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Original Article

Molecular epidemiological analysis of human- and chicken-derived isolates of *Campylobacter jejuni* in Japan using next-generation sequencing

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ABSTRACT

In this research, we analyzed the main sequence types (ST) and ST complexes of human- and chicken-derived isolates of *Campylobacter jejuni* in Japan by using multilocus sequence typing (MLST). We also analyzed lipooligosaccharide biosynthesis locus classes (LOS locus classes) and the numbers of isolates carrying genes coding resistance factors against various antibiotics, and observed their relationships. ST-21 complex was the main ST complex in isolates from humans (n = 38) and chickens (n = 25). None of the isolates showed resistance to imipenem, chloramphenicol, or erythromycin. Few isolates were resistant to ampicillin and streptomycin (1.3%–15%), whereas many showed resistance to tetracycline, ciprofloxacin, and nalidixic acid (38%–48%). Among the ST-21 complex isolates, ST4526 was detected at a very high rate. Those isolates showed resistance to tetracycline and ciprofloxacin, and were susceptible to ampicillin. Among the chicken-derived isolates, 37 of the 38 isolates that showed resistance to ciprofloxacin and nalidixic acid had threonine to isoleucine amino acid substitution in GyrA at codon 86 (T86I). Among the human-derived isolates, 17 of the 47 isolates that showed resistance to ciprofloxacin and 16 of the 48 isolates that showed resistance to nalidixic acid did not have T86I amino acid mutations in GyrA. The human-derived ST-21 complex isolates were classified into LOS locus classes A, B, C, D, and E. The chicken-derived ST-21 complex isolates, with the exception of one isolate, were all classified into LOS locus classes C and D. Among chicken-derived isolates, the most prevalent was ST51 (ST-443 complex) (10 isolates) and all of those were LOS locus class E.

1. Introduction

Campylobacteriosis is a bacterial gastroenteritis caused by ingesting food contaminated by *Campylobacter jejuni* that normally lives in the intestines of chickens and other animals [1]. Molecular epidemiological analysis of *C. jejuni* from human stool samples and from samples of chicken meat and droppings (human-derived and chicken-derived *C. jejuni*) is reported to be useful in identifying the source of contamination and understanding the distribution of clones [2].

The first choice of drugs against campylobacteriosis is the macrolides such as erythromycin. Quinolones such as ciprofloxacin and levofloxacin are also a treatment option, but may not be effective, as about 30% of *C. jejuni* are resistant to quinolones [3]. Therefore, it is important to gain an understanding of antimicrobial resistance of *C. jejuni* clones distributed in Japan.

In addition, it has been reported that lipooligosaccharides (LOS) on the surface of the *C. jejuni* cell may be the cause of neurological symptoms such as Guillain-Barré syndrome after *C. jejuni* infection

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[4]. The genes involved in the biosynthesis of LOS have been grouped into 19 LOS biosynthesis locus classes (LOS locus class), from class A to class S [5]. Of these, LOS locus classes A and B have been identified as causing neurological disorders, and isolates of LOS locus class C are considered highly invasive to intestinal cells [6]. Accordingly, an understating of the distribution of LOS locus classes is also important.

In Japan, there has been only two molecular epidemiological analysis of human- and chicken-derived *C. jejuni* [7,8]. Furthermore, since 2010 there has been only one report on antibiotic susceptibility test results and the relationship with genes that code antibiotic resistance factors [9], and there have been no reports on molecular epidemiological analyses such as LOS locus classifications, multilocus sequence typing (MLST), or their relationships.

The current research was conducted with human- and chicken-derived *C. jejuni* isolated in Japan from 2007 to 2014. We conducted susceptibility tests with several types of antibiotic drugs, and based on the results of genome analysis of the isolates obtained by using a next-generation sequencer, we determined the main sequence types (ST) and ST complexes by MLST. We also analyzed the LOS locus classes and the numbers of isolates carrying genes that code for various antibiotic resistance factors. We carried out a comprehensive analysis and studied the relationships between these factors.

2. Materials and methods

2.1. Used isolates

We studied a total of 185 isolates of *C. jejuni*. We isolated 106 isolates of human *C. jejuni* from stool samples collected from patients who presented with campylobacteriosis-like symptoms at the Toho University Omori Medical Center (91 isolates) or at the Tokyo Metropolitan Bokutoh Hospital (15 isolates) between 2007 and 2014. We isolated 57 isolates from chicken meat sold in Hokkaido (16 isolates), Tokyo (2 isolates), Gifu (21 isolates), Shiga (5 isolates), Hyogo (3 isolates), Yamaguchi (6 isolates), and Fukuoka (4 isolates). Furthermore, we isolated 22 isolates from chicken droppings that we collected at farms in Tohoku (5 isolates), Chubu (2 isolates), Chugoku (1 isolate), Shikoku (5 isolates), and Kyushu (9 isolates). We enriched each isolate on modified charcoal cefoperazone deoxycholate agar medium (Thermo Fisher Scientific, Waltham, MA, USA). After introducing the isolates to the enrichment medium, they were cultured in a microaerobic environment at 42 °C. The trial was conducted with approval from the Research Ethics Board of the Toho University School of Medicine (no. 25091).

