



Aerotolerance and multilocus sequence typing among *Campylobacter jejuni* strains isolated from humans, broiler chickens, and cattle in Miyazaki Prefecture, Japan

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ABSTRACT. *Campylobacter jejuni* is one of the leading causes of human gastroenteritis in Japan. As chickens and cattle are common reservoirs for *C. jejuni*, this microaerophilic, stress-sensitive bacterium can overcome and survive various stress conditions during zoonotic transmission, particularly foodborne, to humans. How *C. jejuni* overcomes stress conditions is, however, unclear. In the present study, 70 *C. jejuni* strains isolated from various sources (26 human, 20 broilers, and 24 cattle isolates) in Miyazaki, Japan, from 2010 to 2012, were subjected to multilocus sequence typing (MLST) and aerotolerance testing (aerobic shaking at 200 rpm). The results demonstrated that *C. jejuni* strains from Miyazaki belonged to 12 clonal complexes (CCs) and 43 sequence types (STs). CC-21 and CC-460 were mainly detected in human clinical strains. Most tested strains were aerotolerant, and only one (1.4%) was deemed sensitive to aerobic stress. Approximately 40% strains survived the 24-hr vigorous aerobic shaking at 200 rpm, and these hyper-aerotolerant strains were more prevalent in broiler and cattle isolates than in human isolates. Phylogenetic analysis divided the strains into five clusters, each showing a different pattern of host association. Thus, we have demonstrated for the first time that *C. jejuni* strains with increased tolerance to aerobic stress are highly prevalent in broilers and cattle in Miyazaki, Japan, and that certain clonal populations are frequently implicated in human infection in this area.

KEY WORDS: aerotolerance, *Campylobacter jejuni*, Japan isolate, multilocus sequence typing (MLST)

J. Vet. Med. Sci.

81(8): 1144–1151, 2019

doi: 10.1292/jvms.19-0228

Received: 26 April 2019

Accepted: 2 June 2019

Advanced Epub: 3 July 2019

Campylobacter jejuni is a leading cause of bacterial gastroenteritis in humans worldwide [13]. *C. jejuni* is a gram-negative, rod spiral-shaped, microaerophilic bacterium found in the gastrointestinal tracts of various animals [16]. In particular, poultry and cattle are considered as the common sources for human infection [17, 36]. *C. jejuni* can also be isolated from farms and natural environments [8, 10, 18]. The common clinical symptoms of campylobacteriosis include bloody diarrhea, fever, and abdominal pain [6]. In Japan, approximately 2,000 cases of campylobacteriosis have been reported annually since 1982 [12], and the number of cases detected between 1991 and 2008 reveals no evidence of decrease when compared to previous domestic reports [29].

As *C. jejuni* is zoonotically transmitted from animals to humans mainly via foodborne routes, the survival of *C. jejuni* under stress conditions outside the host animal is an important factor for its transmission to humans [5]. As a microaerophilic bacterium, *C. jejuni* requires low oxygen levels for growth [41], and high levels of atmospheric oxygen may induce oxidative stress owing to the generation of reactive oxygen species (ROS) [15]. Although a limited number of ROS detoxification enzymes are expressed in *C. jejuni* [4, 31], some strains are aerotolerant (AT) and may efficiently survive under aerobic conditions. Thus, the AT *C. jejuni* strains may pose serious risks of food contamination and human infection [19, 25, 34]. According to a study from Canada, approximately 35.7% of *C. jejuni* isolates from retail chicken meat were found to be highly tolerant to aerobic conditions [25]. *C. jejuni* strains with increased aerotolerance survived longer in chicken meat than the strains deemed sensitive to aerobic conditions [26]. To date, however, aerotolerance in *C. jejuni* has never been investigated in Japan.

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Among the several molecular techniques available for the genotyping of *C. jejuni*, multilocus sequence typing (MLST) based on variations in the sequences of seven housekeeping genes in *C. jejuni* is frequently used for epidemiological studies in Japan and other countries [1, 3, 11, 28, 29, 38, 39]. In the present study, we performed MLST analysis of *C. jejuni* strains isolated from three different sources (i.e., broiler chickens, cattle, and humans) in Miyazaki, Japan. We also measured the levels of aerotolerance among the strains examined. To our knowledge, this is the first study to evaluate aerotolerance among *C. jejuni* strains in Japan.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 70 strains of *C. jejuni* isolated from animal sources (44 strains) and humans (26 strains) were examined. Twenty strains were isolated from the cecal contents of broilers collected at processing plants located in Miyazaki Prefecture, Japan, during 2010–2012. In addition, 24 strains were isolated from fecal samples of cattle, including dairy cows and beef, at Miyakonojo meat inspection center, Miyazaki, Japan in 2012. A total of 26 human-derived strains were isolated from human sporadic cases of uncomplicated enteritis in clinical laboratories in Miyazaki Prefecture during 2010–2011. The bacterial strains obtained were routinely cultured on Blood Agar Base No. 2 (Oxoid, Hampshire, U.K.) supplemented with 5% defibrinated horse blood (Nippon Biotest Laboratories, Tokyo, Japan) at 42°C for 48 hr under microaerophilic conditions (80% N₂, 10% CO₂, 5% O₂, and 5% H₂). The collected strains were stored in Brucella broth (BD Bioscience, Billerica, MD, U.S.A.) containing 5% (v/v) glycerol (Wako Pure Chemical, Osaka, Japan) at –80°C. *C. jejuni* strains were confirmed with specific polymerase chain reaction (PCR), as previously described [40].

