Colonization of chicken flocks by *Campylobacter jejuni* in multiple farms in Japan

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**ABSTRACT** *Campylobacter jejuni* was monitored in 4 chicken farms during the period 2003 to 2006 to elucidate the mechanisms of transmission. Three farms (1 to 3), located at least 14 km from each other, belonged to an integrated poultry company, which also provided the farms with day-old chicks from several hatcheries as well as chicken feed. Another farm (4), which belonged to a different company, was located 270 m from farm 1. A total of 206 *C. jejuni* isolates obtained from the 4 farms were classified into 10 flaA-based RFLP types. Identical RFLP types were found in isolates obtained from chickens originating from multiple hatcheries and reared in different chicken houses on individual farms. Flocks were colonized by strains with 1 or 2 RFLP types in each production cycle, sometimes differing between cycles. Identical RFLP types were found in isolates obtained from the environment around the chicken houses. Using multilocus sequence typing, strains with different RFLP types could be distinguished from each other. Identical RFLP and multilocus sequence typing profiles were found in isolates obtained from farms 1 and 4, and from farms 1 and 2. These results suggest that *C. jejuni* in these farms comes from common sources external to the farms, even if the farms belong to different companies and obtain chicks from different suppliers.

**Key words:** *Campylobacter jejuni*, chicken, farm, flaA, multilocus sequence typing

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**INTRODUCTION**

*Campylobacter jejuni* is a common cause of foodborne acute enteritis in many countries. Previous studies indicate broiler meat as one of the possible sources of infection with *C. jejuni* (Cogan et al., 1999). Thus, a reduction of contamination of poultry products with *C. jejuni* may be critical for prevention of human exposure to the organism (van de Giessen et al., 1998). *C. jejuni* is often found to colonize broiler chickens as a commensal organism without the development of clinical symptoms (Beery et al., 1988). Horizontal transmission is generally considered as the most significant cause of *C. jejuni* colonization in broiler flocks. The environment can also be a source of campylobacters colonizing housed broiler flocks (Jacobs-Reitsma et al., 1995; Bull et al., 2006; Workman et al., 2008). The importance of vertical transmission from parent flocks is unclear (Ring et al., 2005).

Molecular characterization of *C. jejuni* strains is useful to trace the spread of the organism. Sequencing analyses of the flaA gene of *C. jejuni* suggests that contamination of carcass samples collected in the processing environment originated primarily from the intestinal contents of the broiler flock (Hiett et al., 2007). Multilocus sequence typing (MLST) of *C. jejuni* isolates has revealed the potential involvement of transport equipment and poultry transport crates in the transmission of this organism between flocks (Patriarchi et al., 2011) and through the poultry-processing plant (Hastings et al., 2011).
In the present study, we isolated *C. jejuni* in multiple farms that grew flocks of meat chickens. Two of the farms, which were located in close proximity, obtained chicks from different suppliers and reared the birds to market size using chicken feed produced in different companies. To determine possible transmission between the 2 farms, the strains were characterized using flaA-based RFLP (Chuma et al., 1997) and multilocus sequence typing (MLST) analyses (Dingle et al., 2001). One of the 2 farms above belonged to an integrated poultry company together with other farms that were situated more than 14 km away from each other. Because *C. jejuni* was also isolated in those farms, the isolates were characterized to investigate transmission of the organism between farms.