2.2. Antibiotic susceptibility testing

We conducted antibiotic susceptibility testing of *C. jejuni* to determine the minimum inhibitory concentrations (MIC) of ampicillin, streptomycin, tetracycline, chloramphenicol, erythromycin, imipenem, ciprofloxacin, and nalidixic acid. Testing was carried out using commercially available frozen plates (Eiken Chemical, Tokyo, Japan), and followed the broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI) [10]. The performance standards for analysis of MIC, namely the breakpoints for resistance, were ≥16 μg/mL for tetracycline, ≥32 μg/mL for erythromycin, and ≥4 μg/mL for ciprofloxacin (CLSI M45-A2) [11]; ≥16 μg/mL for imipenem (CLSI M100-S26, other non-Enterobacteriaceae) [10]; and ≥32 μg/mL for ampicillin, ≥32 μg/mL for streptomycin, ≥16 μg/mL for chloramphenicol, and ≥32 μg/mL for nalidixic acid (Japanese Veterinary Antibiotic Resistance Monitoring System, JVARM) [3]. *Campylobacter jejuni* subsp *jejuni* ATCC 33560 was used as the quality control strain.

2.3. Whole-genome sequence obtained by next-generation sequencing

The DNA used for whole-genome sequencing was extracted from the pure cultured bacteria by lysis and protein denaturation with phenol/chloroform/isoamyl alcohol (25:24:1) (Nippon Gene, Toyama, Japan), followed by final purification with a QIAquick PCR Purification kit (Qiagen, Hilden, Germany). The DNA library for whole-genome sequencing was first prepared by using a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA), and then 300-bp paired-end sequencing of the prepared DNA library was conducted using a MiSeq next-generation sequencer (Illumina). For assembly of the 300-bp short reads we used the CLC Genomics Workbench 9.0 (Qiagen, Chatsworth, CA, USA). Bacterial species identification was carried out based on the *gyrB* full-length nucleotide sequence (2310 bp) from the draft genome nucleotide sequence (https://www.ncbi.nlm.nih.gov/nuccore/KC408908.1). Multilocus sequence typing (MLST) was conducted with the MLST 1.7 web tool (http://cge.cbs.dtu.dk/services/MLST/) which is based on the PubMLST *Campylobacter* database (http://pubmlst.org/campylobacter/; accessed September 2016). Each sequence type (ST) was assigned to a ST complex, and each ST has a profile comprising the allele numbers at the seven MLST loci; based on the allelic profiles we used eBURST (http://eburst.mlst.net/v3/mlst_datasets/) to calculate the evolutionary descent of the entire ST21 complex in the *Campylobacter* MLST database. A comprehensive search of antibiotic resistance gene acquisition was conducted using ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/).

We searched for the G→T point mutation in the promoter region (57 bp upstream of the annotated start codon of $bla_{\text{OXA-61-like}}$), which is reported to be involved in the regulation of OXA-61-like expression [12]. The gene *gyrA*, which codes for the DNA gyrase subunit A of *C. jejuni*, has a quinolone resistance-determining region (QRDR), and a mutation in this gene confers quinolone-susceptibility to *Campylobacter jejuni* subsp. *jejuni* ATCC 700819 (NCBI Reference Sequence: NC_002163.1). Using Jalview (http://www.jalview.org/) we converted the *gyrA* nucleotide sequences to the amino acid sequences (GyrA), then compared and studied them [13]. To analyze *cmeR*, which is transcriptional repressor for CmeABC as the multidrug efflux pump, mutation in the open reading frame of *cmeR* was analyzed by comparing to data of Lin et al., [14]. We also surveyed the literature for LOS locus research. Each LOS locus has specific genes, so we found and classified the full-length nucleotide sequences from the draft genome sequence [15,16].

3. Results

3.1. Distribution of C. jejuni clones, antibiotic susceptibility, antibiotic-resistance genes, and the relationships between them

The rates of resistance of human-derived *C. jejuni* isolates to various antibiotics were not different from those of chicken-derived isolates. Isolates resistant to imipenem, chloramphenicol, and erythromycin could not be detected. The detection rates of human- and chicken-derived isolates resistant to ampicillin, streptomycin, tetracycline, ciprofloxacin, and nalidixic acid were, respectively, 16.2%, 1.6%, 43.2%, 45.9% and 46.5% (Table 1).

