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ORIGINAL ARTICLE

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Antimicrobial susceptibility and mechanism of quinolone resistance in *Campylobacter jejuni* strains isolated from diarrheal patients in a hospital in Tokyo

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Abstract We determined the minimum inhibitory concentrations of six types of antimicrobial agents for 523 strains of *Campylobacter jejuni* that were isolated from diarrheal patients in a general hospital in Tokyo during the period between 2003 and 2005. It was revealed that 20.2%, 22.9%, 6.7%, and 0.6% of all the *C. jejuni* strains tested were resistant to ciprofloxacin (CPFX), nalidixic acid, ampicillin, and fosfomycin, respectively. All the strains were susceptible to clarithromycin and erythromycin. To elucidate the mechanism of quinolone resistance, in a total of 55 strains selected randomly, we carried out sequence determination and analysis of the quinolone-resistance determining regions (QRDRs) of their *gyrA* and *gyrB* genes. Amino-acid substitution at codon 86 (Thr → IIe) of GyrA was found in all the 37 CPFX-resistant strains. There was no amino-acid substitution in the QRDR of the *gyrB* gene. All of the genomic DNAs of these 55 strains showed distinct pulsed-field gel electrophoresis patterns. Taken together, these results suggested that the quinolone resistance of *C. jejuni* was attributable mainly to the mutation at codon 86 (Thr → IIe) in the QRDR of GyrA, and that this particular mutation and other silent mutations could be found not only in a certain clone of *C. jejuni* but also universally in a wide variety of strains.

Key words *Campylobacter jejuni* · Quinolone · Drug resistance · DNA gyrase · Pulsed-field gel electrophoresis (PFGE)

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Introduction

Campylobacter jejuni can be isolated from a large variety of animals, such as avian species and cattle, and it is known to cause diarrheal diseases and sepsis in humans.[1] The number of infectious diseases caused by *C. jejuni* has been increasing in many countries;[2,3] in Japan, a steady increase has also been shown over the past few years (http://www.mhlw.go.jp/topics/syokuchu/). Sagara et al.[4] reported that, in Japan in the 1980s, no ciprofloxacin-resistant strains were found in *C. jejuni* strains isolated from patients with diarrheal diseases. However, recently, it has been reported that *C. jejuni* strains isolated from three geographically distinct regions in Japan showed a high rate of resistance to quinolone agents.[5] Furthermore, the detection rate of quinolone-resistant strains has continued to increase in other countries, although the rate varies from country to country. Therefore, increasing attention has been paid to the acquisition of resistance to quinolone agents by *C. jejuni* strains.[6,7] The mechanism of action of fluoroquinolones against bacteria is through their specific binding to type II topoisomerase, which cuts and reanneals double-stranded DNA, thereby inhibiting the cutting of the DNA and the reannealing of the separated DNA.[8,9] Type II topoisomerases include DNA gyrase, which functions in the early phase of DNA replication, and topoisomerase IV, which is composed of ParC and ParE subunits and functions in the last phase of DNA replication.

The mechanisms of bacterial resistance to quinolone agents include the following: (i) a decrease in the affinity of quinolone agents to their target molecules, i.e., DNA gyrase (GyrA, GyrB) and topoisomerase IV (ParC, ParE), through amino-acid alteration in these enzymes;[8,9] and (ii) a decrease in the membrane permeation of quinolone agents, which occurs as a result of the upregulation of bacterial inner membrane proteins working as efflux pumps of drugs and/or the downregulation of a bacterial outer membrane protein, called porin, involved in the transport of substances into the bacterial body.[10,11] Recently, many reports have suggested that mutations in quinolone-resistance determin-

ing regions (QRDRs) of DNA gyrase genes are substantially involved in the bacterial acquisition of resistance to quinolone agents.[12–16]

In the present study, we carried out drug susceptibility tests of various drugs in *C. jejuni* strains isolated from patients with recent diarrheal diseases. In addition, in strains resistant to quinolones, we investigated the presence of mutations in their *gyrA* and *gyrB* genes. Furthermore, we performed pulsed-field gel electrophoresis (PFGE) analysis for mutant strains having amino-acid substitution in GyrA to determine whether or not they were of the same lineage.

