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Virulence characterization of *Campylobacter jejuni* isolated from resident wild birds in Tokachi area, Japan

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ABSTRACT. The prevalence of *Campylobacter jejuni* in wild birds is a potential hazard for human and animal health. The aim of this study was to establish the prevalence of *C. jejuni* in wild birds in Tokachi area, Hokkaido, Japan and investigate their virulence *in vitro*. In total, 173 cloacal swabs from individual wild birds were collected for the detection of *Campylobacter* spp. Thirty four samples (19.7%) were positive for *Campylobacter* of which 94.1% (32/34 samples) were *C. jejuni*. Additionally, one *C. coli* and one *C. fetus* were isolated. Seven *C. jejuni* isolates (one from crows and the other from pigeons) had important virulence genes including all three CDT genes (*cdtA*, *cdtB* and *cdtC*) and *flaA*, *flaB*, *ciaB* and *cadF*, and the other isolates were lacking *cdtA* gene. Further studies on *in vitro* virulence-associated phenotypes, such as motility assay on soft agar and invasion assay in Caco-2 cells, were performed. The wild bird *C. jejuni* isolates adhered and invaded human cells. Although the numbers of viable intracellular bacteria of wild bird isolates were lower than a type strain NCTC11168, they persisted at 48-hr and underwent replication in host cells.
KEY WORDS: *Campylobacter,* Japan, virulence, wild bird

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The genus *Campylobacter* is microaerophilic, spiral-shaped, motile Gram-negative bacilli with unipolar or bipolar flagella [33]. It is widely distributed in multiple animal hosts including livestock, wild and companion animals, and also, it is found from environmental sources [8, 22]. Among 17 species of the genus, *C. jejuni* is the leading cause of human bacterial gastroenteritis in developed countries and accounts for ~90% of cases of campylobacteriosis [40]. Patients present with watery and bloody diarrhea accompanied by fever and abdominal pain after a latency period of 2 to 5 days, as well as headaches and nausea [7]. *Campylobacter* infection has also been suggested to be associated with acute polyradiculitis (Guillain-Barré syndrome) [3, 29].

In US, 13,000 hospitalizations attributed to *C. jejuni* including 100 deaths are reported each year [36]. On the other hand, 34 cases per 100,000 inhabitants and 300 cases per 100,000 inhabitants were reported annually in Canada (http://dsol-smed.phac-aspc.gc.ca/dsol-smed/ndis/charts.php?c=pl) and New Zealand [4], respectively. In Japan, *C. jejuni* is also recognized as the prominent foodborne bacterium since 1998, and it has accounted for 300 to 600 cases each year according to the statistical data from Ministry of Health, Labour and Welfare (http://idsc.nih.go.jp/iasr/31/359/tpc359.html).

Humans are infected mainly through consumption of contaminated raw or undercooked food and drinking water [2, 10]. Other potential routes of infection have been documented, such as contact with reservoir animals including farm and companion animals, and environmental exposures including wild birds [16], since this pathogen has the ability to colonize a range of environmental reservoirs and multiple animal hosts [31].

C. jejuni is considered to be a commensal bacterium in poultry where its infection is high, with a rate ranging from 50 to 80% [5, 35]. Bird intestines provide optimal conditions for the growth of thermophilic *Campylobacter*, allowing these avian species to serve as asymptomatic carriers [3, 5, 9].

Wild birds are also hosts to *Campylobacter* species, and because of their mobility, they may cause widespread contamination. *Campylobacter* has been isolated from birds, such as crows and pigeons, sharing the same living area and habitat with humans [12, 17, 19, 21, 26, 38]. Certain cases of contaminated food and drinking water have also been caused by transmission via wild birds [1, 3, 38]. These reports suggest that wild birds may have an impact on *Campylobacter*

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infection in humans. Further, scavenging birds, such as sea gulls and crows, hunting for food in areas with raw garbage, are of concern as they may disseminate the bacteria to humans and livestock through contact or environmental contamination.

Previous studies in Japan have reported the occurrence of *C. jejuni* in wild birds [12, 17, 21, 25–27]. However, the potential virulence of wild bird isolates to humans has not been investigated. In this study, we isolated *Campylobacter* from resident wild birds and examined their virulence characteristics to evaluate the potential health risk to humans and animals.

