

Analysis of Distribution of *Campylobacter jejuni* and *Campylobacter coli* in Broilers by Using Restriction Fragment Length Polymorphism of Flagellin Gene

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ABSTRACT. The incidence of *Campylobacter jejuni* and *Campylobacter coli* in broiler farms was 33.9% (19/56). *C. jejuni*-positive flocks accounted for 20.0% (17/85) and *C. coli*-positive ones was 4.7% (4/85). There were 14 patterns (*fla* type) of restriction fragment length polymorphism (RFLP) of flagellin A gene among these 22 strains of *C. jejuni* and *C. coli* including the standard strain *C. jejuni* ATCC 33560. Different *fla* types of *Campylobacter* were isolated from broilers in different growing cycles on the same farms. Four strains of *C. jejuni* were isolated from four breeder farms and four *fla* types of *C. jejuni* were detected from their progenies reared on growing farms. Three *fla* types of *C. jejuni* detected from the progenies were different from those of each breeder. Also, the other three *fla* types of *C. jejuni* were detected from different progenies of each growing farm during the next growing cycle. These findings indicate that the RFLP analysis may contribute to epidemiological studies of *C. jejuni* and *C. coli* contamination of broilers and suggest the risk of contamination with different types of *Campylobacter* in every growing cycle of broilers on the farm even on the same farm. They also supported that there was little likeliness of the vertical transmission of *C. jejuni* and *C. coli* from breeders to broilers. — **KEY WORDS:** broiler, *Campylobacter jejuni*, flagellin gene typing, PCR, restriction fragment length polymorphism.

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Campylobacter jejuni is recognized as a major cause of acute diarrheal disease in humans throughout the world [22, 23]. Campylobacteriosis is mainly a food-borne infection, and poultry products may play an important role in the transmission to humans [1, 8, 10, 21]. *C. jejuni* contamination of poultry meat during processing has been well documented [2, 11, 18, 24]. During the slaughter, poultry carcasses become contaminated by the release of intestinal contents or transfer of contamination from the surface flora of the birds [11]. Some investigators [3, 6, 12, 20] found that *C. jejuni* had already spread at the farm level; but the epidemiology of *C. jejuni* in broilers and its ecology in farm practices have not been completely defined. The source of *C. jejuni* contamination at the broiler-growing level is not clear. Discrimination of *C. jejuni* strains are considered to be required to know the contamination route from the standpoint of epidemiology.

Heat-stable and heat-labile serotyping of *C. jejuni* have been performed by Penner *et al.* [19] and Lior [13], respectively. However, these procedures are very tedious and costly [7] because they require labor investment and a set of antisera for all serotypes. On the other hand, an advantage of genotyping is that it does not depend on the variable phenotypic expression of bacterial cells but measures stable chromosomal differences [7]. Recently, a molecular typing of *C. jejuni* by restriction fragment length polymorphism (RFLP) analysis of the flagellin gene *flaA* was developed with *C. jejuni* strains obtained from outbreaks of food poisoning, non-outbreak strains and human isolates [16]. RFLP analysis was suggested to be useful as a typing method for epidemiologic investigations.

In the present study, we analyzed for the distribution of *C. jejuni* and *C. coli* by performing the molecular typing of the organisms isolated from broilers and breeders by using

RFLP, and discussed the mechanism of the distribution of *C. jejuni* and *C. coli* contamination in broilers at the farm level.

MATERIALS AND METHODS

Chickens and fecal samples: Chickens (nearly 8-week old) were obtained from growing farms just before the transportation to processing plants. Their cecal contents were collected following anesthesia and exsanguination. Fresh feces of breeders were transported to the laboratory and immediately cultured. Cecal contents of their progenies at the ages from 4-week old to 8-week old were also collected. All of the growing farms and the breeder farms were located in Kagoshima prefecture, Japan.

