Prevalence and antimicrobial resistance of Campylobacter and Salmonella in layer flocks in Honshu, Japan

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ABSTRACT. Campylobacter and non-typhoidal Salmonella are the major causes of bacterial gastrointestinal infections in humans. Although antimicrobial therapy is typically not recommended in many cases of these infections, it may be life-saving in patients with severe symptoms. Since chicken eggs and meat derived from layers are destined for human consumption, we investigated the prevalence and antimicrobial resistance of these two bacterial genera in 82 layer flocks at chicken processing plants in Honshu, Japan. Campylobacter was isolated from 77 flocks (93.9%). Resistance to ampicillin, tetracycline, and ciprofloxacin was documented in 42.3% (30/71), 16.9% (12/71), and 14.1% (10/71) of Campylobacter jejuni, respectively. Multilocus-sequence typing identified ST4389 and ST5262 as the most frequent C. jejuni sequence types. In C. coli, resistance to ampicillin, tetracycline, and ciprofloxacin was found in 20.0% (7/35), 20.0% (7/35), and 25.7% (9/35), respectively. The most frequent sequence type in C. coli was ST8292. Erythromycin resistance was not observed among Campylobacter species. Salmonella was isolated from 14 flocks (17.1%). The two most frequent serovars were Salmonella Corvallis and S. Braenderup. Neither S. Enteritidis nor S. Infantis were isolated. Streptomycin resistance was observed in six isolates (26.1%), and all isolates were susceptible to cefotaxime and ciprofloxacin. Thus, chicken eggs and meat derived from layers are possible sources of these bacterial infections in humans. The antimicrobial susceptibility of these isolates was maintained, reflecting restrictions on the use of antimicrobial agents on layers.

KEYWORDS: antimicrobial resistance, Campylobacter, layer, multilocus-sequence typing, Salmonella


**MATERIALS AND METHODS**

**Sampling**

Four chicken processing plants participated on a voluntary and anonymous basis in this study. These plants processed spent layer and breed chickens, but not broilers. Sampling was conducted between July 2019 and November 2021. On a sampling day, at each plant, a layer flock was selected, and the gastrointestinal tract was collected from five birds after evisceration. A flock is defined as a group of birds raised in the same layer house within the same period. Gastrointestinal tracts were not collected from flocks shipped from the same farm. Each tract was packed in a plastic bag and sent to the National Institute of Health Sciences via express delivery. The specimens were stored at 4°C until examination, which was performed within 24 hr of sampling.

**Campylobacter enumeration**

One gram of cecal content sample was taken from a gastrointestinal tract. Enumeration of *Campylobacter* in cecal samples was performed by serial dilutions (1:9) in buffered peptone water (Oxoid Ltd., Hampshire, UK), and each diluted suspension was plated in duplicate on modified charcoal cefoperazone deoxycholate agar plates (mCCDA; Oxoid). After incubation for 48 hr at 42°C, colony-forming units (CFUs) were counted. Two suspect colonies were cultured by plating them onto mCCDA and speciated using a multiplex polymerase chain reaction (PCR) [15] that identifies *Campylobacter jejuni*, *C. fetus*, *C. coli*, *C. upsaliensis*, *C. hyointestinalis*, and *C. lari*. The mean of duplicate counts was calculated and converted to log_{10} CFU/g of cecal content samples. The enumeration limit was 1.7 log_{10} CFU/g. One *Campylobacter* species per flock was subjected to multilocus-sequence typing (MLST) and antimicrobial susceptibility testing.

**MLST of Campylobacter isolates**

Multilocus-sequence typing was performed according to the seven-loci scheme for *Campylobacter jejuni* and *C. coli*, employing the primer sets and experimental conditions suggested by the *Campylobacter* MLST database (http://pubmlst.org/campylobacter/).

**Salmonella isolation**

For pre-enrichment, one gram of cecal content sample was mixed with 9 mL buffered peptone water and incubated at 37°C for 18 hr. After incubation, 0.1 and 1 mL of the culture was added to 10 mL Rappaport–Vassiliadis broth (Oxoid) and 10 mL Hajna tetrathionate broth (Eiken Chemical, Tokyo, Japan), respectively. After incubation at 42°C for 20 hr, each culture was streaked onto xylene-lysine-deoxycholate agar (Oxoid) and CHROMagar™ Salmonella (CHROMagar, Paris, France) selection plates, followed by incubation at 37°C for 24 hr. Two suspect colonies were biochemically identified, as previously described [21]. *Salmonella* isolates were tested by slide agglutination with *O* antisera (Denka Co., Tokyo, Japan, and SSI Diagnostica, Copenhagen, Denmark). One of each different *O* serogroup isolate per flock was tested for flagella antigens by tube agglutination using *H* antisera (Denka Co.). Serovars were determined based on the reaction between *O* and *H* group antigens according to the Kauffmann–White scheme [9]. One of each different serovar isolate per flock was subjected to antimicrobial susceptibility testing.

