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Long-term observation of antimicrobial susceptibility and molecular characterisation of *Campylobacter jejuni* isolated in a Japanese general hospital 2000–2017

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ABSTRACT

Objectives: *Campylobacter jejuni* (*C. jejuni*) is one of the most common pathogens that causes gastroenteritis. Because there is currently insufficient epidemiological information about the antimicrobial susceptibility and molecular characterisation of clinical isolates of *C. jejuni* in Japan, this study carried out antimicrobial susceptibility testing and multilocus sequence typing (MLST) of clinical *C. jejuni* isolates in Tokyo between 2000–2017.

Methods: Antimicrobial susceptibility to erythromycin and ciprofloxacin was tested using the broth microdilution method in 430 *C. jejuni* clinical isolates collected over 18 years, between 2000–2017, at a Tokyo general hospital. To observe the sequence type (ST) evolution, 82 isolates were chosen from three non-consecutive years (16 isolates from 2000, 25 isolates from 2008, and 41 isolates from 2017) and analysed by MLST as a molecular characterisation test. Mutations in the quinolone resistance-determining region of the *gyrA* and *gyrB* genes were identified.

Results: The rate of resistance to erythromycin was low, but that of ciprofloxacin resistance was 34.9% in 2000–2008 and 41.9% in 2009–2017. The most common clonal complex (CC) identified during the entire period was CC21; ST4526 with ciprofloxacin resistance was highly prevalent in 2017 (6 of 11; 54.5%).

Conclusion: The results indicate that the rate of resistance to quinolone has gradually increased. Since ST4526 was not isolated in 2000 and 2008, it is likely that ST4526 is rapidly increasing in Japan.

1. Introduction

Campylobacter jejuni (*C. jejuni*) is a Gram-negative, microaerophilic, spiral-shaped organism that causes gastroenteritis in humans. It is a public health threat and one of the most common foodborne zoonotic pathogens worldwide [1]. The transmission of *C. jejuni* to humans occurs by ingestion of contaminated food, especially poultry [1,2]. The main symptom of campylobacteriosis is diarrhoeal disease, including bloody stool, fever, and abdominal discomfort [3]. Additionally, severe autoimmune neurological symptoms, namely Guillain-Barré syndrome, may occur after *C. jejuni* infection [3].

Antibiotic treatments are required for severe campylobacteriosis, which manifests as acute diarrhoea and infection in immunocompromised patients. Macrolides and quinolones, such as erythromycin and ciprofloxacin, have been used for campylobacteriosis treatment. While macrolide resistance has been successfully maintained worldwide at low levels, quinolone resistance has been rapidly increasing in many countries, including Japan [4–7]. Therefore, investigation of the antimicrobial susceptibility of *C. jejuni* is critical.

Multilocus sequence typing (MLST) has previously been used for molecular characterisation of *C. jejuni* in other studies [8,9]. Although *C. jejuni* strains isolated from chicken and humans have exhibited high molecular diversity, those belonging to clonal complex (CC) 21 have spread worldwide [4,10,11]. Several studies have reported an increased prevalence of antibiotic resistance in specific genotypes [12,13]. Because long-term studies focusing on clinical isolates of *C. jejuni* are lacking in Japan, the current study

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carried out antimicrobial susceptibility testing and MLST of clinical *C. jejuni* isolated in a Tokyo general hospital between 2000–2017.

2. Materials and methods

2.1. Bacterial strains and culture

This study used 430 non-duplicate clinical isolates of *C. jejuni*. These were isolated from stool specimens collected over an 18-year period between 2000–2017 at the Tokyo Metropolitan Health and Medical Treatment Corporation Toshima Hospital, Tokyo. The isolates were identified using the Hippurate test and PCR method, as previously described [14]. These isolates were maintained in 10% skimmed milk, stored at −80 °C, and grown on trypticase soy agar containing 5% sheep blood (Becton Dickinson) for 24 h at 42 °C under microaerobic conditions (5% O_2 and 10% CO_2 in N_2). Colonies that grew on agar plates were used for antimicrobial susceptibility and molecular testing.

2.2. Antimicrobial susceptibility testing

To determine the MIC for erythromycin and ciprofloxacin, antimicrobial susceptibility testing was performed using the broth microdilution method in 5% lysed horse blood broth, according to the Clinical and Laboratory Standards Institute (CLSI) [15]. The breakpoint MICs of erythromycin and ciprofloxacin as defined by CLSI M48-A3 were: ≤8 susceptible;=16 intermediate; ≥32 resistant and ≤2 susceptible; = 2 intermediate; and ≥4 resistant, respectively. The MICs were determined following a 24 h culture of the bacterial strains under microaerobic conditions at 42 °C. The breakpoints were categorised according to the CLSI M45-A3 criteria [16]. *Campylobacter jejuni* ATCC33560 was used for quality control.