DNA isolation

The bacterial DNA was extracted as previously described [22]. Some bacterial cells were suspended in 25 μ l of sterilized distilled water and treated with 25 μ l of 50 mM sodium hydroxide (NaOH). The suspended bacterial cells were heated at 95°C for 5 min and treated with 4 μ l of 1 M Tris-HCl (pH 7.5) for neutralization. The suspension was centrifuged at 8,000 \times g and 4°C for 5 min. The supernatant was collected and stored at –20°C until analysis of genomic DNA.

Multilocus sequence typing

MLST analysis of *C. jejuni* was performed according to the guidelines on the *Campylobacter* PubMLST (<http://pubmlst.org/campylobacter/>) and a previous report [11]. The seven housekeeping genes *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA* were sequenced in our laboratory. PCR was performed using Taq DNA polymerase (Qiagen, Hilden, Germany), and the PCR products were purified with illustra ExoStar 1-Step (GE Healthcare, Buckinghamshire, U.K.). The sequencing reaction was performed with BigDye terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Austin, TX, U.S.A.), and the samples were run on a 3130 Genetic analyzer (Applied Biosystems). The sequences were analyzed using the *Campylobacter* PubMLST Database. Sequence types (STs) and clonal complexes (CCs) of isolates were determined according to the *Campylobacter* PubMLST. The novel alleles and STs were submitted to *Campylobacter* PubMLST and termed as new STs. The isolate clusters were visualized with a neighbor-joining tree using the neighbor-joining algorithm implementation using PHYLOViZ 2.0 based on the seven allelic profiles [23, 35].

Aerotolerance testing

Aerotolerance testing was performed as per a previous report [25] with some modifications. In brief, *C. jejuni* strains were cultivated in Nutrient Broth No. 2 (Oxoid) with 1.5% Bacto Agar (BD Biosciences) at 42°C for 18 hr under microaerophilic conditions. The cells were harvested and the absorbance at 550 nm was adjusted to 0.05 using Nutrient Broth No. 2. The bacterial suspension was then incubated at 37°C and 200 rpm under aerobic conditions. Samples were obtained at 0, 12, and 24 hr for serial dilutions and counting of culturable cells. *C. jejuni* was considered as aero-sensitive (AS) if the cells grew under aerobic atmosphere with shaking at 200 rpm until 12 hr. The strains that grew for 12–24 hr were considered as AT, while those that grew for more than 24 hr under aerobic conditions were classified as hyper-aerotolerant (HAT) [25]. Three individual runs of the same experiment were performed.

RESULTS

MLST analysis of *C. jejuni* isolates from various sources

A total of 70 isolates from various sources (broilers, cattle, and humans) in Miyazaki, Japan, acquired between 2010 and 2012 were subjected to MLST analysis. As a result, 70 strains were classified into 43 STs within 12 CCs; however, two STs were unassignable to any CC (Table 1). The dominant CCs included CC-21 (n=17; 24.3%), CC-460 (n=14; 20%), and CC-464 (n=8; 11.4%), and two CCs (CC-45 and CC-48) comprised only one isolate (Table 1). The dominant CCs in the human isolates were CC-21 and CC-460, whereas those in the cattle isolates were CC-460 and CC-464 (Table 1). The broiler isolates were evenly distributed among the CCs, and the unassignable CCs were uniquely isolated from broilers (Table 1). These results show that the dominant CCs varied depending on the source.

