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ORIGINAL ARTICLE

Correlations between 23S rRNA genes and erythromycin resistance in *Campylobacter jejuni*

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Abstract There has been no description of the prevalence of intervening sequences (IVSs) within the 23S rRNA genes in clinical *Campylobacter jejuni* isolated from humans, and, moreover, no studies have yet appeared describing the correlation between nucleotide sequences of 23S rRNA genes and erythromycin (Ery) resistance in human isolates. Minimum inhibitory concentration of 49 human clinical *C. jejuni* isolates isolated in Asia, Europe, and North America were determined, and 8 isolates were resistant to Ery (≥ 8 μg/ml; 16%). Following sequencing and alignment analyses, no correlations between the nucleotide sequences of the V domain and the IVSs within 23S rRNA gene sequences and Ery resistance occurred in the human clinical *C. jejuni* isolates examined. In addition, no point mutations occurred at any expected and putative positions in the V domain with the isolates. In addition, no correlations were seen between the occurrence of the IVSs and random amplified polymorphic DNA subtypes of the *C. jejuni* isolates. Therefore, ABC efflux pump and other resistance mechanisms(s) may be involved in the resistance to Ery in the human clinical *C. jejuni* isolates examined in the present study.

Keywords Antibiotic resistance · Clinical *Campylobacter jejuni* · Erythromycin · Intervening sequences · 23S rRNA genes

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Introduction

Thermophilic *Campylobacter* species, primarily *C. jejuni* and *C. coli*, are curved Gram-negative bacteria which are a recognized cause of acute bacterial diarrhea worldwide. Although the genus *Campylobacter* is composed of 17–18 described species, human illness is associated primarily with *C. jejuni* and *C. coli* (Lastovica and Skirrow 2000; Debruyne et al. 2009).

Campylobacter enteritis is considered to be a zoonotic disease, and domestic animals such as poultry, cattle, and pigs can act as sources of infection (Moore et al. 2005). Recently, the acquisition of antibiotic resistance by *C. jejuni* and *C. coli* is of great public health concern. However, erythromycin (Ery) and other macrolides remain the leading choice for the treatment of severe *Campylobacter* infections (Nachamkin et al. 2000). In *C. jejuni* and *C. coli*, resistance to macrolides including Ery has been previously indicated to be associated with nucleotide mutations within the peptidyl

transferase region in V domain of the 23S rRNA (Trieber and Taylor 2000).

In addition, regarding bacterial 23S rRNA genes, the occurrence of intervening sequences (IVSs) (Burgin et al. 1990; Kordes et al. 1994; Conlan et al. 2005) has been demonstrated. In the genus *Campylobacter*, the helix 45 (central) region within 23S rRNA gene was found to carry the IVSs in two of four *C. jejuni*, in both *C. fetus*, and in one of two *C. upsaliensis* strains (Van Camp et al. 1993). IVS is common in *Campylobacter* spp. (59%; $n=21$ *C. jejuni* and $n=11$ *C. coli*) (Trust et al. 1994).

Most recently, among 104 strains of *C. coli* from turkeys, 69 strains harbored IVSs in all three 23S rRNA genes, whereas the other 35 strains lacked IVSs from at least one of the genes (Chan et al. 2007). In addition, Chan et al. (2007) suggested that the absence of IVS in *C. coli* is characteristic of a unique clonal group of Ery susceptible strains and that IVS can be acquired by these strains via natural transformation to Ery resistance (≥8 μg/ml) (Chan et al. 2007).

We have already reported the molecular identification and characterization of IVSs within 23S rRNA genes from more than 200 *Campylobacter* isolates from seven species, including atypical campylobacters (Tazumi et al. 2009). In that paper, we also described that 30 of 56 *C. jejeuni* isolates (54%) and 5 of 11 *C. coli* (45%) carried IVSs in the helix 45 region (Tazumi et al. 2009).