2.3. Multilocus sequence typing MLST and gyr gene sequencing

Multilocus sequence typing was conducted to evaluate the chronological molecular characterisation of *C. jejuni* isolates harvested in this study. To observe the sequence type (ST) change over time, 82 isolates were chosen from three non-consecutive years (16 isolates from 2000, 25 isolates from 2008, and 41 isolates from 2017) and analysed by MLST. The MLST profiles were identified according to the PubMLST *Campylobacter* database (http://pubmlst.org/campylobacter/; accessed June 2018). To determine the genetic lineage of isolates belonging to CC21, the eBURST tool in the *Campylobacter* MLST database was used.

This study also determined quinolone-targeting genes, namely the quinolone resistance-determining region (QRDR), and mutations in the *gyrA* and *gyrB* genes, as previously reported [17]. Thirty-three clinical isolates chosen from those used for MLST and that exhibited ciprofloxacin resistance (4 mg/L) were used for *gyr* gene sequencing. The DNA used for MLST and *gyr* gene sequencing was extracted using the RBC Genomic DNA Extraction Kit (RBC Bioscience). Polymerase chain reaction amplification was performed on a Gene Amp PCR System 9600-R thermal cycler (PerkinElmer). The PCR products were purified using the FastGene Gel/PCR Extraction kit (Nippon Genetics) and sequenced using a 3730 DNA Analyzer (Applied Biosystems). A similarity search for the deduced amino acid sequences was conducted using BLAST. To identify the position of the substitution mutations, peptide alignment of wild-type *C. jejuni* strain ATCC33560 was performed using BioEdit software.

2.4. Transformation

To evaluate the relationship between novel mutations in *gyr* and quinolone resistance, a transformation experiment was conducted as previously reported [18]. A PCR product that contained the *gyr* mutation was obtained using the protocol described in the previous section. The transformants were selected on Mueller-Hinton agar plates supplemented with 0.5 mg/L and 1.0 mg/L ciprofloxacin.

3. Results

The MIC distribution in the 430 clinical isolates of *C. jejuni* for both ciprofloxacin and erythromycin was 0.032–32 mg/L (Table 1). Among these isolates, one exhibited resistance to erythromycin throughout the entire period. In contrast, the ciprofloxacin resistance rates during the periods 2000–2008 and 2009–2017 were 34.9% and 41.9%, respectively (Table 1).

The MLST classified the 82 clinical isolates into 54 STs, of which 14 were novel (after ST9148). Apart from CC21, which was the most common lineage (19 of 82; 23.2%), a high diversity of STs was observed. Within CC21, ST50 was the most common lineage in 2000, and ST4526 with a high ciprofloxacin MIC was the most common lineage in 2017. In contrast, in 2008, CC48 was the most common lineage and was equally composed of ST48, ST38 and ST453 (Table 2). Notably, nine of the 11 isolates that belonged to CC21 and were isolated in 2017 showed quinolone resistance.

Thirty-three clinical strains isolated in 2000, 2008, and 2017 that showed resistance to ciprofloxacin were investigated for amino acid substitutions in QRDRs in GyrA and GyrB. All 33 tested isolates possessed substitutions in QRDRs. Two types of substitutions were found in GyrA (Thr86Ile and Asp90Asn), which have previously been reported [19], and one substitution was found in GyrB (Ala518Thr) (Table 2). Of the 33 isolates tested for QRDR substitutions, 31 ciprofloxacin-resistant isolates shared the Thr86Ile substitution (31 of 33; 93.9%). Among these 31 isolates, four isolates also possessed the Ala518Thr substitution. One isolate that possessed Asp90Asn in GyrA and Ala518Thr in GyrB alone was identified in this study. Although a novel substitution, Ala518Thr in GyrB, was observed, transformants that exhibited an elevated ciprofloxacin MIC in this study were unable to be obtained.

4. Discussion

Antimicrobial resistance of microorganisms, including *C. jejuni*, is a severe global health concern [20]. The antimicrobial resistance of *C. jejuni* is considered to be a serious health concern by the

Table 1
Antimicrobial susceptibility to erythromycin and ciprofloxacin in 430 clinical isolates of *Campylobacter jejuni*.