Materials and methods

Bacterial strains used in the present study

C. jejuni strains were isolated from stool specimens of patients with diarrheal diseases who visited a general hospital in Tokyo during the 3-year period between January 2003 and December 2005. A total of 523 *C. jejuni* strains (132 strains in 2003, 196 strains in 2004, and 195 strains in 2005), as well as *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used in the present study.

Antimicrobial susceptibility test

The minimal inhibitory concentrations (MICs) of various antimicrobial agents were determined by the agar plate dilution method, according to the standard protocol of the Clinical Laboratory Standards Institute (CLSI). The antimicrobial agents used were ciprofloxacin, fosfomycin, ampicillin (CPFX, FOM, ABPC; Meiji Seika Kaisha, Tokyo, Japan), nalidixic acid (NA; Wako Pure Chemical, Osaka, Japan), clarithromycin (CAM; Taisho Pharmaceutical, Tokyo, Japan), and erythromycin (EM; Shionogi, Osaka, Japan). The bacterial strains were initially cultured in Trypticase soy agar (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 5% sheep blood for 48 h under microaerobic conditions (5% oxygen) at 37°C and were suspended in Mueller-Hinton broth (Becton Dickinson) to McFarland turbidity no. 0.5. Twofold serial dilutions of the individual antimicrobial of interest were prepared and the resulting solutions were added to Mueller-Hinton agar (Becton Dickinson) supplemented with 5% sheep blood. The 10-fold dilution of bacterial suspensions (5 µl) were inoculated onto the obtained agar media containing the intended final concentrations of each drug. MICs were determined following a 48-h culture of the bacterial strains under microaerobic conditions at 37°C.

Polymerase chain reaction (PCR)

Partial gene amplification was carried out in the QRDRs of chromosomal DNA. Bacterial strains (one platinum loop) were suspended in sterilized water (300 µl) and heated at 100°C for 10 min, and then used as templates. Oligonucleotide primers for the *gyrA* gene were synthesized based on our own design and those for the *gyrB* gene were synthesized in reference to the report by Fukushima et al.[17] The *parC* primers described by Gibreel et al.[7] were also used (Table 1). Premix *Taq* (Takara Bio, Shiga, Japan) was used as DNA polymerase. Template DNA (1 µl) was added to the PCR reaction mixture following preparation of the mixture at the recommended concentration. DNA amplification was performed in a PCR Thermal Cycler Dice (Takara Bio) with the following parameters: predenaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C (*parC*) or 55°C (*gyrA* and *gyrB*) for 30 s, and extension at 72°C for 1 min, plus final extension at 72°C for 10 min.

Sequence determination

To analyze the base sequences of QRDRs, cycle sequence was carried out using the BigDye Terminator v1.1 Cycle Sequence kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. PCR products (1 µl) obtained as above were used as samples. Forward primers for *gyrA* and *gyrB* were added to the reaction mixture at a final concentration of 3.2 µM. Reactions were carried out in the PCR Thermal Cycler Dice (Takara Bio) with the following parameters: predenaturation at 96°C for 5 min; 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 20 s,, and extension at 60°C for 4 min. Subsequently, dyes were removed using AutoSeq G-50 (GE Healthcare UK, Amersham, Buckinghamshire, UK) followed by sequencing analysis in an automated DNA sequencing system (ABI PRISM 3130xl; Applied Biosystems, Foster City, CA, USA). A similarity search for the deduced amino-acid sequences against DDBJ/EMBL/GenBank sequence databases was conducted using the

Table 1. Primers used in this study

Target gene	Primer name	Oligonucleotide sequence (5′-3′)	GenBank accession no.
gyrA	gyrA-F	GCCTGACGCAAGAGATGGTT	This study
	gyrA-R	TTTGTCGCCATACCTACAGC	
gyrB	gyrB-F	AGCTAGAGAATTAACGCGCA	AB084064
	gyrB-R	ACAGAAATCACATTAAAACGC	
parC	parC-F	AAACCTGTTCAGCGCCGCATT	Gibreel et al.[7]
	parC-R	GGTTATGCGGTGGAATATCG	