



Public Health

NOTE

Characterization of *Campylobacter jejuni* in large-billed crows (*Corvus macrorhynchos*) in Tochigi prefecture, Japan

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ABSTRACT. As free-living crows are a potential source of *Campylobacter* infections in broilers and cattle, we characterized *Campylobacter* spp. isolated from crows using multilocus sequence typing and antimicrobial susceptibility testing. We obtained 82 samples from 27 birds captured at seven different times using a trap set in Tochigi prefecture, Japan. *Campylobacter jejuni* was isolated from 55 (67.1%) of the 82 samples and classified into 29 sequence types, of which 21 were novel. Tetracycline and streptomycin resistance rates were 18.2% and 3.6%, respectively. These results show that most types of *C. jejuni* infecting crows differ from those isolated from humans, broilers, and cattle. Thus, the importance of free-living crows as reservoirs of *Campylobacter* infections in broilers and cattle may be limited.

KEYWORDS: antimicrobial resistance, Campylobacter, crow, sequence type

Campylobacter spp. is the most common cause of human gastroenteritis worldwide [28]. Kumagai *et al.* [12] investigated foodborne outbreaks in Japan caused by *Campylobacter* spp. during 2007–2018 and concluded that primary sources of the disease were chicken (80.3%) and beef products (10.5%). The Japanese Ministry of Agriculture, Forestry and Fisheries has designated *Campylobacter* spp. as a prioritized hazard in food safety risk management, identified chicken and beef as the principal sources of *Campylobacter* spp. in broiler and beef farms [9, 10, 21, 22]. A national survey conducted in 2009 found that the prevalence of *Campylobacter* spp. in Japanese broiler flocks was 47.2% (67/142) [9]. Drinking water, feed, transport crates, cattle, and wildlife have been identified as potential reservoirs of *Campylobacter* spp. in Japanese beef farms was 88.0% (22/25) [10]. Free-living crows have easy access to livestock farms; therefore, they could be a potential source of *Campylobacter* infections in the livestock. The crows breed in urban and agricultural settings where they fly around and forage different locations, including livestock farms. Weis *et al.* [27] detected *Campylobacter* spp. in 66.9% (85/127) of free-ranging American crows (*Corvus brachyrhyncos*). In Japan, Maeda *et al.* [14] reported that *C. jejuni* was the most dominant bacterium in the intestinal microbiota of a large-billed crow (*Corvus macrorhynchos*).

Campylobacter infections in humans typically cause acute self-limiting gastroenteritis, and antimicrobial therapy is not provided in most cases. However, when patients are immunocompromised or have other comorbidities, antimicrobial treatment may be necessary [30]. Therefore, antimicrobial-resistant *Campylobacter* spp. are a serious threat to public health. In this study, we aimed to characterize *Campylobacter* spp. isolated from free-living crows using multilocus sequence typing (MLST) and antimicrobial susceptibility testing.

To capture free-living crows, a mailbox type trap $(2.5 \times 3.0 \times 2.5 \text{ m})$ was built in a private forest in Tochigi prefecture. There was a beef farm within 20 m and several livestock farms including a broiler farm within 3.5 km of the trap. A day before capturing the crows, leftovers from a restaurant were left in the trap. An average of 12 crows were captured every two weeks from May to July 2014. For each crow, a cloacal swab sample was taken using a BBL CultureSwab Plus with Charcoal, Single Swab (Becton

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Dickinson, Sparks, MD, USA). A numbered looping plastic cable tie was loosely attached to each crow's tarsus for identification. The permit to trap jungle crows was obtained from the Tochigi prefecture (No. 0010), and approval for the study was obtained from Utsunomiya University (Animal Experiment Permission No. A11-0012). All procedures in this study were performed in accordance with the ethical guidelines for the Care and Use of Laboratory Animals at Utsunomiya University.