Based on the MLST results, the 185 isolates isolated in this study were classified into 69 STs (18 ST complexes). ST-21 complex was the dominant ST complex, accounting for 35.8% of human-derived *C. jejuni* (38/106 isolates) and 31.6% of chicken-derived *C. jejuni* (25/79 isolates) (Fig. 1). In this study, we registered several novel STs in the PubMLST database: ST8143 (1 isolate), ST8144 (4 isolates), ST8146 (1 isolate), ST8147 (1 isolate), ST8148 (1 isolate), and

Table 1
Antibiotic resistance of *Campylobacter jejuni* isolates in Japan antibiotic isolated from humans at medical facilities (n = 106) and from chicken meat and droppings collected from markets and farms (n = 79).

Antibiotic	Origin of isolate	MIC breakpoint (μg/mL)	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Resistance	
						Number of isolates (%)	
Ampicillin	Human	32	0.5–128	4	64	16 (15.1)	30 (16.2)
	Chicken		0.5–>128	4	32	14 (17.7)	
Imipenem	Human	16	≤0.06–0.12	≤0.06	0.12	0 (0)	0 (0)
	Chicken		≤0.06–0.12	≤0.06	0.12	0 (0)	
Streptomycin	Human	32	≤0.06–>128	1	1	2 (1.9)	3 (1.6)
	Chicken		≤0.06–>128	1	1	1 (1.3)	
Tetracycline	Human	16	≤0.06–>128	0.5	128	41 (38.7)	80 (43.2)
	Chicken		≤0.06–>128	1	128	39 (49.4)	
Chloramphenicol	Human	16	0.5–>16	1	2	0 (0)	0 (0)
	Chicken		0.5–8	1	2	0 (0)	
Erythromycin	Human	32	0.12–2	0.25	1	0 (0)	0 (0)
	Chicken		0.12–1	0.25	0.5	0 (0)	
Ciprofloxacin	Human	4	≤0.06–>32	0.25	16	47 (44.3)	85 (45.9)
	Chicken		≤0.06–>32	0.25	16	38 (48.1)	
Nalidixic acid	Human	32	4–>128	8	128	48 (45.3)	86 (46.5)
	Chicken		2–>128	8	>128	38 (48.1)	

ST8151 (1 isolate) (Table 2). In ST-21 complex, ST4526 was the predominant ST, accounting for 10 of the human-derived *C. jejuni* isolates and 8 of the chicken-derived isolates. All 10 human-derived isolates in ST4526 were resistant to ciprofloxacin, and 6 of those isolates had amino acid substitutions in the QRDR of GyrA, from threonine in position 86 to isoleucine (T86I). Nine tetracycline-resistant isolates were detected, and all of them carried the *tet(O)* gene coding for 30S ribosomal protection protein Tet(O). In ST4526 of the chicken-derived *C. jejuni,* all 8 isolates showed resistance to tetracycline and ciprofloxacin, all carried *tet(O)*, and all also had the T86I amino acid substitutions in the QRDR domain of *gyrA*. In contrast, none of the human- or chicken-derived *C. jejuni* ST4526 isolates had ampicillin resistance.

Among ST51 isolates (ST-443 complex), 1 isolate of human-derived *C. jejuni* and 10 isolates of chicken-derived *C. jejuni* were detected, making this the ST with the highest detection rate among the chicken-derived *C. jejuni* isolates. Among the chicken-derived *C. jejuni* isolates in ST51 (10 isolates), 5 isolates showed ampicillin resistance, 9 isolates showed tetracycline resistance, and 9 isolates showed ciprofloxacin resistance. Furthermore, 9 of the 10 isolates were carrying the GyrA T86I amino acid substitution mutation, but $bla_{OXA\text{-}61\text{-}like}$, which codes for β-lactamase and is associated with ampicillin resistance, and *tet(O)*, which is associated with tetracycline resistance, could not be detected.

Among the STs registered in the PubMLST database up to September 2016, 23 of the human-derived *C. jejuni* isolates and 2 of the chicken-derived *C. jejuni* isolates did not belong to any ST complex (listed in Table 2 as not defined). Of these 23 human-derived *C. jejuni* singleton isolates, we were able to detect the gene that codes for β-lactamase ($bla_{OXA\text{-}61\text{-}like}$) in 15 isolates and characterized them as narrow-spectrum class D β-lactamases.