**Antimicrobial susceptibility testing of Campylobacter and Salmonella isolates**

Minimum inhibitory concentrations were determined by a two-fold broth microdilution method in 96-well microtiter plates (Eiken Chemical). *Campylobacter jejuni* ATCC33560 and *Escherichia coli* ATCC25922 were used as quality control strains for *Campylobacter* and *Salmonella* isolates, respectively.

Antimicrobial susceptibility testing in *Campylobacter* isolates was conducted against ampicillin (0.12–256 mg/L), streptomycin (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). The breakpoints for ampicillin (32 mg/L), streptomycin (32 mg/L), tetracycline (32 mg/L), nalidixic acid (32 mg/L), ciprofloxacin (4 mg/L), and chloramphenicol (16 mg/L) were adopted from the Clinical and Laboratory Standards Institute (CLSI) [3] and Japanese Veterinary Antimicrobial Resistance Monitoring (JVARM) system [22]. The breakpoint for gentamicin (2 mg/L) was specified by the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme [6].

Antimicrobial susceptibility testing in *Salmonella* isolates was conducted against ampicillin (1–128 mg/L), cefazolin (1–128 mg/L), cefotaxime (0.5–64 mg/L), cefotetracycline (1–128 mg/L), kanamycin (1–128 mg/L), tetracycline (0.5–64 mg/L), nalidixic acid (1–128 mg/L), ciprofloxacin (0.03–4 mg/L), colistin (0.12–16 mg/L), chloramphenicol (1–128 mg/L), gentamicin (0.5–64 mg/L), and trimethoprim (0.25–16 mg/L). The breakpoints for ampicillin (32 mg/L), cefalozolin (8 mg/L), cefotaxime (4 mg/L), kanamycin (64 mg/L), tetracycline (16 mg/L), nalidixic acid (32 mg/L), ciprofloxacin (1 mg/L), colistin (4 mg/L), chloramphenicol (32 mg/L), gentamicin (16 mg/L), and trimethoprim (16 mg/L) were adopted from the CLSI [4] and JVARM system [22].

**RESULTS**

A total of 410 cecal content samples were collected from 82 flocks shipped from 82 different farms (eight in Tohoku, thirty-nine in Kanto, thirteen in Koshin-etsu, fourteen in Tokai, two in Kinki, five in Hokuriku, and one in Chugoku regions on Honshu). The age of birds in the flocks ranged from 38 to 180 weeks (average: 96 weeks), with 76.8% of flocks (63/82) being dominated by birds aged from 90 to 110 weeks. *Campylobacter* was isolated from 308 cecal samples (75.1%) collected from 77 flocks (93.9%). In each *Campylobacter*-positive flock, the bacteria were isolated from at least two of five cecal samples. The mean intra-flock prevalence in
Campylobacter-positive flocks was 80.0% (308/385). The mean Campylobacter concentration (log_{10} ± standard deviation CFU/g) in the 308 Campylobacter-positive samples was 5.2 ± 1.6 log_{10} CFU/g, with 90.3% (278/308) of them having more than 3.0 log_{10} CFU/g. Of the 77 Campylobacter-positive flocks, 42 (54.5%) were colonized with C. jejuni, 6 (7.8%) with C. coli, and 29 (37.7%) with both C. jejuni and C. coli.

Resistance to ampicillin, tetracycline, nalidixic acid, and ciprofloxacin in 71 C. jejuni isolates was 42.3 (30/71), 16.9 (12/71), 14.1 (10/71), and 14.1% (10/71), respectively (Table 1). All C. jejuni isolates were susceptible to streptomycin, chloramphenicol, erythromycin, and gentamicin. Seventy-one C. jejuni isolates were classified into 32 sequence types (STs) (Table 2), with the two most frequent being ST4389 and ST5262, which belonged to clonal complex (CC) 464. CC464 accounted for 47.9% (34/71) of all C. jejuni isolates and 76.7% of ampicillin-resistant C. jejuni. ST4389 isolates were observed in 17 flocks carried from five regions (Tohoku, Kanto, Koshin-etsu, Hokuriku, and Chugoku). Moreover, 16 of 17 ST4389 isolates and all ST6704 isolates were resistant to ampicillin.

