	32	25 (100)
Clindamycin	0.06	0.25	4 (9.3)	0.13	16	3 (10)	0.5	16	8 (17.4)	0.5	64	3 (25.0)	0.13	4	0 (0)	0.25	4	1 (4.0)
Erythromycin	0.25	0.5	3 (7.0)	0.25	8	2 (6.7)	0.25	64	11 (23.9)	0.5	64	3 (25.0)	0.25	64	7 (18.4)	1	64	2 (8.0)
Florfenicol	0.5	1	4 (9.3)	1	64	4 (13.3)	1	2	2 (4.3)	0.5	1	0 (0)	0.5	1	0 (0)	1	1	0 (0)
Gentamicin	0.25	0.5	4 (9.3)	0.25	32	4 (13.3)	0.5	1	2 (4.3)	0.5	32	3 (25.0)	0.5	0.5	0 (0)	0.5	32	7 (28.0)
Nalidix acid	64	64	35 (81.4)	64	64	29 (96.7)	64	64	39 (84.8)	64	64	8 (66.7)	64	64	38 (100)	64	64	25 (100)
Telithromycin	0.25	1	0 (0)	0.5	4	0 (0)	0.5	4	0 (0)	1	8	0 (0)	0.5	4	0 (0)	1	0.5	0 (0)
Tetracycline	64	64	34 (79.1)	64	64	26 (86.7)	2	64	33 (71.7)	32	64	9 (75.0)	2	64	16 (42.1)	32	64	15 (60.0)

MIC_{50} and MIC_{90}, the lowest concentration to inhibit 50% and 90% of the isolates tested, respectively; R, resistant isolates (%).

Table 2. Ribosomal mutations and virulence factors in erythromycin-resistant *Campylobacter jejuni* and *Campylobacter coli* isolated from chicken and swine feces and carcasses

Isolate	Sample	MIC (μg/ml)[a]			Nucleotide/amino acid substitution			Virulence factor
		EM	AZI	TEL	23S rRNA[b]	L4[c]	L22[c]	
C. jejuni								
V01-10-A03-008-016	chicken feces	32	1	2	-	V196A	-	*flaA, cdtB, cadF, racR, cdtA, cdtC*
V01-10-A03-008-017	chicken feces	32	1	2	-	V196A	-	*flaA, cdtB, cadF, racR, cdtA, cdtC*
V01-10-A03-008-014	chicken feces	32	2	4	-	T91K,V176I,T177S,V196A	A73T, S109A	*flaA, cdtB, cadF, racR, cdtA, cdtC*
V09-CJ-02	chicken carcass	32	2	4	-	V196A	-	*flaA, cdtB, cadF, cdtA, cdtC*
V06-CJ-04	chicken carcass	32	2	8	-	V196A	-	*flaA, cdtB, cdtA, cdtC*
C. coli								
V01-10-A03-027-027	chicken feces	≥64	32	8	A2075G	V196A	I65V, A74G, S109T, E111A, T114A	*flaA, cadF, ceuE*
V01-10-A03-027-029	chicken feces	64	64	4	A2075G	V196A	I65V, A74G, S109T, E111A, T114A	*flaA, cadF, ceuE*
V01-10-A03-027-031	chicken feces	32	≥64	1	A2075G	V196A	I65V, A74G, S109T, E111A, T114A	*flaA, cadF, ceuE*
A03-008-007	chicken feces	≥64	≥64	4	A2075G	V196A	Q24R, S109A	*flaA*
V01-10-A03-027-026	chicken feces	≥64	≥64	4	A2075G	V196A	I65V, A74G, S109T, E111A, T114A	*flaA, cadF, ceuE*
V01-10-A03-027-025	chicken feces	≥64	≥64	8	A2075G	V196A	I65V, A74G, S109T, E111A, T114A	*flaA, cadF*
V01-10-A03-027-028	chicken feces	≥64	≥64	8	A2075G	V196A	I65V, A74G, S109T, E111A, T114A	*flaA, cadF*
V06-10-S03-027-009	chicken carcass	32	4	4	-	V196A	I65V, A74G, S109T, E111A, T114A	*cadF*
V06-10-S03-027-015	chicken carcass	≥64	≥64	4	A2075G	V196A	-	-
2–4	pig feces	≥64	32	≥8	A2075G	V196A	I65V, A74G, S109T, E111A, T114A	*flaA, cdaF, ceuE*
A02-008-017	pig feces	32	≥64	0.12	A2075G	V196A	I65V, A74G, S109T, E111A, T114A	*flaA*
A02-008-024	pig feces	≥64	≥64	4	A2075G	M192I, V196A	I65V, A74G, S109T, E111A, T114A	*flaA, virB11*
4–1	pig feces	≥64	≥64	8	A2075G	M192I, V196A	I65V, A74G, S109T, E111A, T114A	*flaA*
A02-008-006	pig feces	≥64	≥64	≥8	A2075G	V121A, A140T, M192I, V196A	I65V, A74G, S109T, E111A, T114A	
A02-008-009	pig feces	≥64	≥64	≥8	A2075G	V121A, A140T, M192I, V196A	I65V, A74G, S109T, E111A, T114A	*flaA*
A02-008-010	pig feces	≥64	≥64	≥8	A2075G	V176I,T177S,V184I, M192I, V196A	I65V, A74G, A103V, S109T, E111A, T114A	*flaA, cadF*
A02-008-018	pig feces	≥64	≥64	≥8	A2075G	V196A	Q24R, S109A	
V14-10-S02-028-001	pig feces	≥64	≥64	≥8	A2075G	V121A,V176I,T177S,V184I,M192I,V196A	I65V, A74G, A103V, S109T, E111A, T114A	*flaA, cadF*
V01-10-A02-027-018	pig feces	≥64	≥64	≥8	A2075G	V196A	I65V, A74G, S109T, E111A, T114A	*ceuE*
V11-CC-01	pig carcass	≥64	32	8	A2075G	V121A, M192I, V196A	-	*flaA*
V02-CC-02	pig carcass	64	64	8	A2075G	P28S, V196A	I65V, A74G, S109T, E111A, T114A	*flaA, cdaF*
V11-CC-03	pig carcass	≥64	≥64	8	A2075G	V121A, M192I, V196A	-	*flaA*
A02-008-013	pig feces	32	64	8	A2075G	P28S, V196A	I65V, A74G, S109T, E111A, T114A	*flaA*