The cloacal swab samples were sent under refrigeration to the National Veterinary Assay Laboratory by express delivery and examined within 48 hr of sampling. Campylobacter spp. was isolated by direct plating on modified charcoal cefoperazone deoxycholate agar, as previously described [21]. A maximum of two suspect isolates per sample were identified by PCR [13]. One Campylobacter species per sample was used for MLST and antimicrobial susceptibility testing. MLST was performed according to the seven-loci schemes for Campylobacter spp., employing the primer sets and experimental conditions suggested by the Campylobacter MLST database (http://pubmlst.org/campylobacter/). To assess the phylogenetic diversity of C. jejuni isolates from the crows, seven MLST allelic numbers for 43 sequence types (STs) were analyzed using PHYLOViZ Online (https://online.phyloviz.net/index). The minimum inhibitory concentrations of antimicrobials were determined using the broth microdilution method with dried plates (Eiken Chemical, Tokyo, Japan), following guidelines of the Clinical and Laboratory Standards Institute [5, 6] and reports of the Japanese Veterinary Antimicrobial Resistance Monitoring [16]. C. jejuni ATCC33560 was used as a quality control strain. Susceptibility testing of C. jejuni was conducted against ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, streptomycin, and tetracycline. For tetracycline-resistant isolates, PCR detection of tet genes was performed as previously described [17]. For streptomycin-resistant isolates, two primers (AadE-F1: AAGGCGGGAAAGCCCTAAAATGGC and AadE-R1: CTCTTTCTGATTTTGAGGTATTCAG) were used to amplify the complete coding sequence of *aadE* (GenBank accession no. AY701528) [18]. The cycling conditions for PCR using the primer set were 25 amplification cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by 72°C for 5 min. The nucleotide sequence of the PCR product (998 bp) was determined by direct DNA sequencing.

Sampling was conducted every seven days, and a total of 82 cloacal swab samples were collected. The 82 samples were composed of cloacal swabs from 27 birds. Of these, 21 (77.8%) birds were captured twice or more. All captured birds were largebilled crows. *Campylobacter* spp. were isolated from 55 (67%) of the 82 samples. All isolates were identified as *C. jejuni*. One *C. jejuni* isolate per sample was used for MLST and antimicrobial susceptibility testing. The 55 *C. jejuni* isolates were divided into 29 STs (Table 1). Of the 29 STs, 21 (72.4%) were novel. These 29 STs were phylogenetically different from predominant STs (ST19, ST21, ST22, ST42, ST48, ST50, ST61, ST407, ST918, ST922, ST4253, and ST4626) in human campylobacteriosis [2, 3, 20, 30] (Fig. 1). Although ST45, ST2761, ST8276, and ST6307 belonged to clonal complex (CC) 45, CC952, CC952, and CC1275, respectively, the remaining 25 STs were unassigned. Ten (18.2%) isolates (ST45, one of the four ST2117 isolates, ST2216, ST8277, ST8282, all three ST8289 isolates, and the two ST8290 isolates) were resistant to tetracycline and carried *aadE*. No isolates were resistant to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, or nalidixic acid.

The prevalence of *C. jejuni* on each sampling day ranged from 25.0% (8th July) to 100.0% (1st May) (Table 2). Of the 27 birds captured for this study, 24 (88.9%) had *C. jejuni* in their gastrointestinal contents on at least one sampling day. The remaining three birds (crow codes c16, c17, and c27) were captured only once during this study and did not shed *Campylobacter* spp. in their gastrointestinal contents. Of the 21 birds captured twice or more, only four (19.0%) (crow codes c3, c9, c20, and c25) had *C. jejuni* on every sampling day. Moreover, except for a single bird (crow code c1), none of the *C. jejuni* isolates classified into the same ST were obtained from the same birds on subsequent captures. Interestingly, although the two isolates obtained from crow c1 on 1st and 13th May were classified into the same ST (ST8290), *Campylobacter* spp. was not isolated on 26th May, and the *C. jejuni* isolate obtained on 10th June was classified into another ST (ST6307).

The results of the present study show that genetically diverse *C. jejuni* strains can infect free-living crows. In addition, these findings show that the shedding of *C. jejuni* into the gastrointestinal contents is intermittent and different *C. jejuni* strains can alternately infect a single crow. Shyaka *et al.* [24] collected cloacal swab samples from 139 crows (*Corvus corone* and *Corvus macrorhynchos*) in the Tokachi area of Hokkaido, Japan, from May 2010 to May 2011, and isolated *Campylobacter* spp., most of which were identified as *C. jejuni*, from 27 (20.9%) birds. Okamura *et al.* [20] collected cecal content samples from 123 crows (*78 Corvus corone*, 28 *Corvus macrorhynchos*, and 17 unidentified species) in Aomori prefecture, Japan, from October 2012 to April 2014, and isolated *Campylobacter* spp. from all the samples, most of which were also identified as *C. jejuni*. Between these two studies, the sample types, timing of implementation of the surveys, and survey locations differed. These differences could influence the prevalence of *Campylobacter* spp. In this study, intermittent shedding of *C. jejuni* was observed in most of the crows. Since the timing of shedding differs depending on the presence of *Campylobacter* infection in crows, the prevalence of *Campylobacter* spp. in crows fluctuates widely. The fact that, different *C. jejuni* strains alternately infected a crow is consistent with the results of another study demonstrating that strain-specific immune response by the host is important in controlling a shift in *C. jejuni* strains during the infection period [25].