**MATERIALS AND METHODS**

**Chicken Farms**

Four farms in Japan were monitored for *C. jejuni* during the period between July 2003 and December 2006. Commercial broiler farms 1, 2, and 3 belonged to an integrated poultry company and obtained chicks from 6 hatcheries (F, I, K, M, S, and Y) that also belonged to the poultry company. In each production cycle, day-old chicks were brought from multiple hatcheries and placed in those farms and reared for 47 to 58 d. Chicken feed in these farms was supplied through this company. Employees of the company occasionally visited these 3 farms to inspect birds and hygienic conditions of the farms and to help the farmers at the time of the grow-out. Birds in farm 1 were reared in 13 conventional single-storied chicken houses and two 2-storied windowless houses. In farm 2 where all broilers were reared in 12 windowless houses, fecal samples were collected in 2 out of the 12 houses. At farm 3, 2 out of 5 conventional houses were monitored. Farm 4 belonged to another company, and day-old chicks were brought from another hatchery (hatchery B) that had never supplied chicks to farms 1, 2, or 3. Farm 4 had 12 conventional houses where birds were reared for 82 to 85 d. These flocks consisted of hybrids obtained from crossing local brands with Japanese native breeds, which must be reared more than 80 d according to the Japanese Agricultural Standard System. Between production cycles, litters from chicken houses were removed after grow-out and the houses and equipment were cleaned, washed using water, and dried, followed by disinfection. In farm 1, iodine, potassium monopersulphonate triple salt-based disinfectant, and cresol were used in turn. Chicken houses in farm 2 were disinfected using a mixture of didecyldimethylammonium chloride, 1,2-dichlorobenzene, 4-chloro-3-methylphenol. In farm 3, 1,2-dichlorobenzene and potassium monopersulphonate triple salt-based disinfectant were used in turn. In farm 4, houses were disinfected using cresol, and then didecyldimethylammonium chloride or glutaraldehyde were used.

Farms 1 and 4 were located within 270 m of each other, separated by woods, a field, and a rice field. Both farms had their own incinerators to destroy chicken manure. Farms 2 and 3 were 14 and 30 km, respectively, southeast of farm 1.

**Collection of Fecal Samples**

To grasp overall contamination of flocks in farm 1, fecal samples during production cycles 1 and 2 were collected in multiple chicken houses once a week when birds were 13 to 58 d of age (Table 1). Because *C. jejuni* was isolated when these birds reached 34 to 39 d of age (see Results), samples in cycles 3 to 9 (farm 1) and cycle 10 (farm 2) and cycle 11 (farm 3) were collected after the age of 31 d. Collection of samples was allowed once during each of the cycles in farms through 2 to 4. The surface of freshly dropped feces that did not touch the ground from 5 birds in each of the chicken houses was collected. Fecal samples were kept in Cary-Blair medium at 4°C during transportation to the laboratory for 2 to 4 h.

**Isolation of C. jejuni from Fecal Samples**

Each of the samples was spread onto modified charcoal cefoperazone deoxycholate agar (mCCDA) plates consisting of *Campylobacter* blood-free selective agar base CM0739 (Oxoid Ltd., Cambridge, UK) and CCDA selective supplement SR0155 (Oxoid), and incubated at 37°C for 48 h under microaerobic atmosphere using AnaeroPack Campylo (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan). Two suspect colonies were picked from a plate and screened for *Campylobacter* species by Gram stain, the presence of oxidase, and the absence of growth under aerobic conditions. *Campylobacter jejuni* was identified by the hippuricase gene-based PCR assay (Linton et al., 1997). Boiled lysate was used as the amplification template. Bacteria were suspended in 500 μL of sterile distilled water, heated at 100°C for 10 min, and stored at −20°C until use.

**Isolation of C. jejuni from Environmental Samples**

In farm 1, environmental samples were collected during the period between April 2003 and December 2006 as follows: intestinal contents obtained from 28 rodents captured in 9 houses, 3 insects in 2 houses, a lizard in one house, 11 fecal samples of rodents in 9 houses, 1 fecal sample from a dog and a cat found nearby 2 houses, 25 fecal samples of wild birds nearby 4 houses; 30 feed samples, 6 litter samples, 63 samples of drinking water from pails in 3 houses, 6 dust samples collected in 2 houses; 15 soil samples obtained nearby 6
Table 1. Isolation of *Campylobacter jejuni* from fecal samples obtained on chicken farms and flaA typing results

