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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