However, no description on the prevalence of IVSs in *C. jejuni* organisms isolated clinically from humans are available, and, moreover, no studies have appeared on the correlation between the nucleotide sequences of IVSs within 23S rRNA genes and Ery resistance in human isolates.

Therefore, the present study aims to clarify correlations between the nucleotide sequences of the V domain and the IVS within the 23S rRNA genes and Ery resistance, in human clinical *C. jejuni* isolates. The authors also aimed to classify 49 human clinical *C. jejuni* isolates by using subtyping procedure with random amplified polymorphic DNA (RAPD) analysis.

Materials and methods

Campylobacter isolates and culture conditions

Campylobacter jejuni isolates ($n=49$), which were isolated from human clinical sources in several countries of Asia, Europe, and North America were chosen randomly and examined in the present study and are detailed in Table 1. These *C. jejuni* isolates were cultured on Mueller–Hinton agar (Oxoid, Hampshire, England) containing 5% (v/v) defibrinated horse blood (Nippon Bio-Test, Tokyo, Japan) in microaerobic conditions [5% (v/v) O_2, 10% (v/v) CO_2 and 10% (v/v) H_2].

Determination of MICs

The determination of the MICs (2-168 μg/ml) of Ery was carried out using the agar dilution method, described by Graudreau and Gilbert (1997). To ensure reproducibility, MIC determinations were repeated three times. Ery-resistance was considered as MICs of ≥8 μg/ml (Gibreel and Taylor 2006; Kim et al. 2006: Chan et al. 2007), whereas intermediate resistance was defined as MIC≥2–<8 μg/ml. Susceptibility to erythromycin was defined as MIC<2 μg/ml.

Genomic DNA preparation, PCR amplification and nucleotide sequencing

Genomic DNA was prepared from organisms using a sodium dodecyl sulfate, proteinase K, and cetyltrimethylammonium bromide treatment, and followed by phenol-chloroform extraction and ethanol precipitation (Sambrook and Russell 2001).

In the present study, we designed a PCR primer pair of f-/r-CJV23 (f-CJV23, 5'-GTATAGGGTGTGACGCCTGCCC-3' and r-CJV23, 5'-AGCCAACCTTTGTAAGCCTCCG-3') to amplify the V domain within the 23S rRNA gene sequences (Gibreel and Taylor 2006). f-CJV23 corresponds to the sequences from the nucleotide positions (np) 43,400–43,421 base pairs (bp), 398,281–398,302 and 700,575–700,596, (*C. jejuni* NCTC11168, DDBJ/EMBL/GenBank NC_002163) (np 5,066–5,087 bp for *E. coli rrnB*, J01695) and r-CJV23 from the np 43,858–43,879 bp, 398,739–398,760 and 701,033–701,054 (*C. jejuni* NCTC 11168) (np 5,524–5,545 bp for *E. coli rrnB*), respectively. We have already designed a PCR primer pair of f-/r-Cl23h45, to amplify the helix 45 region within 23S rRNA gene sequences from the *Campylobacter* organisms (Tazumi et al. 2009).

The PCR was performed in 25-μl reaction volumes at 94°C for 5 min, 30 cycles at 94°C for 30 s 55°C for 30 s, 72°C for 30 s, and finally 72°C for 5 min. The PCR products, separated by 1% (w/v) agarose gel electrophoresis in 0.5× TBE, were purified using a QIAquick PCR Purification Kit (Qiagen, Tokyo, Japan). The purified fractions were subjected to cycle sequencing with BigDye Terminator (version 3.1; Applied Biosystems, Tokyo, Japan) and with sequencing primers. Sequence analysis was carried out using the GENETYX Windows software (v.9; GENETYX, Tokyo, Japan).

Subtyping of the *C. jejuni* isolates by using RAPD procedure

Subtyping of the 49 human clinical *C. jejuni* isolates by using RAPD procedure was carried out according to the procedure described by Hilton et al. (1997).