<table>
<thead>
<tr>
<th>Farm</th>
<th>Production cycle</th>
<th>Hatchery</th>
<th>Isolation by house</th>
<th>Isolation by feces</th>
<th>Rearing period</th>
<th>Date of sampling and the isolation results</th>
<th>Ages (d) of birds positive for <em>C. jejuni</em></th>
<th>flaA type (no. of isolates tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>I</td>
<td>2/2</td>
<td>57/140</td>
<td>Jul. 4 to Sep. 4, 2003</td>
<td>Jul. 17</td>
<td>34-55</td>
<td>A3 (78), A5 (33)</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td></td>
<td>1/1</td>
<td>8/15</td>
<td>Sep. 2 to Nov. 2, 2004</td>
<td>Oct. 7*</td>
<td>34-39</td>
<td>A8 (12)</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td></td>
<td>1/4</td>
<td>1/20</td>
<td>Feb. 26 to Apr. 20, 2005</td>
<td>Jan. 18*</td>
<td>51</td>
<td>A3 (2)</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td></td>
<td>1/1</td>
<td>5/5</td>
<td>Jul. 12 to Sep. 12, 2005</td>
<td>Aug. 18*</td>
<td>45</td>
<td>A10 (2)</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td></td>
<td>1/1</td>
<td>5/5</td>
<td>Oct. 12 to Dec. 5, 2005</td>
<td>Aug. 26*</td>
<td>38</td>
<td>A11 (2)</td>
</tr>
<tr>
<td>8</td>
<td>Y</td>
<td></td>
<td>1/1</td>
<td>3/5</td>
<td>Dec. 10, 2005, to Jan. 25, 2006</td>
<td>Nov. 18*</td>
<td>A10 (2)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>K</td>
<td></td>
<td>1/1</td>
<td>2/5</td>
<td>Oct. 20 to Dec. 15, 2006</td>
<td>Jan. 17*</td>
<td>39</td>
<td>A10 (2)</td>
</tr>
<tr>
<td>10</td>
<td>I</td>
<td></td>
<td>2/2</td>
<td>10/10</td>
<td>Oct. 23 to Dec. 17, 2003</td>
<td>Nov. 21*</td>
<td>31</td>
<td>A12 (2)</td>
</tr>
<tr>
<td>12</td>
<td>B</td>
<td></td>
<td>1/1</td>
<td>1/5</td>
<td>Oct. 5 to Dec. 28, 2004</td>
<td>Aug. 8*</td>
<td>16</td>
<td>A8 (3)</td>
</tr>
<tr>
<td>13</td>
<td>B</td>
<td></td>
<td>1/1</td>
<td>1/5</td>
<td>Jun. 6 to Aug. 28, 2005</td>
<td>Oct. 21*</td>
<td>63</td>
<td>A10 (1)</td>
</tr>
<tr>
<td>14</td>
<td>B</td>
<td></td>
<td>1/1</td>
<td>2/5</td>
<td>Sep. 22 to Dec. 12, 2006</td>
<td>Apr. 18*</td>
<td>73</td>
<td>A10 (2)</td>
</tr>
</tbody>
</table>

1 No. of houses positive for *C. jejuni*/no. of houses tested.
2 No. of fecal samples positive for *C. jejuni*/no. of samples tested.
*Asterisk indicates isolation of *C. jejuni*. 

**Table Note:**
- Ages (d) of birds positive for *C. jejuni*.
- flaA type (no. of isolates tested).

**Abbreviations:**
- I: Isolation
- F: Flora
- B: Bacteria
- A: Asterisk
houses: 14 swab samples of rubber boots; fecal samples from 20 chicks, and 3 litter samples for transport of chicks in hatchery 1. Intestinal contents and fecal samples from the animals were directly spread on mCCDA agar plates. Swab samples were enriched using Preston Campylobacter selective enrichment broth (Preston broth) consisting of Nutrient broth No. 2 (CM0067, Oxoid), and Preston Campylobacter growth supplement SR0084 (Oxoid), and Preston Campylobacter selective supplement SR0117 (Oxoid) for 24 h at 42°C. Ten grams of litter samples or 10 mL of water samples were mixed with 90 mL of Preston broth and enriched for 24 h at 42°C. All the enriched cultures were spread on mCCDA agar plates. Swab samples were enriched using Preston Campylobacter selective enrichment broth (Preston broth) consisting of Nutrient broth No. 2 (CM0067, Oxoid) supplemented with 5% (vol/vol) lysed horse blood, Campylobacter growth supplement SR0084 (Oxoid), and Preston Campylobacter selective supplement SR0117 (Oxoid) for 24 h at 42°C. Ten grams of litter samples or 10 mL of water samples were mixed with 90 mL of Preston broth and enriched for 24 h at 42°C. All the enriched cultures were spread on mCCDA agar plates. Identification of C. jejuni was performed as described above.