Isolation of *C. jejuni* and *C. coli*: Samples (about 0.1 g) of chicken cecal contents or feces were enriched in 10 ml of Preston broth consisting of nutrient broth, 7% defibrinated horse blood and a *Campylobacter*-selective supplement (SR117: Oxoid Ltd., Basingstoke, U. K.), and incubated microaerobically at 42°C for 24 hr. Three loopfuls from each enrichment was streaked on CCDA (Oxoid) plates containing a selective supplement (SR085: Oxoid), and incubated under the same conditions as above for 48 hr. One typical colony of *Campylobacter* on each CCDA plate was streaked again on Muler-Hinton agar (Oxoid) plates for cloning. The isolates from the plates were identified by the following tests: Gram staining, cell morphology, catalase and oxidase reactions, motility, indoxyl acetate hydrolysis, resistance to 30 µg of cephalothin, and failure to grow under aerobic condition at 37°C. Hippurate hydrolysis-positive and -negative isolates were identified as *C. jejuni* and *C. coli*, respectively.

Extraction of DNA: The isolated bacteria and *C. jejuni*

ATCC 33560 as the standard strain were cultured in brain heart infusion broth under microaerobic condition at 37°C for 24 hr with shaking. Each bacterial growth was harvested into a 1.5-ml microtube, which was centrifuged at 6,400 rpm for 5 min. The pellet was suspended in 300 µl of a buffer solution (50 mM Tris-HCl and 20 mM EDTA, pH 8.0) containing 2% SDS and pronase (100 µg/ml). The mixture was incubated at 60°C for 1 hr and allowed to stand on ice for 10 min. To the suspension, 130 µl of a saturated NaCl solution was added and it was kept on ice for 5 min. The mixture was centrifuged at 12,000 rpm for 10 min at 4°C and the supernatant was extracted twice with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). The purified DNA was ethanol-precipitated, rinsed with 70% ethanol, and dissolved in 400 µl of a buffer (1 mM Tris-HCl and 0.1 mM EDTA, pH 8.0).

RFLP: A pair of primers amplifies the complete nucleotide region (1,728 bp) coding flagellin A. The sequences of the primers are as follows; FL1: GGATTTTCGTATTAACACAAATGGTGC, and FL2: CTGTAGTAATCTTAAACATTTTG. A PCR reaction mixture (50 µl) contained 10 µl of the purified DNA solution, 10 mM Tris-HCl (pH 8.3), 0.2 mM each deoxynucleotide triphosphate, 10 pmol of each primer, and 1 unit of Tth DNA polymerase (Toyobo Co., Osaka, Japan). This solution was covered with 30 µl of mineral oil (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and subjected to 35 cycles of amplification in a PCR cycler (MJ Research, Watertown, Mass., U.S.A.). Each cycle consisted of 1 min at 94°C, 2 min at 54°C, and 2 min at 74°C. A part of the amplified sample was checked by electrophoresis on a 0.8% agarose gel by ethidium bromide staining.

Two tubes of a sample (total 100 µl) were collected in a microtube containing 300 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). It was extracted twice with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). After 1 µl of glycogen (Boehringer Mannheim GmbH, Mannheim, Germany) was added, it was precipitated with two volumes of ethanol containing sodium acetate, rinsed with 70% ethanol, and dissolved with 13 µl of distilled water. The DNA was digested with the appropriate units of restriction enzyme Dde I (Takara Shuzo Co., Otsu, Japan). The digested DNA was analyzed by electrophoresis in 2% NuSieve GTG agarose gel (FMC BioProducts, Rockland, Me., U.S.A.) at 100 V for 2 hr. A 100-bp DNA

ladder was used as a standard molecular-size marker. DNA bands were visualized with a ultraviolet transilluminator and photographed.

RESULTS

Incidence of *C. jejuni* and *C. coli* in broiler farms: Fifty-six growing farms were surveyed. Eleven, six, and two farms were investigated twice, 3 times, and 4 times, respectively, in different seasons. So 85 broiler flocks were totally investigated. Five cecal contents of broilers were used for each detection of *C. jejuni* and *C. coli*. The number of *C. jejuni*-positive flocks was 17 (20.0%) and that of *C. coli*-positive flocks was four (4.7%). Table 1 shows the number of *C. jejuni*- and/or *C. coli*-positive farms. The positive rate of farms tended to increase in proportion to the trial number of investigation.