Table 1 Human clinical isolates of *C. jejuni* analyzed in the present study

No.	Isolate no.	Source (Country) (Disease)	Helix 45 IVS (Type)	Erythromycin (MIC)	RAPD
1	81-176	Human (USA)	+ (C)	S	1
2	HP5110	Human (Japan)	−	S	2
3	HP5122	Human (Japan)	−	S	2
4	LCDC4483	Human (Canada)	−	S	2
5	CF-85-46	Human (Japan)	−	S	3
6	HP5090	Human (Japan)	+ (C)	S	4
7	HP5095	Human (Japan)	± (B)	I	5
8	HP5100	Human (Japan)	+ (D)	S	6
9	HP5096	Human (Japan)	+ (C)	S	7
10	OH4382 (O19)	Human (Japan) (GBS)	+ (B)	S	4
11	86-357	Human (USA)	+ (A)	R (8 μg/ml)	8
12	86-389	Human (USA)	−	S	5
13	85-3	Human (USA)	+ (B)	I	9
14	D450 (O19)	Human (USA) (GBS)	+ (B)	S	4
15	D3083	Human (USA) (GBS)	+ (B)	R (8 μg/ml)	5
16	MHU003	Human (Japan)	+ (B)	R (16 μg/ml)	4
17	HP5117	Human (Japan)	−	S	5
18	011220	Unknown	−	R (8 μg/ml)	4
19	ST23	Human (USA)	−	I	4
20	CF85-48	Human (Japan)	+ (A)	I	4
21	HP5072	Human (Japan)	+ (B)	I	9
22	HP5083	Human (Japan)	−	S	10
23	HP5075	Human (Japan)	± (B)	I	4
24	HP4941	Human (Japan)	+ (A)	R (8 μg/ml)	5
25	LMG6444	Human (Belgium)	−	I	2
26	HP5084	Human (Japan)	+ (A)	S	5
27	84-196	Human (USA) (GBS)	−	R (8 μg/ml)	4
28	D3468 (O19)	Human (USA) (Diarrhea)	+ (B)	S	4
29	D3215 (O19)	Human (USA) (Diarrhea)	+ (B)	S	4
30	OH4384 (O19)	Human (Japan) (GBS)	+ (B)	S	4
31	D452 (GBS-19, O19)	Human (USA) (GBS)	+ (B)	S	4
32	D445 (O19)	Human (USA) (GBS)	+ (B)	S	4
33	D3141	Human (USA) (Diarrhea)	+ (B)	S	4
34	85-1	Human (USA) (Diarrhea)	−	S	4
35	84-198 (non GBS non19)	Human (USA) (Diarrhea)	−	S	8
36	84-194	Human (USA) (Diarrhea)	−	I	4
37	81116	Human (UK) (Diarrhea)	+ (D)	I	11
38	86-386	Human (USA) (Diarrhea)	−	S	5
39	84-191	Human (USA) (Diarrhea)	−	R (8 μg/ml)	5
40	D3002	Human (USA) (Diarrhea)	+ (B)	S	5
41	R	Human (UK) (Diarrhea)	+ (B)	I	5
42	O	Human (UK) (Diarrhea)	−	I	4
43	M	Human (UK) (Diarrhea)	−	I	4
44	L	Human (UK) (Diarrhea)	−	R (8 μg/ml)	2
45	CUM001	Human (USA) (Diarrhea)	+ (B)	S	5
46	79AH88-88	Human (France) (Diarrhea)	+ (A)	S	4
47	D3088	Human (USA) (Diarrhea)	+ (B)	S	5
48	88-84 (85 AC)	Human (France) (Diarrhea)	−	I	3
49	88-89 (85H)	Human (France) (Diarrhea)	+ (A)	I	2

C Campylobacter, IVS intervening sequence, MIC minimum inhibitory concentration,+ IVS positive, - IVS negative, RAPD random amplified polymorphic DNA, GBS Guillain Barre Syndrome, I intermediate, R resistant, S susceptible