MATERIALS AND METHODS

Sample collection: A total of 173 cloacal swab samples were collected from individual dead birds including 139 crows (*Corvus corone* and *C. macrorhynchos*), 24 pigeons (*Columbia livia* and *Streptopelia orientalis*) and 10 Eurasian tree sparrows (*Passer montanus*). Wild birds were captured by a bird trap for pest control in Tokachi area, Hokkaido, Japan during May 2010 to May 2011. The birds were sacrificed with gas euthanasia. We also collected samples from officially-sanctioned hunted birds during the above period. Birds were taken to the laboratory, and cloacal feces were immediately transferred to commercial transport medium (Eiken Chemical, Tochigi, Japan) with sterile cotton-tipped swabs. Samples were kept at cool temperature and processed for the microbiological isolation within one day after sampling.

Isolation and identification of Campylobacter isolates: One m*l* portion of transport medium was transferred to 9 m*l* of Bolton broth (Oxoid, Hampshire, U.K.). The broth culture was incubated at 42°C under microaerobic condition (AnaeroPack-MicroAero, Mitsubishi Gas Chemical, Tokyo, Japan) for 48 hr, and subsequently, a loopful of culture was streaked on modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plate (Oxoid). The plates were incubated at the same condition for 48 hr. Suspected colonies were subjected to Gram-staining, oxidase test, catalase test and Dryspot *Campylobacter* test (Oxoid). All *Campylobacter* isolates were subjected to 16S rDNA sequencing for inter species identification [38]. The reactions were performed using SYBR Premix Ex Taq Kit (Takara Bio, Otsu, Japan), and amplification was carried out in a LightCycler 480 (Roche Diagnostics, Rotkreuz, Switzerland).

Detection of virulence genes: DNA extraction from *C. jejuni* isolates was performed using PrepMan Ultra Sample Preparation Reagent (Life Technologies Japan, Tokyo, Japan) according to the manufacturer's instruction. Real-time PCR was used to examine the presence of following virulence-associated genes in: *flaA*, *flaB*, *ciaB*, *cadF*, *cdtA*, *cdtB* and *cdtC*. The amplification was performed as described previously [11]. The size of the obtained amplicon was verified using a 2% gel electrophoresis and visualized with UV trans-illumination after ethidium bromide staining.

Bacterial motility: Swarming motility of *C. jejuni* isolates was investigated according to the method described by Scott *et al*. [34]. Briefly, two microliters of grown bacteria were inoculated onto Mueller Hinton II broth (BD, Franklin Lakes, NJ, U.S.A.) with 0.4% agar. The diameter of the resulting swarming colonies was measured following incubation for 23 hr at 42°C in microaerobic conditions. *C. jejuni* strain NCTC11168, which was a clinical isolate and widely used, was used as a control [18].

Gentamicin protection assay: Invasive abilities of *C. jejuni* isolates to human colonic epithelial cell line Caco-2 cells were examined by gentamicin protection assay with slight modifications [14]. Caco-2 cells was maintained in Eagle's Minimum Essential Medium (E-MEM; Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, U.S.A.), penicillin-streptomycin, 100 mM L-glutamine, 100 mM sodium pyruvate and E-MEM amino acid (Sigma) at 37°C with 5% CO_2. The monolayer of Caco-2 cells (1.0×10^5 cells/well) in a 24-well culture plate was infected with *C. jejuni* at MOI of 50, and the culture plates were centrifuged at 3,000 rpm for 5 min to allow inoculated bacteria to adhere on Caco-2 cell surface. After 1 hr, non-adherent bacteria were removed by washing with 9.6 mM phosphate buffered saline (PBS), pH 7.4. The extracellular bacteria were killed by gentamicin (100 μg/m*l*) for 2 hr at 37°C. After washing, the medium was replaced with E-MEM with 10% FBS, and then, the Caco-2 cells were lysed by 500 μl of 0.1% Triton X- 100 (Wako Pure Chemical, Osaka, Japan) in PBS at 1-hr, 24-hr and 48-hr post infection. The intracellular culturable bacteria were counted on mCDDA medium.

Statistical analysis: The significance of differences between the groups was evaluated by one-way analysis of variance (ANOVA). Statistical analysis was performed with Graph Pad Prism software version 5 (Graph Pad Software Inc., La Jolla, CA, U.S.A.). The *P*-values <0.05 were considered significant.