Aerotolerance of *C. jejuni* isolates from Miyazaki

The aerotolerance of *C. jejuni* was determined through their cultivation under aerobic conditions at 200 rpm for 24 hr. Among

Table 1. Multilocus sequence typing of *Campylobacter jejuni* isolates from different sources in Miyazaki, Japan in 2010–2012

Clonal complex (No. of isolate)	Sequence type ^{a)}	Source			Total	Clonal complex (No. of isolate)	Sequence type ^{a)}	Source			Total
		Human	Broiler	Cattle				Human	Broiler	Cattle	
CC-21 (17)	ST-21	1	-	-	1	CC-607 (5)	ST-607	-	1	-	1
	ST-50	2	1	2	5		ST-1707	-	1	-	1
	ST-4253	9	-	-	9		ST-6206	-	-	1	1
	ST-4526	-	2	-	2		ST-6488	-	-	2	2
CC-460 (14)	ST-113	-	-	1	1	CC-353 (4)	ST-2905	-	1	-	1
	ST-460	-	1	-	1		ST-6207	-	-	1	1
	ST-5255	-	1	-	1		ST-6473	-	-	1	1
	ST-6083	-	1	-	1		ST-6492	-	-	1	1
	ST-6472	-	-	2	2	CC-354 (4)	ST-6084	-	1	-	1
	ST-6474	-	-	1	1		ST-6470	-	-	1	1
	ST-6491	-	-	1	1		ST-6471	-	-	2	2
	ST-6568	3	-	-	3	CC-22 (3)	ST-22	1	-	-	1
	ST-6570	3	-	-	3		ST-1739	1	-	-	1
	CC-464 (8)	ST-464	-	1	-	1	ST-4038	-	-	1	1
ST-4389		-	2	2	4	CC-42 (3)	ST-42	2	-	-	2
ST-5268		1	-	-	1		ST-517	-	-	1	1
ST-6209		-	-	1	1	CC-61 (2)	ST-61	2	-	-	2
CC-52 (5)	ST-6486	-	-	1	1	CC-45 (1)	ST-6569	1	-	-	1
	ST-52	-	2	-	2	CC-48 (1)	ST-3503	-	1	-	1
	ST-4265	-	1	-	1	UA (3)	ST-2156	-	1	-	1
	ST-6479	-	-	1	1	ST-6085	-	2	-	2	
	ST-6480	-	-	1	1	Total		26	20	24	70

a) Novel STs found in this study are shown in bold. CC, clonal complexes; ST, Sequence type, UA represents the sequence types unassigned to any CCs.

Table 2. Aerotolerant screening of *Campylobacter jejuni* isolates from different sources in Miyazaki, Japan in 2010–2012

	Human	Broiler	Cattle	Total
Aero-sensitive	0	1 (5%)	0	1 (1.4%)
Aerotolerant ^{a)}	22 (84.6%)	7 (35%)	12 (50%)	41 (58.6%)
Hyper-aerotolerant ^{b)}	4 (15.4%)	12 (60%)	12 (50%)	28 (40%)
Total	26	20	24	70

a) Aerotolerant, viable after aerobically incubated for 12 hr; b) Hyper-aerotolerant, viable after aerobically incubated for 24 hr.

the 70 *C. jejuni* isolates, 28 (40%) were deemed HAT and 41 (58.6%) were AT; only one (1.4%) strain was found to be AS, owing to its inability to thrive under aerobic shaking conditions after 12 hr (Table 2). HAT strains were highly prevalent, mainly in *C. jejuni* isolates from cattle and broilers; however, AT strains were predominant among human isolates (Fig. 1 and Table 2). The AS strain was isolated from a broiler (Fig. 1 and Table 2). Although HAT strains were well distributed in most CCs, all strains in CC-354 were found to be HAT (Fig. 2). High prevalence of AT *C. jejuni* strains was detected in the major CCs (CC-21 and CC-460), and CC-464 comprised *C. jejuni* strains with different levels of aerotolerance (Fig. 2). Thus, the prevalence of HAT *C. jejuni* was affected by the source and CC.

Phylogenetic relationships between *C. jejuni* isolates in Miyazaki

Based on the allelic profiles of MLST, a neighbor-joining tree was generated for the analysis of the phylogenetic relationships among *C. jejuni* strains. The isolates were clustered into five subgroups with a CC-61 singleton, which did not belong to any subgroup (Fig. 3). Clusters V and IV comprised *C. jejuni* isolates from all three different sources (i.e., broilers, cattle, and humans). However, human clinical isolates were predominant in clusters V and IV (Fig. 3A); the strains in cluster V mostly belonged to CC-21, while those in cluster IV were mostly included in CC-22 and CC-42 (Fig. 3A). Cluster I was also associated with human clinical isolates, but the predominant strains in this cluster were from cattle, suggesting that cattle are the dominant source for cluster I (Fig. 3A). Clusters II and III were primarily associated with animal isolates; only one human isolate was part of clusters II and III. In particular, cluster II solely included the isolates from broilers and cattle (Fig. 3A). These results demonstrate that *C. jejuni* strains were phylogenetically related based on the source and that some clusters were closely related to human infection.