a) Abbreviations: EM, erythromycin; AZI, azithromycin, TEL, telithromycin. b) Position according to *Escherichia coli* numbering. c) Position of amino acids changes. DNA sequences of *rplD* and *rplV* genes coding L4 and L22 ribosomal proteins, respectively, were compared with the sequence in the *C. coli* JV20 genome.

resistant isolates (MIC 32 μg/m*l*). A comparison of the amino acid sequences of the ribosomal proteins L4 and L22 in the *C. jejuni* and *C. coli* strains with type strains revealed several different amino acid substitutions and a combination of such substitutions. Four amino acid substitutions in the L4 ribosomal protein and two in the L22 ribosomal protein were observed in *C. jejuni* with intermediate-level resistance to erythromycin (MIC 32 μg/m*l*): T91K (n=1), V176I (n=1), T177S (n=1) and V196A (n=4) in L4, and A73T (n=1) and S109A (n=1) in L22. In erythromycin-resistant *C. coli* (MIC ≥32 μg/m*l*), eight amino acid substitutions in the L4 ribosomal protein and seven in the L22 ribosomal protein were observed: V196A (n=23), M192I (n=8), V121A (n=5), P28S (n=2), V176 (n=2), T177 (n=2), V184 (n=2) and A140T (n=2) in L4, and I65V (n=18), A74G (n=18), S109T (n=18), E111A (n=18), T114A (n=18), Q24R (n=2) and S109A (n=2) in L22.

Prevalence of virulence factors: The erythromycin-resistant *Campylobacter* isolates were analyzed for the presence of 12 virulence markers that are associated with human invasion and infection. Distinguishing features separating *C. jejuni* and *C. coli* were observed. *C. jejuni* isolates possessed more virulence genes than did *C. coli*; all *C. jejuni* isolates carried four to six virulence genes, whereas *C. coli* isolates had zero to three such genes. Almost all *C. jejuni* and *C. coli* isolates possessed the *flaA* gene; however, three gene subunits, *cdtA, cdtB* and *cdtC,* were found in 100% of *C. jejuni* isolates, but in none of the *C. coli* isolates. Furthermore, the *cadF* gene was more prevalent in samples from chickens [*C. jejuni* 80% (4/5) and *C. coli* 77.8% (7/9)] than in samples from pigs [*C. coli* 28.6% (4/14)], irrespective of the bacterial species.