RFLP patterns of *C. jejuni* and *C. coli* isolated from broilers: *C. jejuni* and *C. coli* were isolated from 21 broiler flocks. The RFLP patterns are shown in Fig. 1. Only one RFLP pattern of *Campylobacter* was detected in the cecal contents of broilers from each flock. There were 14 patterns including that of the standard strain *C. jejuni* ATCC 33560 (lane 1). Lanes 5, 8, 19 and 22 were *C. coli*, and the other lanes were *C. jejuni*. Two strains of *C. coli* (lanes 5 and 8) showed the same patterns as those of strains of *C. jejuni*. The most frequent type was *fla*-5 (five strains: lanes 6, 8, 9, 16 and 21). Three strains (lanes 3, 7 and 13) were *fla*-3, two strains (4 and 5) were *fla*-4, and two strains (lanes 12 and 14) were *fla*-8. The strains of lanes 5 and 16 were isolated from the same farm but from different flocks. Also, the strains of lanes 12 and 13 were isolated under the same situation as above. Namely, different *fla* types of *Campylobacter* were isolated from broilers in different growing cycles of the same farm.

RFLP patterns of *C. jejuni* isolated from breeders and their progenies: Four breeder farms and 15 growing farms rearing their progenies were surveyed. Five fresh feces from each breeder farm and five cecal contents of progenies in each flock were used for detection of *C. jejuni* and *C. coli*. Only one RFLP pattern of *C. jejuni* was detected in the feces or the cecal contents of each positive flock. Four strains of *C. jejuni* were isolated from fresh fecal samples obtained from breeders of four breeder farms. The *fla* types of each strain were *fla*-4, *fla*-15, *fla*-4, and *fla*-5, as shown

Table 1. Incidence of *C. jejuni* and *C. coli* contamination in broiler farms and flocks

Trial number	Farms examined	Positive number	Number of flocks	<i>C. jejuni</i> -positive flocks	<i>C. coli</i> -positive flocks
once	31	6 (19.4%)	31	5	1
twice	11	7 (63.6%)	22	7	0
3 times	6	4 (66.7%)	18	3	2
4 times	2	2 (100%)	8	2	1
total	56	19 (33.9%)	85	17	4

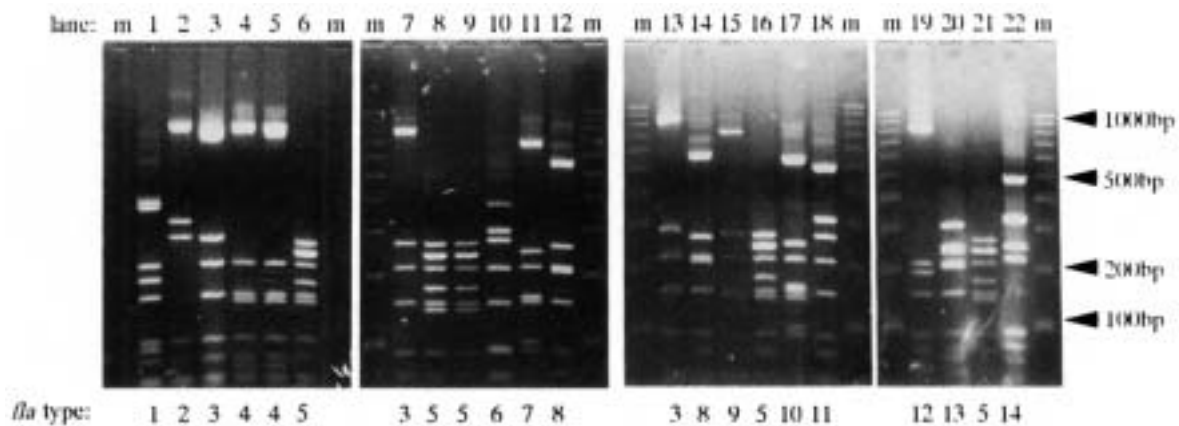


Fig. 1. RFLP patterns of *C. jejuni* and *C. coli* isolated from broilers. Lanes 5, 8, 19, and 22: *C. coli*, the other lanes: *C. jejuni*. m: 100 bp ladder as standard molecular size.