Resistance to ampicillin, tetracycline, nalidixic acid, and ciprofloxacin in 35 C. coli isolates was 20.0 (7/35), 20.0 (10/71), 25.7 (9/35), and 25.7% (9/35), respectively. All C. coli isolates were susceptible to streptomycin, chloramphenicol, erythromycin, and gentamicin. Thirty-five C. coli isolates were classified into 14 STs (Table 3). The most frequent ST was ST8292, which belonged to CC1150. CC1150 accounted for 68.6% (24/35) of C. coli isolates.

Salmonella was isolated from 26 cecal samples (6.3%) collected from 14 flocks (17.1%). The mean intra-flock prevalence in Salmonella-positive flocks was 37.1% (26/70). In each of the eight Salmonella-positive flocks, Salmonella was isolated from only one of five cecal samples. Of the 14 Salmonella-positive flocks, six were infected with two or more serologically different isolates. Twenty-three Salmonella isolates were serotyped into eleven serovars, while one was untypeable (Table 4). The most frequent serovar was S. Corvallis, followed by S. Braenderup and S. Javiana. S. Corvallis was obtained from seven flocks carried from four regions (Kanto, Koshin-etsu, Tokai, and Chugoku). Six Salmonella isolates (26.1%) were resistant to streptomycin, but none were resistant to the other 11 antimicrobials tested.

**DISCUSSION**

We recently reported that Campylobacter prevalence in layer flocks was 84.0%; C. jejuni ST4389 was the most frequent sequence type, and ciprofloxacin resistance was 19.8% [33]. In this study, we found elevated intra-flock Campylobacter prevalence (80.0%) and mean concentration (5.2 log_{10} CFU/g) in cecal contents from Campylobacter-positive flocks at the time of slaughtering. High intra-flock Campylobacter prevalence and cecal concentrations >5 log_{10} CFU/g were also reported in broiler flocks colonized with the microbe [12, 17, 31]. In most cases, the carcasses and meat of broiler flocks colonized with Campylobacter and slaughtered at chicken processing plants are contaminated with the microbe [30, 31], suggesting that the same is likely to occur with layer flocks colonized with Campylobacter. ST4389 and ST5262, the top two STs in C. jejuni identified in this study, accounted for 3.2% (6/188) of isolates from human C. jejuni infection cases in Japan between 2000 and 2017 [25, 40]. ST4389 is a minor ST in C. jejuni isolated from broilers and ST5262 is never isolated from broilers in Japan [1, 16, 26]. Eight STs (ST19, ST354, ST440, ST460, ST918, ST7289, ST4324, and ST6704) of C. jejuni and one ST (ST830) of C. coli were also isolated from human Campylobacter infection cases in Japan [16, 25, 40]. The present results show that there is a possibility that isolates from human Campylobacter infections originate from layers. Interestingly, 68.6% of C. coli isolates belonged to CC1150 in this study. It is well known that most of the C. coli isolated from humans, cattle, pigs, and broilers worldwide belong to CC828; CC1150 is rarely isolated from humans and these animals [5, 7, 14, 19, 23, 24, 27, 28, 42, 43]. In particular, C. coli isolates belonging to CC1150 have never been obtained from humans, cattle, pigs, broilers, or wild birds in Japan [2, 34, 35]. Therefore, the CC1150 lineage, and particularly ST8292, might be more adaptable to Japanese layers than the CC828 lineages.

Resistance to antimicrobials other than ampicillin in C. jejuni and C. coli isolated from layers was almost the same or lower than that reported for broilers via the JVARM system [22]. For example, resistance to tetracycline and ciprofloxacin in C. jejuni isolated from broilers in 2017 was 46.3 and 44.8%, respectively, which is approximately 3-fold the results from this study. The use of antimicrobials is not allowed during the laying period in Japan due to residual contamination in eggs. However, ampicillin is excluded from this ban because the concentration of ampicillin residue in chicken eggs during and after administration does not exceed the maximum allowable (0.01 mg/kg) [10]. Therefore, ampicillin can be used in layers more easily and safely than other antimicrobials, which in turn may favor the selection of ampicillin-resistant C. jejuni in layer farms [33].