3.2. Quinolone susceptibility of C. jejuni and association with mutations in the quinolone resistance-determining region (QRDR) and cmeR mutation

With regard to chicken-derived *C. jejuni* isolates, of the 38 isolates that showed resistance to ciprofloxacin and nalidixic acid, all except 1 isolate (MIC values: ciprofloxacin, 8 μg/mL; nalidixic acid, >128 μg/mL) had GyrA T86I amino acid substitution mutations

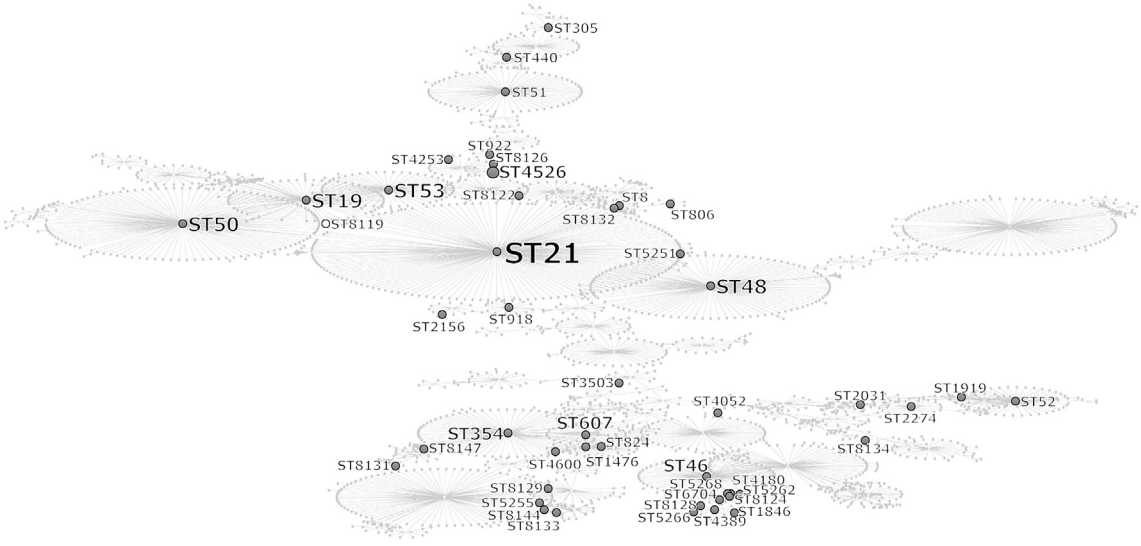

Fig. 1. eBURST diagram depicting the evolutionary descent of the entire ST21 complex in the *Campylobacter* PubMLST database. The database was accessed on 1 September 2016. Gray points and ST numbers are those detected in this study. Of the *Campylobacter jejuni* isolates isolated from human and chicken samples in this study, 34.1% (63/185 isolates) were included in the ST21 complex.

Table 2
Multilocus sequence typing (MLST), LOS locus class, antibiotic resistance, harboring acquired antimicrobial resistance genes and QRDRs amino acid substitution in GyrA of the 185 *Campylobacter jejuni* isolates in Japan.