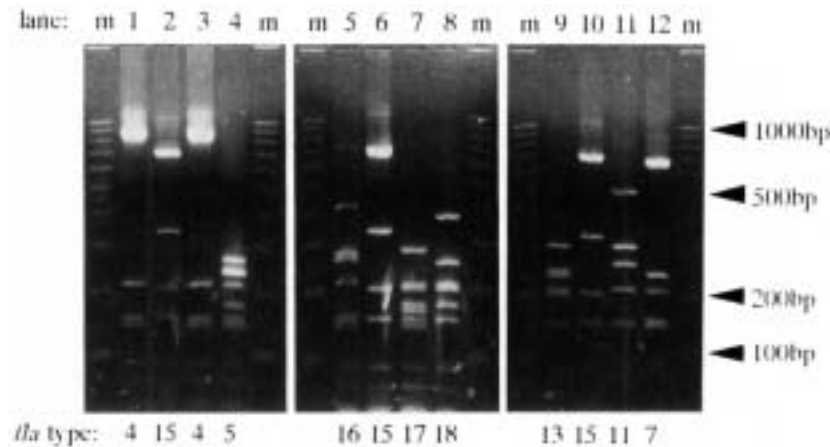


Fig. 2. RFLP patterns of *C. jejuni* isolated from breeders and broilers. m: 100 bp ladder as standard molecular marker. Lanes 1 to 4: strains isolated from breeders. Lanes 5 to 8: strains isolated from progenies of each breeder. Lanes 9 to 12: strains isolated from other progenies reared for the next growing cycle on the same farms.

in Fig. 2. Each progeny was transferred to various growing farms after the chickens were hatched in a commercial hatchery. Four types, *fla*-16, *fla*-15, *fla*-17, and *fla*-18 (Fig. 2) of *C. jejuni*, were isolated from the progenies reared on four growing farms among 15 farms. After the progenies were transferred to processing plants, the other flocks of chickens were introduced into each of four growing farms for the next cycle of growing. The intervals of the cycles were about 3 weeks. These second flocks were progenies different from the first flock of each growing farm. The *fla* types of the isolates from four growing farms were *fla*-13, *fla*-15, *fla*-11, and *fla*-7 as shown in Fig. 2. The relationship of *fla* type between the breeders and their progenies are summarized schematically in Fig. 3.

DISCUSSION

At the growing-farm level, chickens are thought to be easily contaminated with *C. jejuni* by horizontal

transmission [3, 6]. Once some chickens turned positive for the organism, the contamination would spread easily through the flock. For this reason, we considered that five chickens from each broiler flock may be enough for the investigation of the incidence of *C. jejuni* and *C. coli*. The contamination rate of farms examined only once was 19.4%, whereas those of farms examined more than twice were much higher. This finding suggests that *C. jejuni* and *C. coli* contamination would be detected by repeated examinations of different cycles of broilers even on the farms that appeared to be clean at the first examination. It may imply that each farm involves the risk of contamination more or less.