DISCUSSION

In the present study, *Campylobacter* isolates from animals and carcasses that were tested against nine antimicrobials were most commonly resistant to ciprofloxacin and nalidixic acid (81.4–96.7% for *C. jejuni* and 66.7–100% for *C. coli*). We noted higher resistance to (fluoro) quinolones in *C. coli* from pigs and *C. jejuni* from chickens than has been reported by European Union countries (Spain 90.9% and 94.5%, respectively; Hungary 52.6% and 86.1%; Switzerland 41.1% and 40.7%; France 46.3% and 56.9%; and the Netherlands 10.9% and 67.3%) [7]. Although most of the *C. jejuni* isolates were susceptible to erythromycin, *C. coli* isolates from pigs (23.9%) and pig carcasses (25.0%) showed a relatively high rate of resistance. This finding agreed with the results of other studies [7, 22]. Generally, resistance to macrolides is more prevalent in *C. coli* isolates of pig origin than in *C. coli* from chickens or *C. jejuni* from pigs or chickens [7, 22]. In Korea, fluoroquinolones (enrofloxacin) and macrolides (tylosin) are routinely given to chickens and pigs to prevent and treat enteric and respiratory diseases, respectively [12]. This practice, which is also followed by other countries, may favor the selection of resistant bacteria [6, 7].

In the present study, mutations in highly resistant strains were identified at position 2,075 in the 23S rRNA gene in *Campylobacter* spp. The primary mechanism of macrolide resistance was due to a single point mutation in the 23S rRNA gene, as previously reported by researches in Poland [2, 3, 18] and Korea [19]. Mutations in five *C. jejuni* and one *C. coli* showing intermediate-level resistance (MIC 32 μg/m*l*) were not identified at position 2,075 in the 23S rRNA gene. Thus, a further study on low-level resistance mechanisms, such as the CmeABC efflux pump and mutation of other ribosomal proteins, is required.

The 50S ribosomal subunit proteins L4 and L22, encoded for by the *rplD* and *rplV* genes, respectively, were characterized in erythromycin-resistant isolates [2, 18]. Amino acids spanning positions 63–74 are reported to be the most important target regions of the L4 protein [3]. Mutations within this region confer high-level macrolide resistance in various bacterial species [3]. In the present study, four and eight amino acid substitutions in the L4 ribosomal protein were identified in *C. jejuni* and *C. coli*, respectively. The mutations at positions, 196 and 121, were reported by previous studies [3, 18]; however, the present study is the first to report mutations at positions, at 28, 91, 140, 192, 176, 177, 184 and 841. In the L22 ribosomal protein, we noted two amino acid substitutions in *C. jejuni* and seven in *C. coli*, respectively. Although in the present study, erythromycin-susceptible strains were not included for mutations, such amino acid substitutions were reported in both susceptible and resistant isolates in other studies [3, 11, 18]. Thus, these substitutions may have little direct involvement in erythromycin resistance in *Campylobacter* spp. So far, the significance of the amino acid substitutions in the ribosomal proteins L4 and L22 remains unknown and warrants further evaluation.

The presence of virulence factor genes in erythromycin-resistant *Campylobacter* isolates varied by bacteria species and source. The *C. jejuni* isolates carried more virulence genes than did the *C. coli* isolates that we tested. Although further studies on the relationship between virulence genes in bacteria and pathogenicity in the host are needed, our results may explain why *C. jejuni* is a more common cause of human infections (90–95%) than *C. coli* (5–10%) [20]. The most common virulence gene in both *C. jejuni* and *C. coli* was a flagellin-coding *flaA* gene. Motility expression via the flagella is essential for cell adhesion and invasion to achieve infection [10, 14, 20].

The second most common virulence gene in this study was *cadF*, which is responsible for adhensin, and the fibronectin-binding protein involved in invasion, influencing microfilament organization in host cells [1, 20]. Furthermore, this gene had a high prevalence in *Campylobacter* isolates in human cases and chicken carcasses [15]. In the present study, this gene in *C. coli* was more prevalent in isolates from chickens than those from pigs. Although the presence or absence of key genes in *Campylobacter* spp. cannot be used to predict the virulence of strains [4], further studies on virulence genes in *C. coli* from different origins are needed in order to develop effective intervention strategies to prevent transmission of resistant strains via the food chain.

We discovered a high rate of antimicrobial resistance in both *C. jejuni* and *C. coli*, with a mutation in the 23S rRNA gene mainly responsible for erythromycin resistance in *Campylobacter* isolates and more virulence genes in *C.*

jejuni than in *C. coli*. The effect of the amino acid substitutions in the L4 and L22 proteins on macrolide resistance and the relationship between the presence of virulence genes and pathogenicity require further evaluations. To prevent the transmission to humans of resistant and virulent *Campylobacter* spp. via the food chain, we urge more prudent use of critically important antimicrobials, such as fluoroquinolones and macrolides, in swine and poultry production, as well as constant monitoring of resistance among *Campylobacter* isolates in animals and animal products.

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