ST complex	Sequence type	Number of human isolates ($n = 106$)												
		Number of isolates	LOS locus class					Ampicillin-resistant	Tetracycline-resistant	Ciprofloxacin-resistant	*bla*OXA-61-like with no mutation in the promoter region	*bla*OXA-61-like with G→T mutation in the promoter region[a]	*tet(O)*	T86I amino acid substitution in GyrA
			class A	class B	class C	class D	class E							
21	8	2	0	0	0	1	1	2	2	0	0	1	2	0
	19	8	2	0	2	3	1	0	1	8	6	0	0	5
	21	5	1	0	0	1	3	0	3	2	4	0	2	3
	50	7	0	1	4	2	0	0	6	3	5	0	3	3
	53	1	1	0	0	0	0	0	0	0	1	0	0	0
	806	1	0	0	1	0	0	0	1	0	0	0	0	0
	4253	1	0	0	0	0	1	0	0	0	1	0	0	0
	4526	10	4	2	2	1	1	0	9	10	10	0	9	6
	5251	0	0	0	0	0	0	0	0	0	0	0	0	0
	8119	0	0	0	0	0	0	0	0	0	0	0	0	0
	8122	0	0	0	0	0	0	0	0	0	0	0	0	0
	8126	2	1	0	0	1	0	0	2	1	1	0	2	1
	8132	1	1	0	0	0	0	0	0	0	1	0	0	0
Subtotal		38	10	3	9	9	7	2	24	24	29	1	18	18
464	464	1	0	0	0	1	0	1	0	1	0	1	0	1
	4108	2	0	0	2	0	0	0	0	0	0	0	0	1
	4389	2	0	0	1	0	1	1	1	0	0	0	1	0
	5262	0	0	0	0	0	0	0	0	0	0	0	0	0
	5268	0	0	0	0	0	0	0	0	0	0	0	0	0
	6704	3	0	0	1	1	1	2	0	0	1	0	0	0
	8124	0	0	0	0	0	0	0	0	0	0	0	0	0
Subtotal		8	0	0	4	2	2	4	1	1	1	1	1	2
354	354	3	2	0	1	0	0	0	0	0	0	0	0	2
	1476	1	0	0	1	0	0	0	0	0	0	0	0	1
	5721	2	1	0	0	0	1	0	0	0	0	0	0	1
	8131	1	0	0	1	0	0	0	0	0	0	0	0	0
	8147	0	0	0	0	0	0	0	0	0	0	0	0	0
Subtotal		7	3	0	3	0	1	0	0	0	0	0	0	4
443	51	1	0	0	1	0	0	0	0	0	0	0	0	0
	440	2	2	0	0	0	0	0	0	0	0	0	0	0
	5266	1	0	0	0	0	1	0	0	1	0	0	0	1
Subtotal		4	2	0	1	0	1	0	0	1	0	0	0	1
48	48	0	0	0	0	0	0	0	0	0	0	0	0	0
	918	4	2	0	0	1	1	0	1	3	3	0	1	3
	3503	1	0	0	1	0	0	0	1	0	1	0	0	0
Subtotal		5	2	0	1	1	1	0	2	3	4	0	1	3
42	42	3	1	0	2	0	0	0	1	0	0	0	0	0
257	257	1	0	0	1	0	0	0	1	0	0	0	0	0
	824	1	0	1	0	0	0	0	0	0	0	0	0	0
	8129	1	0	1	0	0	0	0	0	0	0	0	0	0
Subtotal		3	0	2	1	0	0	0	1	0	0	0	0	0
460	5255	1	0	1	0	0	0	0	0	0	0	0	0	0
	8144	2	2	0	0	0	0	0	0	1	0	0	0	1
Subtotal		3	2	1	0	0	0	0	0	1	0	0	0	1
574	305	0	0	0	0	0	0	0	0	0	0	0	0	0
	2031	2	1	0	1	0	0	0	1	1	1	0	1	1
Subtotal		2	1	0	1	0	0	0	1	1	1	0	1	1
22	22	1	0	0	0	0	1	0	0	0	1	0	0	0
353	4052	1	0	0	1	0	0	0	0	1	0	0	0	1
	5259	0	0	0	0	0	0	0	0	0	0	0	0	0
	8133	1	0	0	1	0	0	0	0	0	0	0	0	0
Subtotal		2	0	0	2	0	0	0	0	1	0	0	0	1
45	45	0	0	0	0	0	0	0	0	0	0	0	0	0
	137	0	0	0	0	0	0	0	0	0	0	0	0	0
	583	1	0	0	0	1	0	0	0	0	0	0	0	0
	5263	0	0	0	0	0	0	0	0	0	0	0	0	0
Subtotal		1	0	0	0	1	0	0	0	0	0	0	0	0
49	8134	1	1	0	0	0	0	1	1	1	0	1	1	0
283	4063	1	0	0	1	0	0	0	0	0	1	0	0	0
362	2993	1	0	0	0	0	1	0	0	1	0	0	0	1
403	933	1	0	0	0	1	0	0	1	0	0	0	1	1
52	52	1	0	0	0	0	1	0	0	0	0	0	0	0
	1919	1	0	0	0	0	1	1	1	1	0	1	1	1
	4265	0	0	0	0	0	0	0	0	0	0	0	0	0
Subtotal		2	0	0	0	0	2	1	1	1	0	1	1	1
607	607	0	0	0	0	0	0	0	0	0	0	0	0	0
	4600	0	0	0	0	0	0	0	0	0	0	0	0	0
Subtotal		0	0	0	0	0	0	0	0	0	0	0	0	0