RESULTS

Prevalence of Campylobacter spp. in wild birds in Obihiro: A total of 19.7% (34/173) samples from wild-living birds, including 20.9% (27/139) from the crows, 25% (6/24) from the pigeons and 10% (1/10) from the sparrows, were positive for *Campylobacter* spp. (Table 1). Of the 27 crow-originated isolates of *Campylobacter* spp., 25 (92.6%) were identified as *C. jejuni*, 1 (3.7%) as *C. coli* and 1 (3.7%) as *C. fetus*. Six samples from pigeons and one sample isolated from Eurasian tree sparrows, all *Campylobacter*-positive, were all confirmed as *C. jejuni*.

Detection of virulence genes using real-time PCR: Thirty-three isolates of *C. jejuni* from wild birds were screened for the presence of virulence genes. Real-time PCR was used to seven important *C. jejuni* virulence-related genes namely *cdtA*, *cdtB* and *cdtC*, genes related with the cytotoxin expression, *flaA*, *flaB* and *cadF*, linked with adherence and colonization; and *ciaB*, associated with invasion (Table 2). Seven *C. jejuni* isolates (six isolated from pigeons and one from a crow) harbored all the 7 virulence genes investigated. Other remaining 25 isolates (24 from crows and 1 from a Eurasian

Table 1. Prevalence of *C. jejuni* in wild birds in Obihiro

Birds	Total no. of samples	No. of samples positive for *Campylobacter* spp. (%)	Isolated species and proportion (%)	
Crow (*Corvus corone* and *C. macrorhynchos*)	139	27 (20.9)	*C. jejuni*	25 (92.6)
			C. coli	1 (3.7)
			C. fetus	1 (3.7)
Pigeon (*Columba livia* and *Streptopelia orientalis*)	24	6 (25.0)	*C. jejuni*	6 (100.0)
Eurasian tree sparrow (*Passer montanus*)	10	1 (10.0)	*C. jejuni*	1 (100.0)
Total	173	34 (19.7)	*C. jejuni*	32 (15.5)
			C. coli	1 (10.6)
			C. fetus	1 (10.6)

tree sparrow) were positive for *cdtB*, *cdtC*, *flaA*, *flaB*, *cadF* and *ciaB*, but *cdtA* gene (Table 2).

Motility assay on soft-agar plate: Since 7 isolates (C38, P3, P5, P6, P8, P9 and P10) were found to be positive for the 7 virulence genes, flagella-mediated motility of them was studied using a standard soft-agar assay. One isolate (C38, from crow sample) showed a high motility after 23-hr incubation, whereas no motility was observed in other isolates (Fig. 1).

Invasiveness and proliferation of C. jejuni isolates from wild birds: To understand the *C. jejuni* isolates invasion and proliferation ability in an epithelial cell-line, 7 isolates harboring all 7 virulence-associated genes (C38, P3, P5, P6, P8, P9 and P10) and 2 isolates lacking *cdtA* gene (C1 and C2) were used in the human intestinal Caco-2 cell-line invasion assay. As observed 1-hr post-infection (Fig. 2), isolates from wild birds showed a high ability to invade Caco-2 cells at a comparable level as with NCTC11168. All isolates tested adhered and invaded into Caco-2 cells, and persisted within the cells for 48 hr. No significant difference was observed between the isolates. After 24 hr, all the tested isolates showed 1.2 log-reduction in the internal bacteria number counts. At 48 hr, isolates from wild birds showed a slight increase (average, 0.2 Log) and underwent replication. The number of bacteria of wild bird isolates at 24 hr and 48 hr was significantly lower than those of NCTC11168 (Fig. 2).

Table 2. Real-time PCR results of virulence-related genes in *C. jejuni* isolated from wild birds

Birds (no. of samples)	*cdtA*	*cdtB*	*cdtC*	*flaA*	*flaB*	*ciaB*	*cadF*
Crow (24)	–	+	+	+	+	+	+
Crow (1)	+	+	+	+	+	+	+
Pigeon (6)	+	+	+	+	+	+	+
Eurasian tree sparrow (1)	–	+	+	+	+	+	+

DISCUSSION

The present study yielded the following new findings. First, our study is the first report of *Campylobacter* spp. prevalence in resident wild birds of Hokkaido area. Second, we examined virulence-associated activities of wild bird *C. jejuni* isolates in comparison with those of type strain