Although, a large number of *C. jejuni* isolates from humans, broilers, and cattle in Japan have been genetically characterized by MLST [2, 3, 11, 19, 20, 29], the STs of *C. jejuni* isolates in those studies considerably differ from the *C. jejuni* isolates in crows captured in the present study. Although, CC21 is the most frequent lineage in humans, broilers, and cattle in Japan [2, 3, 11, 19, 20, 29], it was not isolated in the present study. Of the 55 *C. jejuni* isolates obtained in the present study, 35 (63.6%) were classified into 21 novel STs. These isolates might be more adaptable to crows than to humans, broilers, and cattle. ST6307, the most frequent ST in this study, has never been isolated from humans and livestock in Japan, although ST6307 is associated with wild birds and environmental water overseas according to the PubMLST database of *C. jejuni/coli* isolates (https://pubmlst.org/

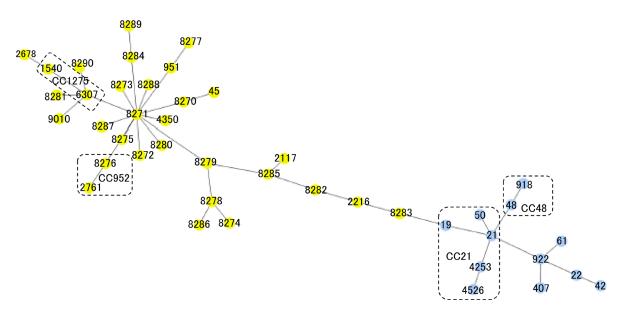


Fig. 1. Phylogenetic tree of *Campylobacter jejuni* isolated from crows (yellow) and humans (blue). Numbers represent sequence types. CC represents clonal complex.

Table 1.	Sequence types and antimicrobial resistance profiles
of Ca	mpylobacter jejuni isolates