Number of chicken isolates ($n = 79$)												
Number of isolates	LOS locus class					Ampicillin-resistant	Tetracycline-resistant	Ciprofloxacin-resistant	*bla*OXA-61-like with no mutation in the promoter region	*bla*OXA-61-like with G→T mutation in the promoter region[a]	*tet(O)*	T86I amino acid substitution in GyrA
	class A	class B	class C	class D	class E							
1	0	0	1	0	0	1	1	0	0	1	1	0
3	0	0	3	0	0	0	3	3	3	0	3	3
2	0	0	2	0	0	0	2	1	2	0	1	0
3	0	0	3	0	0	1	2	2	2	0	1	2
1	0	0	1	0	0	0	0	0	1	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
1	1	0	0	0	0	0	0	1	0	0	0	1
8	0	0	0	8	0	0	8	8	7	0	8	8
1	0	0	1	0	0	0	0	0	1	0	0	0
4	0	0	4	0	0	0	4	0	4	0	0	0
1	0	0	1	0	0	0	0	0	1	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
25	1	0	16	8	0	2	20	15	21	1	14	14
0	0	0	0	0	0	0	0	0	0	0	0	0
2	2	0	0	0	0	0	0	0	0	0	0	0
2	0	0	2	0	0	2	1	0	0	0	0	0
1	0	1	0	0	0	0	0	0	0	0	0	0
4	3	1	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	0	0	0	0	0	0	0	0	0	0
11	6	3	2	0	0	2	1	0	0	0	0	0
2	2	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	1	0	0	0	1	0	0	0	1
0	0	0	0	0	0	0	0	0	0	0	0	0
1	1	0	0	0	0	0	0	1	0	0	0	1
4	3	0	0	1	0	0	0	2	0	0	0	2
10	0	0	0	0	10	5	9	9	0	0	0	9
1	0	0	0	0	1	0	1	0	0	0	1	0
1	0	0	1	0	0	0	0	1	0	0	0	1
12	0	0	1	0	11	5	10	10	0	0	1	10
1	1	0	0	0	0	1	1	0	1	0	1	1
3	1	2	0	0	0	1	1	2	0	0	1	1
1	1	0	0	0	0	0	0	0	0	0	0	0
5	3	2	0	0	0	2	2	2	1	0	2	2
1	1	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
3	1	2	0	0	0	0	0	0	0	0	0	0
2	2	0	0	0	0	0	0	0	0	0	0	0
5	3	2	0	0	0	0	0	0	0	0	0	0
1	1	0	0	0	0	1	1	1	0	1	1	1
0	0	0	0	0	0	0	0	0	0	0	0	0
1	1	0	0	0	0	1	1	1	0	1	1	1
1	0	0	0	1	0	0	0	1	1	0	0	1
0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	0	0	0	0	0	2	0	0	0	2
0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	1	0	0	0	0	0	2	0	0	0	2
3	0	0	0	0	3	0	1	0	2	0	1	0
1	0	0	0	0	1	0	1	1	1	0	1	1
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	1	1	1	1	0	0	0	1
5	0	0	0	0	5	1	3	2	3	0	2	2
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	1	0	0	0	1	0	0	0	1
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	1	0	0	1	1	0	0	1	1
2	0	0	0	2	0	0	1	2	0	0	1	2
2	0	0	0	2	0	0	0	0	0	0	0	0
1	0	0	1	0	0	0	0	0	0	0	0	0
3	0	0	1	2	0	0	0	0	0	0	0	0

(*continued on next page*)

Table 2 (*continued*)

ST complex	Sequence type	Number of human isolates ($n = 106$)												
		Number of isolates	LOS locus class					Ampicillin-resistant	Tetracycline-resistant	Ciprofloxacin-resistant	*bla*OXA-61-like with no mutation in the promoter region	*bla*OXA-61-like with G→T mutation in the promoter region[a]	*tet(O)*	T86I amino acid substitution in GyrA
			class A	class B	class C	class D	class E							
ND	407	8	2	0	3	2	1	1	3	7	6	0	2	5
	922	2	0	1	0	1	0	0	0	0	2	0	0	1
	2156	4	0	0	1	0	3	4	0	0	0	4	0	0
	2274	1	0	0	1	0	0	0	1	1	1	0	1	1
	3632	1	1	0	0	0	0	1	0	1	0	1	0	1
	4324	1	0	1	0	0	0	0	0	0	1	0	0	0
	6308	1	1	0	0	0	0	0	0	0	0	0	0	0
	8128	2	0	0	1	0	1	2	2	2	0	0	0	2
	8130	1	0	0	1	0	0	0	1	0	0	0	1	0
	8143	0	0	0	0	0	0	0	0	0	0	0	0	0
	8146	0	0	0	0	0	0	0	0	0	0	0	0	0
	8148	1	0	0	1	0	0	0	0	0	0	0	0	0
	8151	1	1	0	0	0	0	0	1	1	0	0	0	0
Subtotal		23	5	2	8	3	5	8	8	12	10	5	4	10
Total		106	27	8	33	17	21	16	41	47	46	9	28	44

[a] A to G mutation in the 57 bp upstream of initiation codon; ND, Not defined.

(Fig. 2). In the case of human-derived isolates, however, 17 of the 47 isolates that showed resistance to ciprofloxacin and 16 of the 48 isolates that showed resistance to nalidixic acid did not have T86I amino acid mutations in GyrA. Also, 13 human isolates that had GyrA T86I amino acid mutations were not resistant to either ciprofloxacin or nalidixic acid. One *C. jejuni* of the human isolates showed nalidixic acid and ciprofloxacin resistant. This isolate had aspartic acid to asparagine amino acid substitution in GyrA at position 90. One *C. jejuni* of the human isolates had two nucleotides insertion (guanine and adenine) at nucleotide position 369 and 370 in *cmeR*. However, this isolate showed susceptible to nalidixic acid and ciprofloxacin.

