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NOTE

Bacteriology

Campylobacter spp. prevalence and fluoroquinolone resistance in chicken layer farms

Yoshimasa SASAKI[1,2)]*, Taketoshi IWATA[3)], Masashi UEMA[1)], Kenzo YONEMITSU[4)], Shizunobu IGIMI[5)] and Hiroshi ASAKURA[1,2)]

[1)]National Institute of Health Sciences, Kanagawa, Japan
[2)]The United Graduate School of Veterinary Science, Gifu University, Gifu, Japan
[3)]Division of Zoonosis Research, National Institute of Animal Health, National Agriculture and Food Research Organization, Ibaraki, Japan
[4)]Murayama Branch, National Institute of Infectious Diseases, Tokyo, Japan
[5)]Tokyo University of Agriculture, Tokyo, Japan

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ABSTRACT. Chicken is a major source of human campylobacteriosis. Chicken meat originates not only from broilers but also from spent layers; however, few reports have documented the prevalence and antimicrobial resistance of *Campylobacter* spp. in layers in Japan. Therefore, we investigated the prevalence and antimicrobial susceptibility of *Campylobacter* spp. in 47 layer farms in Japan. Fecal samples were collected from the youngest and oldest flocks on the farm, and *Campylobacter* spp. was isolated from 46/47 (97.9%) farms. Among the *C. jejuni* isolates, the resistance rates to ampicillin, tetracycline, and ciprofloxacin were 29.6%, 22.2%, and 19.8%, respectively. The ciprofloxacin resistance rate (7.3%) in *C. jejuni* isolated from old flocks was significantly ($P<0.01$) lower than that in young flocks (32.5%).

KEYWORDS: *Campylobacter*, chicken layer, fluoroquinolone resistance, multilocus sequence typing

Campylobacter spp. is the most common cause of human gastroenteritis worldwide [20]. The annual number of food poisoning outbreaks in Japan caused by *Campylobacter* spp. was higher than 200 between 2014 and 2018 [22]. *Campylobacter* spp. infections typically cause acute self-limiting gastroenteritis, and antimicrobial therapy is usually not provided. However, when patients are immunocompromised or have other comorbidities, antimicrobial treatment may be necessary [21]. Erythromycin is often used as a first-line treatment for human campylobacteriosis; however, in the absence of a microbiological diagnosis, fluoroquinolones are used to treat *Campylobacter* enteritis [5, 15, 16, 23]. Therefore, fluoroquinolone-resistant *Campylobacter* spp. represents a serious threat to public health.

Raw or undercooked chicken meat is a major source of *Campylobacter* spp. infection [4, 10, 22]. The Japanese Ministry of Agriculture, Forestry, and Fisheries has designated *Campylobacter* spp. as a prioritized hazard in food safety risk management, identified chicken meat as the principal source of *Campylobacter* spp. infection in humans, and monitored the prevalence of *Campylobacter* spp. in broiler flocks [6, 17–19]. However, chicken meat originates not only from broilers but also from spent layers. According to the Meat Inspection and Other Return Survey available on the website for Japanese government statistics (https://www.e-stat.go.jp/en, statistics code 00450192), spent layers annually account for approximately 10% of the chickens slaughtered at chicken processing plants in Japan [11]. Additionally, Hattori *et al.* [8] reported cases of *Campylobacter* enterocolitis in children due to raw chicken egg consumption.

According to Japanese Veterinary Antimicrobial Resistance Monitoring (JVARM) reports [12–14], the fluoroquinolone resistance rate in *Campylobacter* spp. isolated from broilers was higher than that in commercial layers. The use of fluoroquinolones in commercial layers during the laying period has not been approved in Japan because of residual fluoroquinolones in eggs. However, fluoroquinolones can be used in immature hens before laying eggs, as well as in broilers. In Japan, 17-week-old hens are transferred from grower to layer houses, where they continue to lay eggs for more than 47 weeks. Therefore, fluoroquinolone resistance in *Campylobacter* spp. isolated from older birds might be lower than that in younger birds because of the longer absence

*Correspondence to: Sasaki, Y.: ysasaki@nihs.go.jp, Division of Biomedical Food Research, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki, Kanagawa 210-9501, Japan
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of antibiotic selection pressure. In this study, we investigated the prevalence and antimicrobial susceptibility of *Campylobacter* spp. isolates from young and old layer chickens. The results of our study could be useful for managing the risk of campylobacteriosis caused by the consumption of chicken meat and eggs.