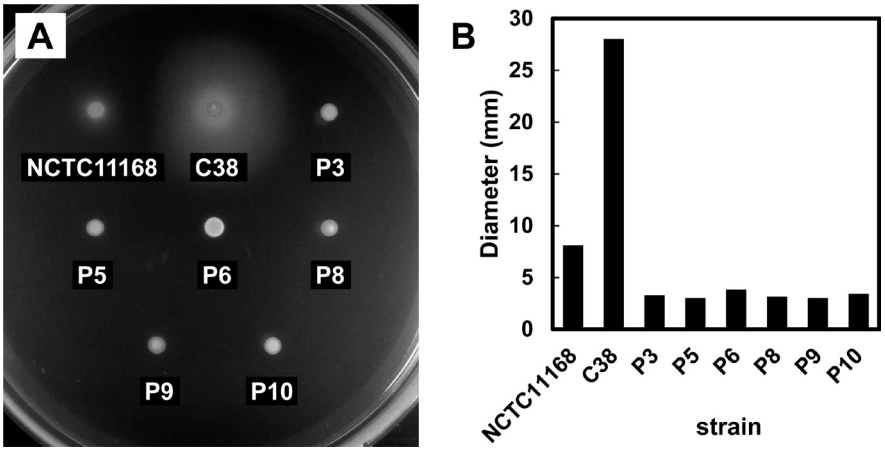

Fig. 1. Motility of *C. jejuni* isolates (C38, P3, P5, P6, P8, P9 and P10) on soft agar plate. (A) Photograph of swarming colonies of *C. jejuni* strains on 0.4%-agar Mueller Hinton II plate after 23-hr incubation. (B) Diameter of swarming colonies of the *C. jejuni* isolates after 23-hr incubation. The clinical isolate NCTC11168 was used as a control.

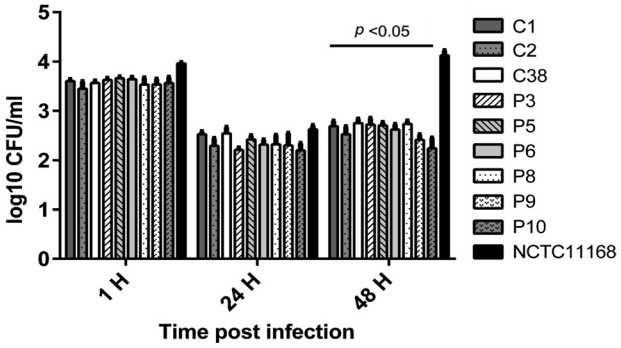

Fig. 2. Gentamicin protection assay in Caco-2 cells. Caco-2 cells were incubated with *C. jejuni* as described in Materials and Methods. After for 1-, 24- or 48-hr incubation, bacterial cells in Caco-2 cells were counted on mCDDA. Experiment was done in triplicate. NCTC11168 was used as a control. Significant difference of the wild-bird isolates to NCTC11168 at 48-hr incubation: $P<0.05$.

NCTC11168. To our knowledge, no reports are available that demonstrate virulence properties of *C. jejuni* isolates from wild birds.

Overall prevalence of *Campylobacter* spp. in wild birds in this study was 19.7%. The prevalence of *Campylobacter* in this study showed average level when compared with the results from other studies, which described *Campylobacter* spp. in wild birds in the range of 3–80% around the world [1]. The species identification showed that most isolates were *C. jejuni* in our study as the same with other domestic and overseas studies, although *C. coli* and *C. fetus* were found in one sample each. *C. jejuni* and *C. coli* account for 95% of campylobacteriosis in humans, while *C. fetus* is of veterinary importance due to its ability to colonize the intestinal mucosa and/or urogenital tract in cattle, causing abortion and sterility. *C. fetus* can also infect humans, especially elderly and immunocompromized individuals [20, 37].

