The Dynamics of Antimicrobial-Resistant *Campylobacter jejuni* on Japanese Broiler Farms

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**ABSTRACT.** We investigated for dynamics of *Campylobacter* clones on 2 different managerial broiler farms. *Campylobacter* isolates were differentiated by resistance typing and molecular typing methods. On farm I, the same *C. jejuni* clones resistant to fluoroquinolone and oxytetracycline were isolated after one and half years again and another susceptible clone was invaded. The susceptible clone was isolated again after half year. Broiler flocks on the farm may be repeatedly infected with a few *C. jejuni* clones. On farm II, new clones including antimicrobial resistant one, were often invaded. The change of predominant *C. jejuni* clone in each flock on both the farms was observed, in the absence of antimicrobial selective pressure.

**KEY WORDS:** antimicrobial resistance, broiler chicken, *Campylobacter jejuni.*

**NOTE** Public Health

Thermotolerant *Campylobacter* frequently causes campylobacteriosis, which is an enteric disease in humans. Most cases of campylobacteriosis are epidemiologically linked to the consumption of poultry products [10]. Molecular typing methods, such as flagellin gene typing (fla-typing) [11, 15] and pulsed-field gel electrophoresis (PFGE) [3, 19], have been applied to discrimination among *Campylobacter* isolates. However, the transmission routes of *Campylobacter* spp to chickens have not clearly been defined [1, 9, 13, 17, 22]. Persistence of specific *Campylobacter* clones on broiler farms through successive flock rotations has been reported [14, 16].

An increase in the number of *Campylobacter* isolates resistant to fluoroquinolone (FQ) in patients and animals has been reported [2, 18, 21, 23, 25, 27, 28]. Several epidemiological studies have indicated a relationship between the use of FQ in veterinary medicine and an increase in FQ-resistant *Campylobacter* [2, 23, 25].

The Japanese Veterinary Antimicrobial Resistance Monitoring Program (JVARM) started in 1999 [26], has been monitoring changes in antimicrobial resistance of bacteria including *Campylobacter* spp [6]. In the present study, longitudinal investigation was performed on two broiler farms in order to identify the persistent specific clones.

Two broiler farms (Farms I and II), where FQ-resistant *C. jejuni* isolates were obtained, although no quinolone had been used according to JVARM in 2001, were selected. On both the farms, following slaughter, the litter was removed and the houses were cleaned, disinfected and left to dry; subsequently, new chicks were stocked.

Farm I is a domestic industry and has eight open-sided chicken houses. Farm I was never empty even though each house was empty for a period of one month or longer, prior to stocking new chicks. For growth promotion, the feed was supplemented with colistin and enramycin until the chicks became 21-days-old, and with colistin, enramycin and salinomycin from the 22-days-old to approximately 8-weeks-old. However, antimicrobial agents have not been used for therapy at least for 2 years and quinolones have never been used until now.

Farm II is a commercial industry and has a total of 18 chicken houses. Two chicken houses were windowless and two-storied. The other 16 chicken houses were open-sided and one-storied. New chicks were stocked to each chicken house in rotation at around the same time. The entire farm was empty for approximately 3 weeks. The investigation on farm II was performed in a windowless house (No. II-O). The antimicrobial agents for growth promotion or therapy were never administered to the flocks in windowless houses.

On farm I, from May to July 2003, 10 fecal samples of flock 2 from a chicken house (No. I-C) were collected at 3-week intervals, from the time of stocking to the time of slaughter. In January 2004, 10 fecal samples of each of six flocks (flocks 3 to 8) in each chicken house (Nos. I-A to I-F) were collected; the remaining two chicken houses were empty.

On farm II, from April to June 2003, 10 or more fecal samples of flock 2 were collected from the chicken house (No. II-O) at approximately 3-week intervals, from the time of stocking to the time of slaughter. From July to August 2003 (flock 3) and in January 2004 (flock 4), 5 or 10 fecal samples were collected twice or 3 times from the same chicken house.
The *Campylobacter* spp. were isolated by direct inoculation to modified CCDA (Oxoid, UK) [6]. Isolation was performed by inoculation for enrichment in Preston broth (Oxoid, UK) only during the period from July to August 2003. Preston broth incubated for 24 hr at 37°C was streaked on modified CCDA. Farm I yielded 70 to 100% of *Campylobacter* in the feces of chickens that were either 3-weeks old or older. Farm II yielded 60 to 100% of *Campylobacter* isolates in the feces of chickens that were either 5-weeks-old or older (Table 1). All isolates were identified as *C. jejuni* by PCR [8].