3.3. Association between C. jejuni ST and LOS locus class

The 185 *C. jejuni* isolates were grouped into 5 LOS locus classes: A, B, C, D, and E (Table 2). The breakdown of the LOS locus classes of the human-derived isolates in ST-21 complex is as follows: 10 isolates in class A, 3 isolates in class B, 9 isolates in class C, 9 isolates in class D, and 7 isolates in class E. The chicken-derived *C. jejuni* isolates were grouped in the following LOS locus classes: 1 isolate in class A, 16 isolates in class C and 8 isolates in class D. In ST4526 of ST-21 complex there was a difference between the human- and chicken-derived *C. jejuni* isolates: the human-derived isolates are in LOS locus classes A, B, C, D, and E, whereas all 8 chicken-derived isolates are in LOS locus class D. In ST-464 complex, the human-

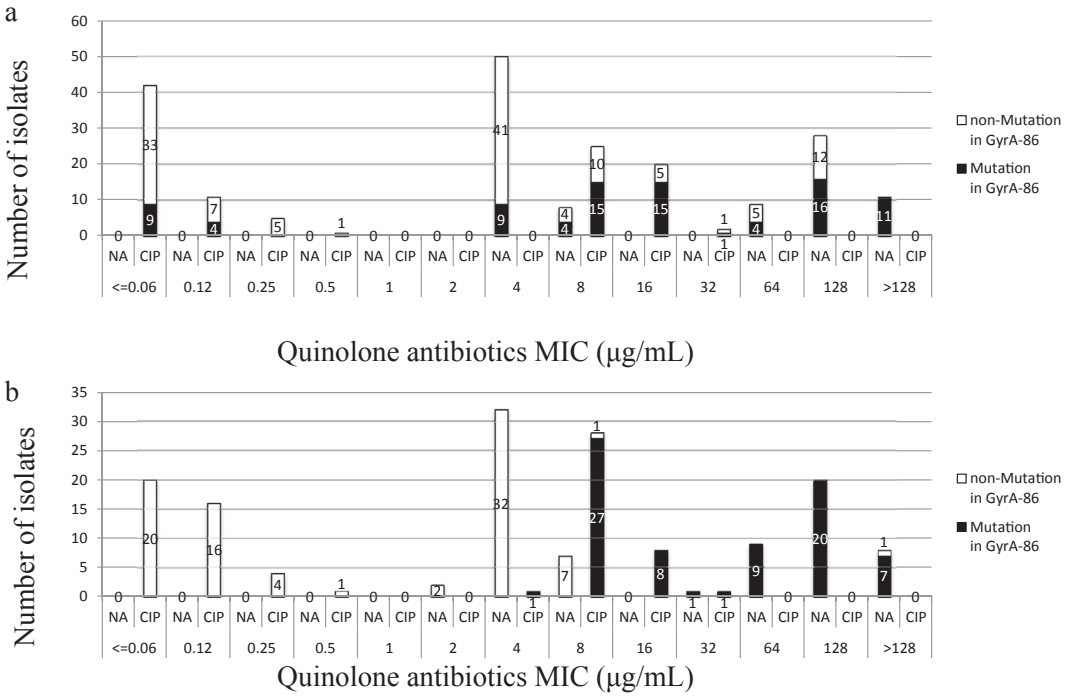

Fig. 2. Relationship between mutation in the quinolone resistance-determining region of *Campylobacter jejuni* isolates and MIC of quinolone antibiotics. (a) *C. jejuni* isolated from human samples. (b) *C. jejuni* isolated from chicken samples. NA, Nalidixic acid; CIP, Ciprofloxacin.

Number of chicken isolates ($n = 79$)												
Number of isolates	LOS locus class					Ampicillin-resistant	Tetracycline-resistant	Ciprofloxacin-resistant	*bla*OXA-61-like with no mutation in the promoter region	*bla*OXA-61-like with G→T mutation in the promoter region[a]	*tet(O)*	T86I amino acid substitution in GyrA
	class A	class B	class C	class D	class E							
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	1	0	1	1	1	0	0	1	1
1	0	0	1	0	0	1	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	1	1	0	2	1	1	0	0	1	1
79	19	8	21	15	16	14	39	38	25	3	20	37

derived isolates were in LOS locus classes C, D, and E, but the chicken-derived isolates were in LOS locus classes A, B, and, C. Furthermore, chicken-derived isolates were predominantly detected in ST51 (ST-443 complex), and all 10 of those isolates were grouped in LOS locus class E.

4. Discussion

From the results of our analysis, we concluded that ST-21 complex was the most prevalent of the human- and chicken-derived *C. jejuni* clones distributed in Japan. It is already known that human-derived *C. jejuni* clones in Japan are mainly ST-21 complex and that broiler chickens also carry ST-21 complex clones, suggesting that eating poultry could cause infection in humans [7]. It has also been reported that ST4526 of ST-21 complex is the diffusing clone in Japan [17]. Our research also confirmed the prevalence of both human- and chicken-derived *C. jejuni* isolates. The human- and chicken-derived *C. jejuni* isolates have major clones in common, and it seems that *C. jejuni* infects humans mainly via chicken meat.