### Typing Methods

Restriction fragment length polymorphism analysis of the flaA gene was undertaken by digestion of the PCR product with Ddel based on the method described previously (Chuma et al., 1997). The flaA types obtained were designated following the previous report (Ishihara et al., 2006). Multilocus sequence typing of representative isolates with different RFLP types was performed according to the method of Dingle et al. (2001). Sequence types (ST) and clonal complexes (CC) were assigned with an integrated automated link to the public PubMLST data bank (http://pubmlst.org/campylobacter). The CC are defined as a group of independent isolates that share at least 4 alleles (Dingle et al., 2001). Campylobacter jejuni strains with flaA types A3 and A4 that were previously reported (Ishihara et al., 2006) were also used for MLST. Representative C. jejuni isolates with different RFLP types were serotyped using a commercially available kit (Denka-Seiken, Tokyo, Japan) according to the scheme described by Penner and Hennessy (1980) at the Microbiology Division, Kyoto Prefectural Institute of Public Health and Environment, Japan. This kit contains 21 individual sera and 4 types of combination sera, (e.g., O:1,44; O:4,13,16,43,50; O:6,7; and O:23,36,53).

Three strains (Cj15–58, Cj15–46, and Cj15–166) previously obtained from chicken feces on farm 1 (Ishihara et al., 2006) and a strain (Cj17–53) obtained from chicken feces on a commercial egg-producing farm, which was located 47 km northeast of farm 1, were also used as references.

### RESULTS

A total of 206 isolates were obtained from 535 fecal samples collected on 4 farms during the study period (Table 1). In cycles 1 and 2, C. jejuni was first isolated when the birds reached 34 to 40 d of age and was continuously isolated for the remainder of the grow-out. Campylobacter jejuni isolates were obtained from 38% (152/400) of samples collected in cycles 1 and 2. Almost all the fecal samples obtained in the subsequent cycles 3 to 9 in farm 1 except cycle 4 and those obtained in farms 2 and 3 gave positive results. One or 2 samples obtained from each of the cycles in farm 4 were positive for C. jejuni.

The RFLP analysis of the flaA gene revealed 10 different Ddel-digested patterns (Figure 1) among the 206 isolates. Four (types A3, A4, A5, and A6) of the 10 patterns were identical to those reported previously (Ishihara et al., 2006). Others were arbitrarily designated as types A8 to A13.

Virtually all isolates obtained in cycle 1 were subjected to RFLP analysis of the flaA gene (Table 1). As a result, isolates obtained from flocks kept in 2 chicken houses on August 7 and 14 belonged to type A3. After that, types A3 and A5 were found in isolates obtained from both the houses. Therefore, representative C. jejuni isolates in cycle 2 though 9 were examined for flaA typing. In cycles 2, C. jejuni was isolated from flocks kept in 12 houses. Isolates with types A3 and A6 were obtained from 7 and 3 houses, respectively. Isolates with both types A4 and A6 were obtained from the remaining 2 houses. All isolates obtained in cycle 3 were typed as A8 except for one (A9). Different flaA types were found in isolates obtained between subsequent cycles 1 through 5. On the other hand, isolates with an identical type (A10) were obtained from broiler chickens, originating from different hatcheries F, K, M, and Y, between subsequent cycles 6 through 8. Strains with RFLP type A6 were isolated from chickens on farm 2 in December 2003 and those with this type were also obtained on farm 1 in January 2004. Type A13 was solely found in isolates obtained from farm 3. Different flaA types (A8, A10, and A11) were found in isolates obtained between subsequent cycles 4. These types (A8,
A10, and A11) were also found in isolates obtained on farm 1 as described above.

In farm 1 in 2003, *C. jejuni* was isolated from 5 water samples in June, and from 6 water samples and 2 rubber boots in August. The organism was not isolated from other environmental samples, including the hatchery samples. Of these isolates, a total of 9 isolates from the chickens’ drinking water and the boots obtained in August 2003 were subjected to RFLP typing. Five water isolates were classified as type A3, and each of 2 isolates from water and the boots were classified as type A5. These types were identical to those found in isolates obtained from fecal samples collected on the same farm (farm 1) in August 2003 (Table 1).