The minimal inhibitory concentration (MIC) determinations were carried out on Mueller-Hinton agar (MHA, Oxoid, UK) supplemented with 5% sheep blood in accordance with the NCCLS guidelines [12]. The plates were incubated for 48 hr at 37°C in a microaerobic atmosphere consisting of 85% N2, 10% CO2 and 5% O2 (O2-CO2 Incubator CPO 1800 series, Hirasawa Co., Ltd., Tokyo). *C. jejuni* ATCC33560 and *C. coli* ATCC33559 were used for quality control. The breakpoints were the same as those adopted by JVARM: for nalidixic acid, ≥32 µg/ml; for enrofloxacin, ≥2 µg/ml; for oxytetracycline, ≥16 µg/ml; for dihydrostreptomycin, ≥32 µg/ml; and for erythromycin, ≥32 µg/ml. Since MICs of colistin for *Campylobacter* were not tested by JVARM, the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) breakpoint, being ≥64 µg/ml for colistin [4] was used.

*C. jejuni* isolates obtained on the two farms in the present study and JVARM in 2001 were discriminated by flaA-typing and PFGE. In general, one isolate per sample was tested; however, when the isolates showed different resistance patterns, two isolates from one fecal sample were tested. The complete nucleotide region (1,728 bp) of the flagellin gene (*fla A*) was amplified by PCR, as previously described [1], with some modifications [7]. Ten micro litres of PCR prod-

### Table 1. Changes of resistance patterns and genotypes of *C. jejuni* isolates

<table>
<thead>
<tr>
<th>Farm</th>
<th>No. of the chicken house</th>
<th>Flock</th>
<th>Time</th>
<th>Age</th>
<th>Rate of isolation</th>
<th>Resistance pattern</th>
<th>fla A type</th>
<th>PFGE type</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>unknown</td>
<td>1b)</td>
<td>Sep. 2001</td>
<td>70-day</td>
<td>1/1</td>
<td>NA, ERFX, OTC (1)²</td>
<td>A1 S1 (1)³</td>
<td></td>
</tr>
<tr>
<td>I-C</td>
<td>2</td>
<td>May-July 2003</td>
<td>2005</td>
<td>0-day</td>
<td>0/10</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-week</td>
<td>8/10</td>
<td>NA, ERFX, OTC (8)</td>
<td>A1 S1 (7), S2 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-week</td>
<td>9/10</td>
<td>NA, ERFX, OTC (5)²</td>
<td>A1 S2 (5), S3 (5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-A</td>
<td>3</td>
<td>Jan. 2004</td>
<td>2-day</td>
<td>0/10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>I-B</td>
<td>4</td>
<td>Jan. 2004</td>
<td>2-week</td>
<td>0/10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>I-C</td>
<td>5</td>
<td>Jan. 2004</td>
<td>5-week</td>
<td>9/10</td>
<td>Susceptible (9)</td>
<td>A2 S3 (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-D</td>
<td>6</td>
<td>Jan. 2004</td>
<td>7-week</td>
<td>10/10</td>
<td>Susceptible (10)</td>
<td>A2 S3 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-E</td>
<td>7</td>
<td>Jan. 2004</td>
<td>9-week</td>
<td>7/10</td>
<td>Susceptible (7)</td>
<td>A2 S3 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-F</td>
<td>8</td>
<td>Jan. 2004</td>
<td>11-week</td>
<td>10/10</td>
<td>Susceptible (10)</td>
<td>A2 S3 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>unknown</td>
<td>1b)</td>
<td>Sep. 2001</td>
<td>32-day</td>
<td>1/1</td>
<td>NA, ERFX, OTC, DSM (1)</td>
<td>A7 S11 (1)</td>
<td></td>
</tr>
<tr>
<td>II-O</td>
<td>2</td>
<td>Apr.-Jun. 2003</td>
<td>3-day</td>
<td>0/10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-week</td>
<td>0/13</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-week</td>
<td>16/17</td>
<td>Susceptible (1)</td>
<td>A3 S7 (1)</td>
<td>A4 S4 (11), S5 (2), S6 (1), S8 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-week</td>
<td>15/15</td>
<td>Susceptible (11)²</td>
<td>A3 S7 (11), S4 (5), S6 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>July-Aug. 2003</td>
<td>5-week</td>
<td>9/10</td>
<td>Susceptible (9)</td>
<td>A3 S7 (9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-week</td>
<td>8/10</td>
<td>Susceptible (3)</td>
<td>A3 S7 (3)</td>
<td>A5 S9 (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>Jan. 2004</td>
<td>5-week</td>
<td>3/5</td>
<td>Susceptible (3)</td>
<td>A6 S10 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-week</td>
<td>5/5</td>
<td>Susceptible (5)</td>
<td>A6 S10 (5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-week</td>
<td>5/5</td>
<td>Susceptible (5)</td>
<td>A6 S10 (5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Nalidixic acid (NA), enrofloxacin (ERFX), oxytetracycline (OTC), and dihydrostreptomycin (DSM).
b) Each flock 1 was studied on JVARM in 2001.
c) (), Number of fecal samples which isolates of the resistance pattern or PFGE type were obtained from.
d) Isolates of one fecal sample showed different resistance patterns or PFGE types.
ucts were digested with 1 U of DdeI (Invitrogen, U.S.A.) at 37°C, and the digested DNA was analysed by electrophoresis in 4% NuSieve 3:1 agarose gel (Cambrex Bio Science Rockland, Inc. U.S.A.) at 100 V. The procedures of restriction endonuclease digestion with Smal and PFGE were described previously [19], and were used with minor modifications. The electrophoresis condition was an initial switch time of 6.8 s and a final switch time of 38.4 s (gradient 6 V/cm and an included angle of 120°) at 14°C for 20 hr in a CHEF-DR III System (Bio-Rad, U.S.A.). The gel was stained with ethidium bromide and photographed using the GelDoc 2000 System (Bio-Rad, U.S.A.).