Isolation period	Antimicrobial agent	MIC (mg/L)											Number of isolates	Resistance rate (%)
		0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32		
2000–2008	Erythromycin		1	33	80	63	29	8				1	215	0.47
2009–2017		1	6	60	82	54	11	1					215	0
2000–2008	Ciprofloxacin	2	14	70	34	14	2	4	3	14	46	12	215	34.9
2009–2017		3	32	60	24	5	1		3	45	28	14	215	41.9

Table 2
Multilocus sequence typing and quinolone resistance-determining region changes in 82 *Campylobacter jejuni* isolates.

Isolation year	Clonal complex	Sequence type	Ciprofloxacin MIC (mg/L)	GyrA	GyrB	Isolation year	Clonal complex	Sequence type	Ciprofloxacin MIC (mg/L)	GyrA	GyrB
2000	21	50	0.25			2017	21	4526	8	Thr86Ile	-
		50	16	Thr86Ile	-			4526	8	Thr86Ile	-
		50	0.125					4526	8	Thr86Ile	-
								4526	8	Thr86Ile	-
	48	918	16	Thr86Ile	Ala518Thr			4526	16	Thr86Ile	-
		48	0.125					4526	32	Thr86Ile	-
								806	0.064		
	443	440	16	Asp90Asn	-			4253	8	Thr86Ile	-
		440	0.25					8075	8	Thr86Ile	-
								9158	0.125		
	42	42	0.125					9162	32	Thr86Ile	-
		42	0.064								
							464	4389	0.125		
	49	3720	0.5					4389	0.125		
		49	16	Thr86Ile	-			4389	8	Thr86Ile	-
								5262	16	Thr86Ile	-
	22	9148	0.125					9157	0.064		
	61	5161	0.25					9161	0.064		
	460	5269	0.064								
	unclassifiable	407	0.25				48	918	0.064		
	unclassifiable	9149	32	Thr86Ile	-			918	0.064		
2008	21	8	0.25					918	0.125		
		2789	0.25					38	0.064		
		9152	0.125								
		21	0.064				22	22	16	Thr86Ile	-
		8	0.5					567	0.125		
	48	453	16	Thr86Ile	Ala518Thr		61	61	0.064		
		48	0.125					61	0.064		
		48	0.25								
		38	32	-	Ala518Thr		443	443	0.064		
		38	2					9156	0.125		
		453	16	Thr86Ile	Ala518Thr						
							353	3911	0.064		
	22	22	16	Thr86Ile	-			4052	32	Thr86Ile	-
		22	0.25								
		22	32	Thr86Ile	-		607	607	0.064		
								9160	8	Thr86Ile	-
	353	5	16	Thr86Ile Val149Ile	-						
		4896	32	Thr86Ile	-		42	42	0.064		
	443	440	0.25				45	6722	8	Thr86Ile	-
		51	0.25								
							354	354	0.25		
	unclassifiable	922	32	Thr86Ile	-		446	446	32	Thr86Ile	-
	unclassifiable	922	0.125				460	8144	0.125		
	unclassifiable	922	0.125				unclassifiable	922	16	Thr86Ile	-
	257	257	0.5				unclassifiable	9159	8	Thr86Ile	Ala518Thr
	354	9151	0.064				unclassifiable	9163	16	Thr86Ile	-
	464	4108	1				unclassifiable	9164	0.5		
	unclassifiable	2247	32	Thr86Ile	-		unclassifiable	9165	0.064		

Centers for Disease Control and Prevention in the USA; therefore, focus on the antimicrobial resistance of *C. jejuni* has become more critical.

The current results indicate that about 40% of *C. jejuni* clinical isolates collected over the study period showed resistance to ciprofloxacin. According to a previous study in Japan by Bakeli et al., 20.2% of *C. jejuni* clinical isolates between 2003–2005 showed resistance to ciprofloxacin [17]. Asakura et al. demonstrated that ciprofloxacin resistance rates of *C. jejuni* derived from humans and chickens in 2005–2006 and 2010–2011 were 36% and 64%, respectively [21]. The current results showed that the rates of ciprofloxacin resistance during two periods, 2000–2008 and 2009–2017, were 34.9% and 41.9%, respectively. These previous studies and the current results indicate that the rate of quinolone resistance in *C. jejuni* isolated in Japan may have gradually risen. Conversely, one erythromycin-resistant isolate was identified in the current study, and few or no erythromycin-resistant isolates were observed in previous studies [10,17,21], suggesting that macrolide-resistant *C. jejuni* is rare in Japan. Quinolone resistance was found in 86.7% of *C. jejuni* clinical isolates from human stool samples in China [7]. In Australia from 1999–2001, a study reported that 2% of *C. jejuni* isolates were resistant to ciprofloxacin [22]. Therefore, the quinolone susceptibility of *C. jejuni* isolates may differ according to geographical location, with an intermediate quinolone resistance rate in Japan compared with that of other countries.