Fourteen strains representing each of the 10 RFLP types were analyzed by MLST together with reference strain Cj17–53 isolated from a commercial egg-producing farm, whose RFLP type was designated as A14. Identical MLST allele combinations were observed between pairs of isolates with the same RFLP types (Table 2). That is, strains Cj15–269 and Cj16–133, which belonged to RFLP type A6, were assigned to ST45 and CC45. Similar observations were found in pairs of isolates with types A8, A10, or A11. Additionally, different allele combinations were seen between isolates with distinct RFLP types. Very similar allele combinations not assigned to the same clonal complex (CC, based on the database) clustered around isolates with RFLP types A11, A12, and A13 obtained from farms 1, 1, and 3, respectively.

Of the 15 strains with different RFLP types, a strain with RFLP type A4 and 2 strains with type A10 were serotyped as O:2 (Table 2). Approximately half of the strains tested were serologically untypeable.

## DISCUSSION

Our finding that the flocks in farm 1 were first infected with *C. jejuni* at 30 to 39 d of age is consistent with previous findings that commercial broiler chickens less than 2 to 3 wk old are rarely colonized naturally (Berndtson et al., 1996; Jacobs-Reitsma et al., 1995) and that the prevalence within an infected flock was age dependent (Evans and Sayers, 2000).

The present results suggest that colonization of the flock by *C. jejuni* occur after the placement of chicks into broiler chicken houses. Isolates with an identical RFLP type (type A10) were recovered from multiple flocks that had originated from different hatcheries and that were reared during successive production cycles between July 2005 and January 2006 in farm 1, suggesting that *C. jejuni* from the previous flock may reside in the farm. On the other hand, strains with RFLP type A5 were isolated from 2 flocks of broiler chickens reared in different houses on farm 1. Isolates with both RFLP types were also obtained from water pails and rubber boots in the present study, suggesting that these play a role in transmission of *C. jejuni* within chicken houses although possible involvement of these factors...
in transmission among different houses was unclear. A high level of genotype diversity was exhibited between \textit{C. jejuni} isolates recovered from chickens reared conventionally (Wilson et al., 2009).

In this study, strains assigned as clonal complexes CC21 and CC45 were recovered. These CC are predominant in \textit{C. jejuni} isolates originating from human and poultry (Dingle et al., 2001; Korczak et al., 2009; Griekspoor et al., 2010; Hastings et al., 2010). However, more than half of the MLST allelic combinations found in this study were not associated with existing CC. The ST determined by MLST and RFLP types based on the \textit{flaA} gene revealed a one-to-one correspondence among the strains used in the present study (Table 2). Clonal complexes of \textit{C. jejuni} can also be predicted by \textit{flaA} typing (Djordjevic et al., 2007). Thus, both the MLST and RFLP results may be useful for investigating the epidemiology of \textit{Campylobacter} in poultry production.

Identical MLST profiles and RFLP types were found in strains isolated in October 2004 and again in August 2005 from birds reared in farms 1 and 4, which were located within 270 m, obtained chicks from different hatcheries and grew them using chicken feed supplied from different companies. These results suggest that the flocks on those farms were colonized by common sources external to the facilities, although the routes of infection were not identified. However, identical and very similar MLST allele combinations were found in isolates obtained from farms 1, 2, and 3 that belonged to an integrated poultry company, obtained chicks from hatcheries that also belonged to the company, and grew the birds using feed supplied from the same company. These results do not rule out the possibility that contamination of day-of-hatch chicks occurred because Byrd et al. (2007) reported recovery of \textit{C. jejuni} from broiler hatchery trayliners. It is also possible that \textit{C. jejuni} was carried to those farms because employees or trucks of the company occasionally visited the farms.

In conclusion, flocks may be colonized by \textit{C. jejuni} after placement of chicks because identical \textit{flaA} types were found in isolates obtained from birds originating from different hatcheries. The typing results of the isolates imply that \textit{C. jejuni} strains within the farm are carried over from the previous flock. Colonization of flocks by \textit{C. jejuni} may also be caused by common sources when the farms are located in close proximity, even if these farms belonged to different poultry companies and obtained chicks and feed from different suppliers.

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