Clonal	Sequence	Nunber of	Antimicrobial
complex	type	isolates	resistance profile
CC45			
	ST45	1	TC (1)
CC952			a
	ST2761	2	Susceptible (2)
~~~~~	<u>ST8276</u>	1	Susceptible (1)
CC1275	ST6307	8	Susceptible (8)
Unaccionad	310307	0	Susceptible (8)
Unassigned	ST951	2	Susceptible (2)
	ST2117	4	Susceptible (3), TC (1)
	ST2216	1	TC (1)
	ST2678	1	Susceptible (1)
	ST4350	1	Susceptible (1)
	<u>ST8270</u>	1	Susceptible (1)
	<u>ST8271</u>	2	Susceptible (2)
	<u>ST8272</u>	1	Susceptible (1)
	<u>ST8273</u>	1	Susceptible (1)
	<u>ST8274</u>	2	Susceptible (2)
	<u>ST8275</u>	1	Susceptible (1)
	<u>ST8277</u>	1	SM+TC (1)
	<u>ST8278</u>	2	Susceptible (2)
	<u>ST8279</u>	3	Susceptible (3)
	<u>ST8280</u>	3	Susceptible (3)
	<u>ST8281</u>	1	Susceptible (1)
	<u>ST8282</u>	1	SM+TC (1)
	<u>ST8283</u>	1	Susceptible (1)
	<u>ST8284</u>	2	Susceptible (2)
	<u>ST8285</u>	1	Susceptible (1)
	<u>ST8286</u>	2	Susceptible (2)
	<u>ST8287</u>	3	Susceptible (3)
	<u>ST8288</u>	1	Susceptible (1)
	<u>ST8289</u>	3	TC (3)
	<u>ST8290</u>	2	TC (2)

Novel sequence types are underlined. Parentheses show the number of isolates. TC; tetracycline, SM: streptomycin.

bigsdb?db=pubmlst campylobacter isolates). The ST6307 isolates were obtained from eight different crows from 1st May to 10th June and all isolates were susceptible to all the antimicrobials tested, suggesting that the ST6307 clone circulates in crows in Tochigi prefecture. Similarly, ST2117, the second most frequent ST in this study, has never been isolated in Japan, although ST2117 is associated with four human sporadic cases in Scotland according to the PubMLST database. Okamura et al. [20] reported that C. jejuni isolates belonging to ST2761, ST9010, and ST1540 were obtained from free-living crows in Aomori prefecture, Japan. Among these, ST2761 was isolated in this study as well, and the remaining two were phylogenetically more related to C. jejuni isolated from crows in this study than those isolated from human campylobacteriosis (Fig. 1). ST45 isolates belonging to CC45 have also been obtained from sporadic human cases and broilers in Japan. CC45 is a generalist lineage with broad host ranges [15, 23].

All C. jejuni isolates obtained in the present study were susceptible to ciprofloxacin. In contrast, ciprofloxacin resistance in C. jejuni has been prevalent in humans, broilers, and cattle in Japan [3, 9, 10, 16, 19, 29]. In this study, low prevalence rates of C. jejuni isolates resistant to tetracycline and/or streptomycin were observed. The two antimicrobial resistance genes, tetO and *aadE*, are known to occur on transmissible plasmids in Enterococcus faecalis and Streptococcus agalactiae [4, 31]. Some reports show high sequence similarity in plasmids between C. jejuni and Gram-positive bacterial genera [8, 26]. Moreover, Nirdnoy et al. [18] reported that pCG8245 carrying tetO and aadE was mobilized using a P incompatibility group plasmid from C. jejuni to Escherichia coli. These reports suggest that C. jejuni exchanges transmissible plasmids with E. coli and Gram-positive bacterial genera. Therefore, these antimicrobial resistance genes could be transmitted from bacteria found in the gastrointestinal tracts of crows. All C. jejuni isolates classified as ST8289 were resistant to tetracycline and found in three crows, suggesting that antimicrobial-resistant C. jejuni strains circulate in crow populations under conditions of no antibiotic selection pressure.

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Crow		Sampling date					
code	1-May	13-May	26-May	10-Jun	24-Jun	8-Jul	22-Jul
c1	ST8290 ^a	ST8290	NI ^b	ST6307	NI	NI	NI
c2	ST6307	ST8279	NI	ST8284	ST4350	NI	NI
c3	ST8280	ST8276	ST8286	ST8289		ST8282	
c4	ST6307	NI	ST8289	NI			
c5	ST8280	NI	ST2678			NI	ST8287
c6	ST8280	NI					
c7	ST8286	NI					
c8	ST8271		ST6307	NI	ST8274		
c9	ST8275		ST6307	ST8271	ST2761		
c10	ST6307			ST8278			
c11		ST6307	ST8279	ST8273	ST8281	NI	
c12		NI	ST8288		ST2761		
c13		ST951	ST8272				
c14		NI	ST2216	ST45			
c15		NI	ST8289		ST951		
c16		NI					
c17		NI					
c18		NI	ST2117				
c19			ST2117	ST8283		NI	NI
c20			ST2117			ST8287	ST8285
c21			ST6307	ST8270		NI	
c22			NI	NI	ST8279		ST8277
c23				ST8278			
c24				ST8287			
c25				ST8284	ST8274		
c26					ST2117		
c27							NI
Prevalence	ce 100.0%	33.3%	82.4%	80.0%	90.0%	25.0%	42.9%

Table 2. I	Isolation and sec	juence types of	Campylobacter	<i>jejuni</i> in captured crows
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^aSequence type of *C. jejuni* isolate. ^bNot isolated.

In conclusion, although various types of *C. jejuni* infect crows living in Tochigi prefecture, Japan, most of these strains differ from those isolated from humans, broilers, and cattle in Japan. Therefore, the importance of free-living crows as reservoirs of *Campylobacter* infection in broilers and cattle may be limited. However, *Campylobacter* spp. in free-living crows should be continuously monitored because crows can have generalist lineages in their gastrointestinal tracts.

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

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