Forty-seven layer farms voluntarily participated anonymously in this study. The farms were located in 24 cities in 10 prefectures in Japan. Each farm had more than 4,000 birds. Of the 47 layer farms, 31 implemented induced molting and planned to raise their layer flocks until an average age of 99 weeks (77 to 104 weeks). The remaining 16 layer farms did not implement induced molting and planned to raise their layer flocks until an average age of 79 weeks (64 to 93 weeks). Sampling was conducted by veterinarians between October 2017 and September 2019; however, sampling could not be performed in the spring season (from March to May in 2018 and 2019) owing to financial reasons. Sampling was conducted twice on six farms after an interval of at least eight months. On each farm, two flock age groups (the youngest and oldest flocks) were selected. For each flock, three fecal samples were collected from three feces on manure conveyor belts using cotton swabs (SEEDSWAB No. 1; Eiken Chemical, Tokyo, Japan). The samples were shipped to the National Institute of Health Sciences via express delivery under refrigeration. At the laboratory, the samples were refrigerated at 4°C until examination, which was performed within three days of sampling.

Each swab head was directly rubbed over the surface of modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid, Basingstoke, UK) and microaerobically incubated at 42°C for 48 ± 2 hr using the AnaeroPack-Microaero (Mitsubishi Gas Chemicals, Tokyo, Japan) (direct culture). After rubbing, the swab head was inoculated into 10 ml Preston enrichment medium (Oxoid) and incubated at 42°C for 22 ± 2 hr (enrichment culture). After incubation, a loopful of the resulting culture was plated onto mCCDA plates and microaerobically incubated at 42°C for 48 ± 2 hr. A maximum of two suspected isolates per sample were identified using multiplex polymerase chain reaction [9]. When suspected isolates were obtained from both the direct and enrichment cultures, those from the direct culture were used. One of each different *Campylobacter* species per flock was regarded as a representative isolate of the flock and subjected to antimicrobial susceptibility testing (ampicillin: 0.12–256 mg/l, streptomycin: 0.12–128 mg/l, erythromycin: 0.12–128 mg/l, tetracycline: 0.12–128 mg/l, nalidixic acid: 0.12–128 mg/l, ciprofloxacin: 0.03–64 mg/l, chloramphenicol: 0.12–256 mg/l, and gentamicin: 0.12–256 mg/l). Antimicrobial minimal inhibitory concentrations were determined using the broth microdilution method with dried plates (Eiken Chemical), as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [2]. The breakpoints (ampicillin: 32 mg/l, streptomycin: 32 mg/l, erythromycin: 32 mg/l, tetracycline: 16 mg/l, nalidixic acid: 32 mg/l, ciprofloxacin: 4 mg/l, and chloramphenicol: 16 mg/l) adopted by CLSI [1] and JVARM [14] were used; except for the tests involving gentamicin (2 mg/l), which was specified by the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme [3]. *C. jejuni* ATCC33560 was used as the quality control strain for the rest of the antimicrobials. The sequence type of one *C. jejuni* isolate per flock was determined using multilocus sequence typing (MLST). MLST was performed according to the seven-loci scheme for *C. jejuni*, employing the primer sets and experimental conditions suggested by the *Campylobacter* MLST database (http://pubmlst.org/campylobacter/). All statistical analyses were performed using R version 4.1.2. Differences between proportions were tested using Fisher's exact test, where *P*-values of <0.05 were considered statistically significant.

Campylobacter spp. isolates were obtained from 46 (97.9%) out of 47 farms. In addition, a second sampling was conducted on six farms from which *Campylobacter* spp. was isolated, and *Campylobacter* spp. was isolated from all the six farms. *C. jejuni* isolates were obtained from 40 young and 41 old flocks (Supplementary Table 1). *C. coli* isolates were obtained from 12 young and 16 old flocks. No statistically significant difference ($P>0.05$) in the prevalence of *Campylobacter* spp. was observed between the two age groups. Eighty-one *C. jejuni* and 28 *C. coli* isolates were subjected to antimicrobial susceptibility testing. All the *Campylobacter* spp. isolates were susceptible to erythromycin, chloramphenicol, and gentamicin. Among the *C. jejuni* isolates, the highest resistance rate was observed against ampicillin (29.6%; Table 1). Of the 24 ampicillin-resistant *C. jejuni* isolates, 18 (75.0%) were susceptible to ciprofloxacin. The resistance rates against nalidixic acid and ciprofloxacin in *C. jejuni* isolated from old flocks were significantly lower than those in *C. jejuni* isolated from young flocks ($P<0.01$). In contrast, no statistical difference was found in the ampicillin and tetracycline resistance rates of *C. jejuni* isolates between young and old flocks. Among the *C. coli* isolates, the resistance rates against ampicillin, tetracycline, nalidixic acid, and ciprofloxacin were 17.9% (5/28), 17.9% (5/28), 14.3% (4/28), and 14.3% (4/28), respectively. No statistical difference ($P>0.05$) was observed in the antimicrobial resistance rates of the *C. coli* isolates between the young and old flocks.