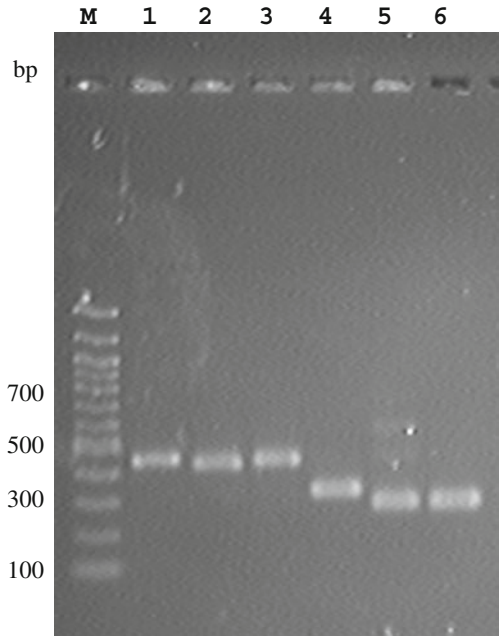

Fig. 1 Agarose gel electrophoresis analyses of PCR amplified products of helix 45 regions within 23S rRNA genes with human clinical *C. jejuni* isolates. *M* 100 bp DNA ladder; *lane 1 C. jejuni* 86-357 [Helix 45 IVS (Type) +(A)]; *lane 2*. 81116 [+(D)]; *lane 3* HP5090 [+(C)]; *lane 4* HP5100 [+(D)]; *lane 5* HP5095 [±(B)]; *lane 6* HP5122 [-]

Results and discussion

In the present study, we first determined the MICs of Ery with all 49 human clinical *C. jejuni* isolates, by using the agar dilution method, and the results are summarized in Table 1. Eight (16%) isolates were resistant to the Ery (8–16 μg/ml) and 14 (29%) had intermediate resistance. (MIC 2–<8 μg/ml) (Table 1).

We then carried out PCR amplification of the V domain within the 23S rRNA gene sequences by using a primer pair of f-/r-CJV23 with the 49 *C. jejuni* isolates (data not shown), nucleotide sequencing and alignment analyses. Consequently, following nucleotide sequence alignment analyses, no point mutations were identified to occur at any expected or putative positions for the Ery resistance (*Escherichia coli rrnB* np 5,308 bp, A → G and np 5,309 bp, A → G) (J01695; Brosius et al. 1978) in the V domain within the 23S rRNA gene sequences from all the eight *C. jejuni* Ery-resistant isolates examined (data not shown).

When PCR was then carried out with the 49 isolates using a primer pair of f-/r-Cl23h45 to amplify the helix 45 regions within the 23S rRNA gene sequences, amplicons were generated with all the isolates. Electrophoretic profiles of PCR amplicons are shown in Fig. 1. These may differ in size due to variation in IVSs, as well as in their presence/absence. Following nucleotide sequencing and alignment analyses, 29 *C. jejuni* isolates (59%) were shown to carry IVSs in the helix 45 region (Table 1). Four kinds of sequence data of IVSs from 29 *C. jejuni* isolates were aligned in Fig. 2 for comparison, although these were already shown elsewhere (Tazumi et al. 2009), and the IVS types (A–D) are shown in Table 1. Thus, only four *C. jejuni* isolates (86-357, D3083, MHU003, and HP4941; 14%) of 29 isolates which carryied IVSs in the helix 45 region were resistant to Ery. Type E sequence lacking IVSs (*C. jejuni* HP5110, LCDC4483 and 011220) was also shown for comparison. In addition, when we examined 27 human clinical *C. jejunmi* isolates, which were susceptible to erythromycin, 17 of these were shown to harbor IVSs in the helix 45 region (Table 1). This is in marked contrast to the findings of Chan et al. (2007), who examined *C. coli* isolates.

All 49 human clinical *C. jejuni* isolates were subtyped to the genotype level using RAPD analysis and the isolates were classified into 11 subtypes, as shown in Table 1. Representative examples of RAPD profiles are shown in Fig. 3.