Seven different fla A types and 11 different PFGE types were identified. All isolates were susceptible to erythromycin and colistin. Most C. jejuni isolates that were resistant to nalidixic acid, enrofloxacin and oxytetracycline were from flock 2 on farm I, had A1 type of fla A and S1 or S2 type of PFGE (Table 1). Similarly, the FQ-resistant isolates from flock 1 on farm I, determined by JVARM in 2001, had A1 and S1 genotypes. All isolates susceptible to all antimicrobial agents tested from farm I had A2 type of fla A and S3 type of PFGE. Two isolates from a 9-week-old chicken on farm I were resistant to nalidixic acid, enrofloxacin and oxytetracycline and had A2 type of fla A and S2 type of PFGE. One susceptible isolate had the same genotype as Wedderkopp et al. reported [29].

Twenty-four C. jejuni isolates that were susceptible to all the antimicrobial agents tested from flocks 2 and 3 on farm II had A3 type of fla A and S7 type of PFGE. Thirteen susceptible C. jejuni isolates obtained from flock 4 had A6 type of fla A and S10 type of PFGE. Twenty-one oxytetracycline-resistant isolates from flock 2 on farm II had A4 type of fla A and S4, S5, S6 or S8 type of PFGE. Five C. jejuni isolates resistant to nalidixic acid, enrofloxacin and oxytetracycline obtained from flock 3 had A5 type of fla A and S9 type of PFGE. FQ-resistant isolates obtained by JVARM in 2001 had A7 type of fla A and S11 type of PFGE (Table 1). At least 7 clones were observed among three flocks on farm II during 9 months. It was believed that there would be several sources and vehicles such as wild birds and vermin of Campylobacter around farm II [24].

The present studies on the two farms commonly showed the change of predominant C. jejuni clones in each flock. A similar change of the predominant clone [13] and the colonisation phenotypes [20] in chickens were reported. Humphrey et al. [5] have reported that a small proportion of FQ-resistant Campylobacter spp. was observed on broiler flocks prior to treatment and the percentage of FQ-resistant isolates increased during treatment with FQ. The FQ-resistant Campylobacter subtypes which emerged either during or after treatment did not necessarily belong to the same subtypes which had been prevalent before treatment [5].