Eighty-one *C. jejuni* isolates were divided into 44 sequence types (STs) using MLST. *C. jejuni* was isolated from both young

Table 1. Antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* isolates

Species	Flocks	No. of isolates	No. of resistant isolates (%)				
			ABPC	SM	TC	NA	CPFX
C. jejuni	Total	81	24 (29.6)	1 (1.2)	18 (22.2)	16 (19.8)	16 (19.8)
	Young flocks	40	13 (32.5)	1 (2.5)	10 (25.0)	13 (32.5)*	13 (32.5)*
	Old flocks	41	11 (26.8)	0 (0.0)	8 (19.5)	3 (7.3)*	3 (7.3)*
C. coli	Total	28	5 (17.9)	0 (0.0)	5 (17.9)	4 (14.3)	4 (14.3)
	Young flocks	12	1 (8.3)	0 (0.0)	2 (16.7)	1 (8.3)	1 (8.3)
	Old flocks	16	4 (25.0)	0 (0.0)	3 (18.8)	3 (18.8)	3 (18.8)

ABPC: ampicillin, SM: streptomycin, TC: tetracycline, NA: nalidixic acid, CPFX: ciprofloxacin. *$P<0.05$ with Fisher's exact test.

and old flocks on 31 farms, and the STs of the two isolates from 27/31 farms were different from each other. In addition, in four (farm codes b, c, e, and f) of the six farms where sampling was conducted twice, STs of three or four isolates (obtained by the two samplings) were different from each other (Supplementary Table 2). Of the 44 STs, 17 were isolated from two or more flocks (Supplementary Table 3). Four STs (ST5081, ST9767, ST9774, and ST9776) were isolated from the same farm. The three main STs identified were ST4389, ST6704, and ST2789, and their antimicrobial resistance was analyzed. The eight ST4389 isolates were obtained from eight farms in six cities, and all of them were resistant to ampicillin. Six of the seven ST6704 isolates, obtained from seven farms in six cities, were resistant to ampicillin. ST4389 and ST6704 belonged to the clonal complex (CC) 464, which was the most frequent accounting for 28.4% (23/81) of the clonal complexes. Six other STs (ST5262, ST5731, ST9769, ST9770, ST9771, and ST9785) belonged to CC464. The ampicillin resistance rate in ST4389 and ST6704 isolates was 93.3% (14/15), whereas the resistance rate of the rest of the CC464 lineage was 12.5% (1/8). Three of the five ST2789 isolates, which were obtained from five farms in five cities, were resistant to ciprofloxacin. ST2789 belonged to CC21, which was the second most frequent clonal complex and accounted for 16.0% (13/81) of *C. jejuni* isolates.

The results showed that the prevalence of *Campylobacter* spp. in layer flocks in the summer, autumn, and winter seasons could be high. Additionally, the STs of the two *C. jejuni* isolates from 27/31 farms, where both young and old flocks tested positive for *C. jejuni,* were diverse, suggesting the existence of multiple *C. jejuni* lineages in a layer farm. A layer farmer raises flocks of different ages to stabilize egg quality and production throughout the year. Layer farmers usually ship the oldest flock to slaughterhouses and replace it with a new flock. Therefore, if the new flock is infected with *Campylobacter* spp. before its introduction to a layer house, *Campylobacter* spp. could be present in their feces for a long time, and other flocks reared on the same farm could repeatedly get infected. Moreover, repeated introduction of grower flocks infected with *Campylobacter* spp. could lead to the maintenance of high contamination levels, resulting in diversity in *Campylobacter* spp. STs.

The ciprofloxacin resistance (7.3%) in *C. jejuni* isolated from old flocks was significantly lower than that (32.5%) in *C. jejuni* isolated from young flocks. In Japan, fluoroquinolones can be used in immature hens before laying eggs. However, they cannot be used in layers because they are not approved by the government of Japan for use during the laying period.

Although the maximum residue level (MRL) of ampicillin in chicken eggs is set at 0.01 mg/kg in Japan, residue concentrations of ampicillin in chicken eggs during and after administration do not exceed the MRL, and no withdrawal period has been established [7]. Therefore, ampicillin can be used more easily and safely in grower and layer flocks than other antimicrobial agents, including fluoroquinolones, tetracyclines, and aminoglycosides. In this study, the highest antimicrobial resistance rate was observed for ampicillin in both *C. jejuni* and *C. coli* isolates. In particular, the two most frequent STs, ST4389 and ST6704, were resistant to ampicillin. These results indicate that ampicillin-resistant *Campylobacter* spp. isolates can be selected using ampicillin.

In conclusion, we found that the prevalence of *Campylobacter* spp. in layer flocks is high. The prevalence of *Campylobacter* spp. in chicken products derived from spent layers and in egg should be investigated to estimate their risk to human health.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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