Fig. 2 Nucleotide sequence alignment analyses in the helix 45 region within 23S rRNA gene sequences from human clinical *C. jejuni* isolates. Analyses of IVS type A, B, C, and D were carried out. Isolates lacking IVSs (E) were also analyzed for comparison. *Numbers at the left and right* refer to the nucleotide positions determined. *Dots* indicate identical bases; changes are explicity indicated; *dashes* are deletions; identical positions in all cases are marked by *asterisks*

```
A    1:ACTTGCACACAACTTAGATTATTTAAGTTTAGAATATGAGAAACTAAGTTATATGTTTAG 60
B    1:......G.....................................................-..... 58
C    1:......G.....................................................----------.. 48
D    1:.....................................-------------------------- 35
E    0:------------------------------------------------------------ 0

A   61:TTATATTTTTACTGATTTTTATAGAGTAAAGATAGAAATAAAACTTAGTAAAATCAGTAA 120
B   59:..................................................................... 118
C   49:..................................................................... 108
D   35:------------------------------------------------------------ 35
E    0:------------------------------------------------------------ 0

A  121:AAATATTCTTAGACTAAAGTTAAGTAGTTTAAGTTGTGTGC-AAGT                165
B  119:............G.............................-....                163
C  109:............G.............................-....                153
D   36:---------------------.....................-....                59
E    1:---------------------...CT..AG.CT.A..T.A.T....                25
                             ***  **  *  * ** * * ****
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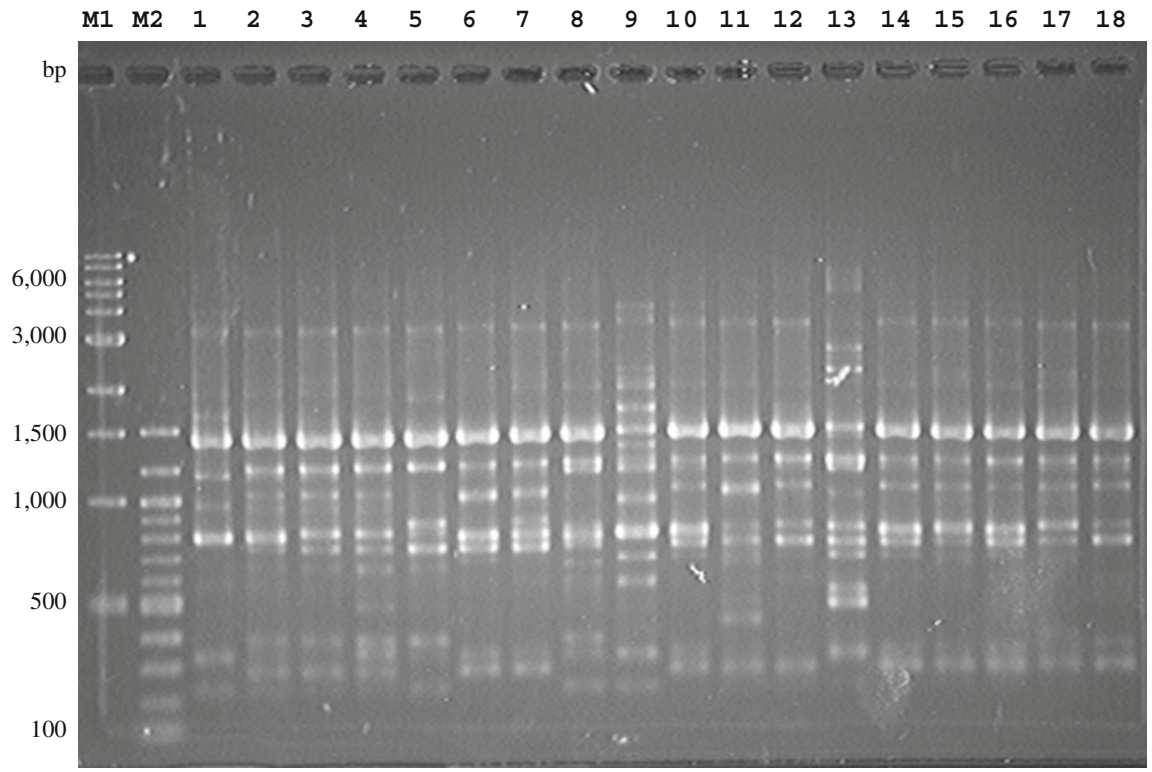

Fig. 3 RAPD profiles obtained from isolates of the human clinical *C. jejuni* isolates by using a primer of the 1254 (5'-CCGCAGCCAA-3') (Hilton et al. 1997). *Lane M1* 1 kbp ladder; *lane M2* 100 bp ladder; *lane 1 C. jejuni* 81-176; *lane 2* HP5110; *lane 3* HP5122; *lane 4* LCDC4483; *lane 5* CF-85-46; *lane 6* HP5090; *lane 7* HP5095; *lane 8* HP5100; *lane 9* HP5096; *lane 10* OH4382 (O19); *lane 11* 86-357; *lane 12* 86-389; *lane 13* 85-3; *lane 14* D450 (O19); *lane 15* D3083; *lane 16* MHU003; *lane 17* HP5117; *lane 18* 011220

Previously, Chan et al. (2007) have indicated that the majority (66/69) of *C. coli* strains from turkeys in North Carolina that harbored IVSs in all 23S rRNA genes were resistant to Ery (≥8 μg/ml), whereas none of the 35 strains with at least one IVS-free 23S rRNA gene were resistant (Chan et al. 2007). They also suggested that the absence of IVS in *C. coli* from turkeys is characteristic of a unique clonal group of Ery-susceptible strains (Chan et al. 2007).