Our research yielded other results, such as the findings that both human- and chicken-derived *C. jejuni* isolates are susceptible to imipenem, chloramphenicol, and erythromycin; that the frequency of isolation of ampicillin- and streptomycin-resistant isolates is low; and that about half of the isolates show resistance to tetracycline, ciprofloxacin, and nalidixic acid. It is reported that resistance of *C. jejuni* isolates to antibiotics is determined by the selection pressure exerted by antibiotics used on poultry farms [18]. The results of our antibiotic susceptibility testing agreed with results previously reported for human- and chicken-derived *C. jejuni* isolates [3,9]; however, there are some contradictory results regarding the use of veterinary antibiotics in broiler chickens, the results of sales of antibiotic additives for chicken feed, and the rates of detection of resistant isolates in Japan. To be specific, sales volumes of antibiotics to broiler farms from 2009 to 2014 were highest for tetracyclines, followed in order by macrolides, penicillins, aminoglycosides, and quinolones, with carbapenems and chloramphenicol not being sold [19]. We did not detect isolates resistant to antibiotics that were not being sold, and we did detect isolates resistant to antibiotics that had good sales results. Although the sales volumes of penicillins and macrolides were higher than that of quinolones, the isolation rate of resistant to penicillins and macrolides isolates were lower than that to quinolones. Development of resistance in *C. jejuni* against quinolones is reported to be low in terms of biological cost and reduced fitness [20,21], and it is speculated that carriers of resistant isolates are continuously present regardless of the small amount of quinolones antibiotic exposure. According to the previously reports, spontaneous mutation frequency associated with quinolones and macrolides-resistant in *C. jejuni* $9.3 \pm 1.2 \times 10^{-9}$–$2.9 \pm 1.4 \times 10^{-6}$ and $<5.4 \times 10^{-10} - 3.0 \times 10^{-9}$ [22,23], respectively. Since the frequency of acquisition for macrolides-resistance is approximately 1000 times lower than that of quinolones-resistance. Therefore, it seems that the consumptions of macrolides and the isolation frequency of macrolides-resistance in *C. jejuni* were not correlate.

The results of this study showed an association between some STs and antibiotic susceptibility. However, although an association was found between antibiotic susceptibility with respect to quinolones and the presence of resistance genes in chicken-derived *C. jejuni* isolates, no such association could be found in human isolates. Although this result agrees with prior reports [9], our study did not reveal any clear reason why the human isolates with GyrA T86I amino acid substitution and *cmeR* mutation were susceptible to quinolones. It is reported that in *C. jejuni* when an amino acid mutation substitutes threonine with alanine in position 86 of GyrA, the results are nalidixic acid resistance and ciprofloxacin susceptibility [24]. The possible existence of amino acid substitutions other than T86I in the QRDR domain or another factor that affects quinolone susceptibility is suggested.

We also studied the association between ST and LOS locus class in *C. jejuni* isolates in Japan and found a tendency for ST to be associated with LOS locus class in the chicken-derived isolates but no such tendency in the human-derived isolates. Other studies on human- and chicken-derived *C. jejuni* isolates have indicated an association between ST and LOS locus class, but they have not recognized an association in specific clones [1,25,26]. Our research likewise could not find a relationship between ST and LOS locus class in human-derived isolates but no such tendency in the human-derived isolates. The clinical samples in our study were all collected in Tokyo, so the human-derived isolates that we isolated may have genetic backgrounds peculiar to the region. That is a limitation of this study, and we cannot rule out the possibility that the quinolone susceptibility found in the human-derived isolates with GyrA T86I amino acid mutations may also be due to genetic background. We focused only on human- and chicken-derived

C. jejuni in this study, but other *C. jejuni* with specific genetic backgrounds, such as those from cattle and other animal species, can also be isolated from humans [17]. In the future, we need to conduct genomic-level analyses of *C. jejuni* from another animal species.

This study confirmed that the majority of human- and chicken-derived *C. jejuni* isolates shared a common genetic background and similar antimicrobial susceptibilities. However, it should be noted that isolates presenting an association between ST and antibiotic susceptibility were limited in number. Differences between human- and chicken-derived *C. jejuni* were also demonstrated by the results of ST and LOS locus class analysis and by the relationship between antibiotic susceptibility and possession of antibiotic resistance genes. These results suggest that *C. jejuni*, which poses a health hazard for humans, is rich in genetic diversity and could derive from sources other than chickens such as cattle and another animal species.

Conflict of interest

No conflict of interest to declare.

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