In this study, S. Corvallis and S. Braenderup were predominant in layer flocks, and neither S. Enteritidis nor S. Infantis were isolated from any flocks. A national survey of Salmonella spp. in Japanese layer farms conducted between 2007 and 2008 reported

| Table 1. Antimicrobial resistance in *Campylobacter* and *Salmonella* isolated from layer flocks |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Species        | No.            | ABPC | CEZ | CTX | SM | KM | TC | NA | CPFX | CL | CP | EM | GM | TMP |
|----------------|----------------|------|-----|-----|----|----|----|----|------|----|----|----|----|-----|
| *C. jejuni*    | 71             | 30   | 2   | 0   | 0  | 0  | 12 | 10 | 10   | 0  | 0  | 0  | 0  | 0   |
|                | 7               | 7    | 0   | 0   | 0  | 6  | 0  | 0  | 0    | 0  | 0  | 0  | 0  | 0   |
| *C. coli*      | 35             | 7    | 2   | 0   | 0  | 0  | 0  | 0  | 0    | 0  | 0  | 0  | 0  | 0   |
| *Salmonella*   | 23             | 0    | 0   | 0   | 0  | 0  | 6  | 0  | 0    | 0  | 0  | 0  | 0  | 0   |

ABPC: ampicillin, CEZ: cefazolin, CTX: cefotaxime, SM: streptomycin, KM: kanamycin, TC: tetracycline, NA: nalidixic acid, CPFX: ciprofloxacin, CL: colistin, CP: chloramphenicol, EM: erythromycin, GM: gentamicin, TMP: trimethoprim, NT: not tested.
that the top five serovars were S. Cerro, S. Braenderup, S. Infantis, S. Corvallis, and S. Enteritidis [29]. This study suggests that the prevalence of S. Cerro, S. Infantis, and S. Enteritidis has decreased over the last 10 years, while S. Corvallis and S. Braenderup have been preserved in layer farms. According to the Infectious Agents Surveillance Report (https://www.niid.go.jp/niid/en/iasr/510-surveillance/iasr/graphs/2297-iasrgbe.html), while S. Enteritidis, S. Infantis, S. Schwarzengrund, S. Thompson, and S. Typhimurium have been the predominant serovars isolated from patients with Salmonella infection, S. Corvallis and S. Braenderup ranked within the top ten serovars isolated from patients with Salmonella infection in 2020 and 2021. Among 235 local broiler chicken products sampled from retail stores between 2018 and 2021, the prevalent serovars were S. Schwarzengrund, S. Infantis, and S. Manhattan; in contrast, S. Corvallis and S. Braenderup were not isolated [32]. Shimojima et al. [36] investigated the presence of Salmonella in 1,378 local meat products (576 chicken, 406 pork, and 396 beef products) and 993 imported meat products (281 chicken, 393 pork, and 319

| Table 2. Sequence types and antimicrobial resistance profiles of Campylobacter jejuni isolates |
|-----------------|----------------|----------------|
| CC   | ST  | ARP                              | No. |
| 21   | 19  | ABPC+NA+CPFX                      | 1   |
|      |     | NA+CPFX                          | 1   |
| 2789 |     | ABPC                             | 1   |
|      |     | NA+CPFX                          | 1   |
|      |     | Susceptible                      | 2   |
| 9775 |     | Susceptible                      | 1   |
| 10208|     | Susceptible                      | 1   |
| 11365|     | Susceptible                      | 1   |
| 45   | 11367| TC                               | 1   |
| 48   | 918  | Susceptible                      | 3   |
| 353  | 400  | NA+CPFX                          | 1   |
| 10207|     | Susceptible                      | 1   |
| 10013|     | ABPC+TC+NA+CPFX                  | 1   |
| 11366|     | ABPC                             | 1   |
| 354  | 354  | Susceptible                      | 1   |
| 653  |     | Susceptible                      | 1   |
| 443  | 440  | NA+CPFX                          | 1   |
|      |     | Susceptible                      | 3   |
| 460  | 460  | Susceptible                      | 1   |
| 460  | 460  | Susceptible                      | 1   |
| 11026|     | TC                               | 1   |
| 464  | 4389 | ARPC                             | 13  |
|      |     | ABPC+TC                          | 3   |
|      |     | Susceptible                      | 1   |
| 5262 |     | Susceptible                      | 7   |
|      |     | TC+NA+CPFX                       | 1   |
| 6704 |     | ABPC                             | 4   |
| 9769 |     | Susceptible                      | 2   |
| 10209|     | ABPC+TC                          | 1   |
| 11015|     | ABPC+NA+CPFX                     | 1   |
| 11024|     | ABPC                             | 1   |
| Unassigned | 1972| ABPC+TC+NA+CPFX                  | 1   |
|      |     | Susceptible                      | 1   |
| 4324 |     | Susceptible                      | 1   |
| 4622 |     | Susceptible                      | 1   |
| 10205|     | Susceptible                      | 1   |
| 10206|     | ABPC+TC                          | 1   |
| 10401|     | Susceptible                      | 1   |
| 11014|     | ABPC+TC+NA+CPFX                  | 1   |
| 11023|     | TC                               | 1   |
| Total|     |                                  | 71  |