The major mechanisms of quinolone resistance, including those in *C. jejuni*, have been well studied. Among them, QRDR substitutions are the most common and found in almost all microorganisms. In Gram-negative bacteria, DNA gyrase, which is composed of GyrA and GyrB, has a higher affinity for quinolones than topoisomerase IV, which is composed of ParC and ParE; thus,

mutations in DNA gyrase are critical for quinolone resistance [23]. Moreover, several studies have demonstrated that *C. jejuni* lacks *par* genes [19]; therefore, they cannot be a source of quinolone resistance. Instead, a single mutation, Thr86Ile in GyrA, leads to high-level resistance to quinolones [19]. In agreement with previous studies, the Thr86Ile substitution in GyrA was most commonly observed in ciprofloxacin-resistant *C. jejuni* isolates in this study (31 of 33; 93.9%). Although the Ala518Thr substitution in GyrB was also observed in quinolone-resistant isolates (5 of 33; 15.2%), a transformation experiment with the Ala518Thr substitution in GyrB was notable to reproduce ciprofloxacin resistance in the recipient. Consequently, the Ala518Thr substitution in GyrB may not be involved in quinolone resistance. Because there is little information regarding GyrB substitutions in quinolone resistance, the relationship between GyrB substitutions and quinolone resistance should be further investigated. On the other hand, since other quinolone-resistance mechanisms, such as the CmeABC efflux system, were not investigated in this study, the involvement of resistance factors other than QRDR substitutions cannot be excluded.

In the current study, one erythromycin-resistant isolate was obtained in 2001. The mechanisms of macrolide resistance in *C. jejuni* have been well studied globally. The major mechanism of macrolide effect on *C. jejuni* is the target mutations such as 23S rRNA gene mutation (i.e., A2074 and A2075) and ribosomal proteins (L4-encoding *rplD* and L22-encoding *rplV*) [24]. More recently, the presence of the *erm(B)* gene, which modified the macrolide target of 23S rRNA and resulted in macrolide resistance, was reported [24]. Although the current study did not analyse the mechanism of macrolide resistance, the correlation between these resistance mechanisms was considered.

In the current study, in which 82 tested isolates were grouped into 54 types of STs including 14 novel STs, the results revealed that *C. jejuni* clinical isolates exhibited high molecular diversity. Among 82 isolates, eight types of STs (ST407, ST922, ST2247, ST9149, ST9159, ST9163, ST9164, and ST9165) did not belong to any major CC; CC21 was the most common molecular lineage over the entire period. Among CC21 isolates, quinolone-resistant ST4526, which was not isolated in 2000 and 2008, became predominant in 2017. The first ST4526 identified in Japan was reported by Asakura et al. in 2012 [10]. They also reported that ST4526 was most frequently isolated after ST50 among CC21 isolates. More recently, Ohishi et al. also revealed that ST4526 was the most predominant in human-derived and chicken-derived isolates in Japan from 2007–2014 [25]. Considering that the current results showed that ST4526 was not isolated in 2000 and 2008, the rapid increase in ST4526 may have recently occurred in Japan.

This study had certain limitations. Although the study period was longer than in previous studies, *C. jejuni* clinical isolates were investigated from one hospital. To more comprehensively study the evolutional changes in the STs of clinical isolates, isolates from other medical institutions should be investigated.

In conclusion, the results indicate that the antimicrobial resistance of *C. jejuni* to quinolones may have gradually evolved over the past couple of decades, while erythromycin susceptibility remained unaffected. The results also revealed that quinolone-resistant ST4526 strains belonging to CC21 may have predominantly spread in Japan. To date, long-term observation of antimicrobial susceptibility and molecular characterisation of clinical campylobacter isolates were not carried out in Japan; the current results agree with previous findings. Furthermore, antimicrobial-resistant *C. jejuni* clinical isolates are expected to spread in the near future. Therefore, continuation of this study of antimicrobial susceptibility is planned with inclusion of a more detailed molecular characterisation of *C. jejuni* clinical isolates.

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Competing interests

None.

Ethical approval

Not required.

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