The isolation rates reported in previous Japanese studies on *C. jejuni* carriage in wild birds are variable. Kinjo *et al*. [25] found that 23.7% of tested pigeons (n=329) were infected by *C. jejuni* on a two-year survey. Fukuyama *et al*. [17] have reported a lower overall rate than ours. In fact, 7.9% (n=700) in all sampled birds and 13.5% (n=378) in pigeons were observed. Ito *et al*. [21] have described an overall rate of 14% (n=313), but a higher infection rate in crows, 34% (n=32) possibly due to their association with a municipal garbage dump. The infection rate varied between these studies and ours, possibly due to different sample sizes, sampling methods and investigated localities. Higher percentage of infection was found in crows and pigeons than in the Eurasian tree sparrows. The occurrence of *C. jejuni* in various wild bird species might have a link with their diet. In fact, the main food sources for sparrow are seed, grain and insects, while the scavenging birds, such as crows and pigeons, are known to feed in garbage [21]. The relationship between *Campylobacter* infection and wild birds diet seems to be further confirmed by results reported by Ito *et al*. [21] and Kapperud and Rosef [23]. They found that herbivorous wild birds were also infected, but at a significantly lower rate than other omnivorous scavenger, such as crows and gulls. These factors made the crows and pigeons to be more exposed to isolates from food animals and humans as their habitats are closely shared.

To gain insight to the pathogenicity of *C. jejuni* isolates from wild birds, we characterized the isolates for the presence of major virulence-associated genes, such as *cadF*, *cdtA*, *cdtB*, *cdtC*, *ciaB*, *flaA* and *flaB*. The virulence genes screened to assess the pathogenesis of *C. jejuni* have been reviewed by Dasti *et al*. [13] and Young *et al*. [41]. The gene *cadF* encodes an outer membrane protein that interacts with host cells to bind to the extracellular matrix protein fibronectin and thus is important for colonization. The cytolethal distending toxin (CDT) is encoded by *cdtA*, *cdtB* and *cdtC*, and their expression is required for their cytotoxicity effect [32]. The tripartite CDT complex triggers the arrest of eukaryotic cells in the G2 phase of the cell cycle causing apoptosis of the concerned cell [39]. The flagellin proteins encoded by *flaA* and *flaB* have been recognized as important factors in *C. jejuni* motility and invasion [30, 33]. Thirty-two isolates of *C. jejuni* from wild birds were screened for the presence of virulence genes, and seven *C. jejuni* isolates (1 crow isolate and all 6 isolates from pigeons) were positive for all tested genes. The other 25 isolates were lacking *cdtA* in the virulence gene profile. This might be due to the complete absence of *cdtA* or occurrence of mutations in respective gene.

Motility is critical for many of *C. jejuni* pathogenesis properties, such as host colonization, secretion of virulence genes and host-cell invasion [41]. *C. jejuni* isolates from wild birds showed a reduced motility with exception of C38, which showed a high motility phenotype (Fig. 1A and 1B). On the other hand, PCR results confirmed the presence, in all isolates from wild birds, of *flaA* and *flaB*, the two major flagellin genes of *C. jejuni*. These results suggest that the defects in swarming motility are caused by lack or loss-of-function of other flagellar genes, or lack of chemotactic ability. It has been postulated that naturally, *C. jejuni* can produce non-motile deletion copies of wild type strain that are more suitable to environments where the flagellar expression would be unnecessary otherwise [24]. These phenotypes may restore their motility, once favorable conditions are met.

Another objective of our study was to determine the invasive capabilities of *C. jejuni* isolates from wild birds to the human intestinal epithelial cell-line, Caco-2. At 1-hr post-infection, intracellular *C. jejuni* of tested isolates was comparable, followed by an identical decrease at 24-hr post infection for all isolates including the clinical isolate. However, at 48-hr post infection, isolates from wild birds showed a similar slight increase (i.e. multiplication inside Caco-2 cells), except for the clinical isolate that showed a sharp significantly different increase. It has been reported that clinical isolates are hyper invasive compared to isolates from asymptomatic individuals [6, 15]. It also has been hypothesized that the virulent capacity of *C. jejuni* is attenuated by the lysosome system which prevents the bacteria to survive nor multiply inside cultured cells [28]. The tested isolates of *C. jejuni* from wild birds were invasive phenotype. Although

weaker, the invasion of Caco-2 cells by the isolates of this study was observed and this highlights their potential as human pathogens.

To our knowledge, this study is the first study to investigate virulence-associated factors of *C. jejuni* isolates from wild birds. While the type strain NCTC11168 was greater in adherence and invasion capacities to Caco-2 cells than those of wild bird isolates, they invaded the human intestinal epithelial cells, persisted and underwent replication in host cells. Since little is known about the link between wild bird origin of *Campylobacter* and human and livestock-associated strains, further studies, such as hemolysis and cytotoxicity assay, *in vivo* infection studies and molecular epidemiological studies, need to be performed to elucidate the pathogenicity of wild bird isolates and their impact on human and animal health.

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