| Table 3. Sequence types and antimicrobial resistance profiles of Campylobacter coli isolates |
|-----------------|----------------|----------------|
| CC   | ST  | ARP                              | No. |
| 828  | 830  | TC+NA+CPFX                       | 1   |
| 10392|     | Susceptible                      | 2   |
| 10393|     | TC                               | 1   |
| 11021|     | TC                               | 1   |
| 1150 2637 |     | ABPC+NA+CPFX                     | 1   |
| 4107  |     | ABPC+TC+NA+CPFX                  | 1   |
| 8292  |     | ABPC                             | 2   |
| 9025  |     | Susceptible                      | 11  |
| 10402|     | ABPC+NA+CPFX                     | 1   |
| 11013|     | Susceptible                      | 2   |
| 11027|     | Susceptible                      | 1   |
| Unassigned 10385 |     | Susceptible                      | 1   |
| 10403|     | Susceptible                      | 1   |
| 10438|     | ABPC+NA+CPFX                     | 2   |
|      |     | NA+CPFX                          | 2   |
| Total|     |                                  | 35  |

| Table 4. Serovars and antimicrobial resistance profiles of Salmonella isolates |
|-----------------|----------------|----------------|
| O serogroup | Serovar | ARP | No. |
| O:4 | Typhimurium | Susceptible | 1 |
| O:7 | Braenderup | SM | 1 |
| O:7 | Braenderup | Susceptible | 3 |
| O:7 | Mbanda | SM | 1 |
| O:7 | Thompson | SM | 1 |
| O:7 | Potsdam | Susceptible | 1 |
| O:7 | Richmond | SM | 1 |
| O:8 | Corvallis | Susceptible | 7 |
| O:9 | Javiana | SM | 1 |
| O:13 | Untypeable | Susceptible | 1 |
| O:18 | Cerro | Susceptible | 1 |
| O:35 | Alachua | SM | 1 |
| O:40 | Jahannesburg | Susceptible | 1 |
| Total |     | 23 |

CC: clonal complex, ST: sequence type, ARP: antimicrobial resistance profile, ABPC: ampicillin, TC: tetracycline, NA: nalidixic acid, CPFX: ciprofloxacin.

CC: clonal complex, ST: sequence type, ARP: antimicrobial resistance profile, ABPC: ampicillin, TC: tetracycline, NA: nalidixic acid, CPFX: ciprofloxacin.
beef products) between 2009 and 2017; however, neither S. Corvallis nor S. Braenderup were isolated. Therefore, S. Corvallis and S. Braenderup isolated from human Salmonella infections likely originate from layers.

In conclusion, we found that the prevalence of Campylobacter in layer flocks remained high throughout the investigation period, and S. Corvallis and S. Braenderup were the predominant Salmonella serovars. Some of the clinical isolates of Campylobacter and Salmonella infections in humans likely originate from layers. Depending on the characteristics of the clinical isolate, it cannot be estimated whether the isolate originated from the egg or meat of the layers. The possibility that meat derived from layers with these bacteria may be a source of infection in humans could not be completely ruled out in the present results. To estimate the health risk associated with Campylobacter and Salmonella infections in humans, the prevalence of these two genera in chicken meat derived from layers should be investigated. All Salmonella isolates were susceptible to ciprofloxacin and cefotaxime; all Campylobacter isolates were susceptible to erythromycin, and ciprofloxacin resistance was lower in layers than in Campylobacter isolated from broilers [22]. The antimicrobial susceptibility of these isolates was maintained, reflecting restrictions on the use of antimicrobial agents on layers.

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

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