Thirteen *fla* types were shown among 21 isolates from broiler flocks, suggesting that the RFLP analysis is useful enough to investigate the epidemiology of *C. jejuni* and *C. coli* in broilers at the farm level. The most predominant type was *fla*-5 in this study of broilers on the farms, but further studies may be needed to discuss the apparent predominance of the *fla* type of *C. jejuni* and *C. coli* in

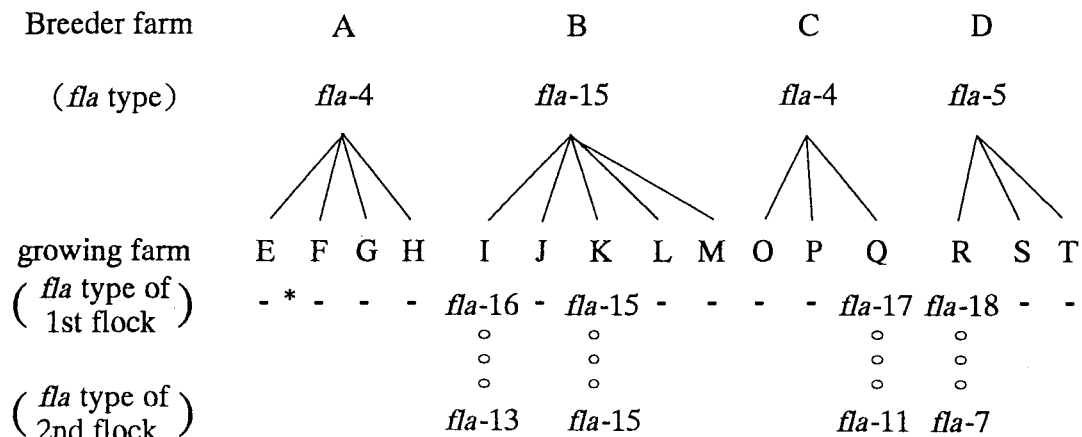


Fig. 3. Relationship in *fla* type between breeders and their progenies. The first flocks were the progenies of each breeder. The second flocks were introduced 3 months later than the first ones and were different progenies from the first ones. *: Neither *C. jejuni* nor *C. coli* was isolated.

broilers. Although the strains of lanes 5 and 16 (Fig. 1) were isolated from the same farm, the RFLP patterns were different from each other. The strains of lanes 12 and lane 13 (Fig. 1) isolated from the same farm showed also different patterns. Essentially identical results were demonstrated in each member of three pairs of strains [namely, lanes 5 and 9; lanes 7 and 11; and lanes 8 and 12 in Fig. 2]. These findings suggest that each broiler flock would have a chance to be contaminated with different types of *C. jejuni* or *C. coli* if the growing cycle is different even on the same farm.

According to the study of the *C. jejuni* contamination through egg shells, vertical transmission in chickens is considered to be highly improbable [5, 17, 9]. On the other hand, the eggs of Japanese quails were indicated to get contaminated with *C. jejuni* while passing through the oviduct [14]. Although the gene of *C. jejuni* was detected by PCR in cecal contents of some 18-day-old chicken embryos, no live bacterium was isolated by culture with enrichment [4]. We studied vertical transmission by comparing *fla* types of *C. jejuni* isolated from breeders and their progenies. Although the first flocks were the progenies of each breeder, *fla* types of three flocks (growing from I, Q, and R in Fig. 3) were different from those of each breeder. This result proves clearly that *C. jejuni* isolated from these three progenies were not derived from their breeders. The *fla* type of one of the first flocks (growing farm K) was the same as that of the breeder, and that of the second flock was also the same. On closer inspection of this farm, we found that the shipping score and the sanitary condition had been exceptionally bad and the farm had eventually quitted rearing broilers. So the bacteria might have been anchored heavily to the surroundings. In this study, the possibility that *C. jejuni* isolated from one flock of broiler was derived from the breeder can not be denied. According to the findings of all 15 growing farms, however, vertical transmission from breeders to broilers was considered to be unlikely. It has been recognized that *fla*

types and serotypes overlapped in some cases [15, 16]. It should be necessary to use other methods including gene typing and serotyping for more detailed analysis if the *fla* types were the same.

In conclusion, it is suggested that RFLP analysis is valuable for the epidemiologic studies of distribution of *C. jejuni* and *C. coli* and there may be a risk of contamination with different types of *C. jejuni* and *C. coli* in every growing cycle of broilers on the farms even on the same farm.

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