In the present study, however, no correlations occurred between the nucleotide sequences of the IVSs within the 23S rRNA genes and Ery resistance in the 49 human clinical *C. jejuni* isolates obtained in Asia, Europe, and North America. In addition, in the eight Ery-resistant clinical *C. jejuni* isolates, no point mutations were identified at the expected and putative positions in the V domain within the 23S rRNA gene sequences for Ery resistance.

Thus, no correlations between the nucleotide sequences of the V domain and the IVSs within the 23S rRNA genes and Ery resistance occurred in human clinical *C. jejuni* isolates. Although, in the present study, eight clinical *C. jejuni* isolates were shown to be resistant to Ery (approximately 16%), no point mutations occurred at any expected and putative positions in the V domain within the 23S rRNA gene sequences. Thus, although possible correlations between the multilocus sequence typing-based sequence types, the presence of IVSs, and the Ery susceptibility of *C. coli* from turkeys have already been described (Chan et al. 2007), no correlations were identified between the occurrence of IVSs within the 23S rRNA genes and the RAPD subtypes in the 49 human clinical *C. jejuni* isolates examined in the present study.

C. jejuni and *C. coli* strains that grew on media amended with 8 μg/ml of Ery have been classified as resistant (Kim et al. 2006; Chan et al. 2007). In addition, it has already been described that the Ery-resistant *Campylobacter* isolates could be divided into two groups: low level (LLR; Ery MICs ranging from 8 to 16 mg/l with no mutation in the target gene) and high level (HLR; Ery MICs over 128 mg/l, in which a point mutation was always detected in the 23S rRNA gene) (Gibreel and Taylor 2006). Our present results are consistent with the former observation. Therefore, ABC efflux pump and other resistance mechanisms may be involved in the resistance to Ery in the human clinical *C. jejuni* isolates examined in the present study, as already described (Payot et al. 2004).

In conclusion, following nucleotide sequencing and alignment analyses, no correlations between the nucleotide sequences of the V domain and the IVSs within the 23S rRNA gene sequences and Ery resistance occurred in the human clinical *C. jejuni* isolates examined. In addition, no point mutations were identified to occur at any expected and

putative positions in the V domain within the 23S rRNA gene sequences in the clinical isolates. Also, no correlations were identified between the occurrence of the IVSs and the RAPD subtypes.

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