HAT *C. jejuni* strains were distributed among the five clusters; however, cluster II comprising only animal isolates exhibited the highest prevalence of HAT strains (Fig. 3A and 3B). The prevalence of HAT *C. jejuni* in cluster I was similar to that in cluster III, while the distribution of sources differed between the two clusters (Fig. 3A and 3B). These results suggest that HAT strains may be more frequently associated with animal isolates than human clinical strains.

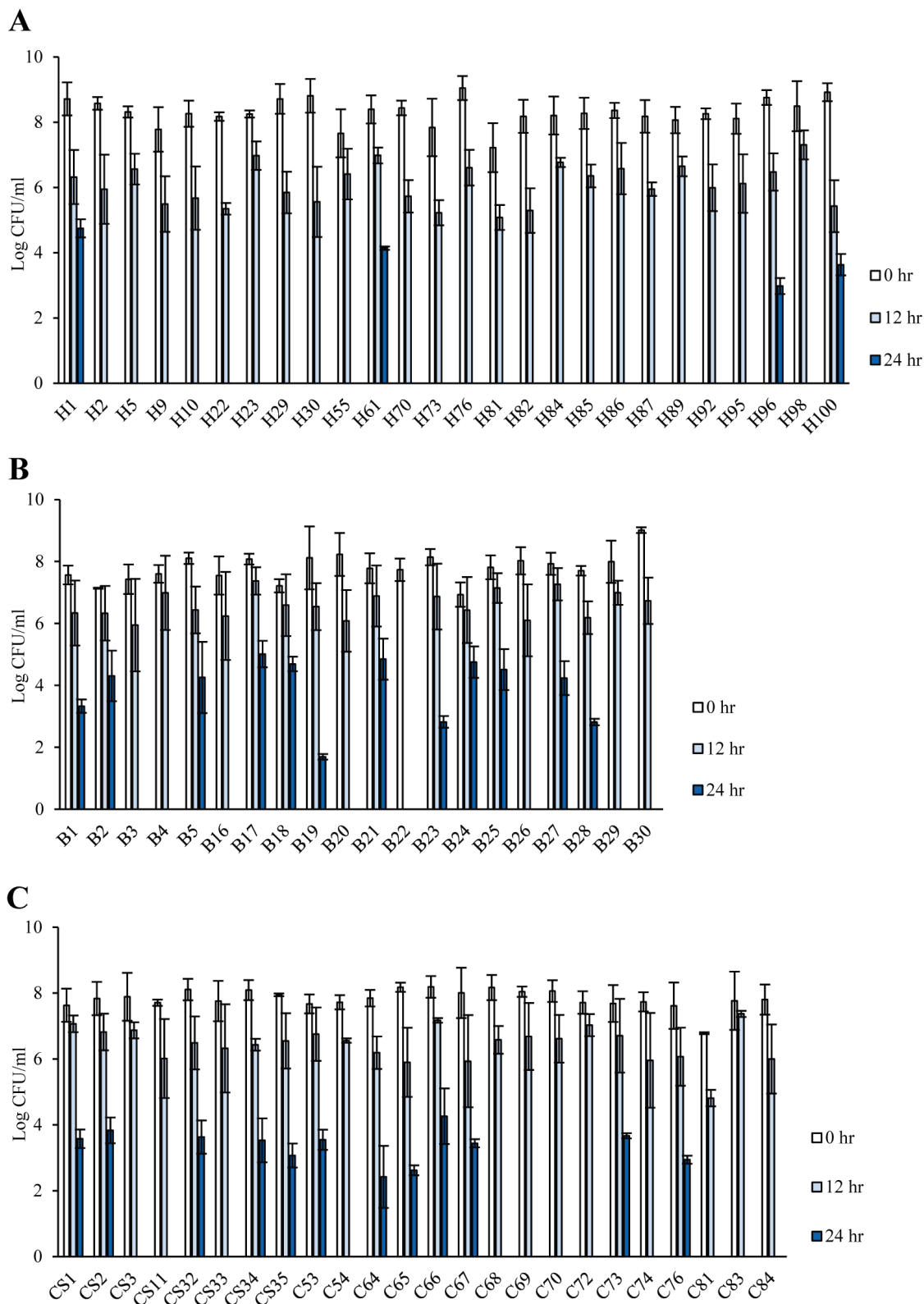


Fig. 1. The aerotolerance levels of *Campylobacter jejuni* strains isolated from different sources in Miyazaki, Japan. Culturable cells were counted after incubation for 0, 12, and 24 hr at 37°C and 200 rpm under aerobic conditions. Isolates from humans (A), broiler chickens (B), and cattle (C). The results show the means and standard deviations of three individual experiments.

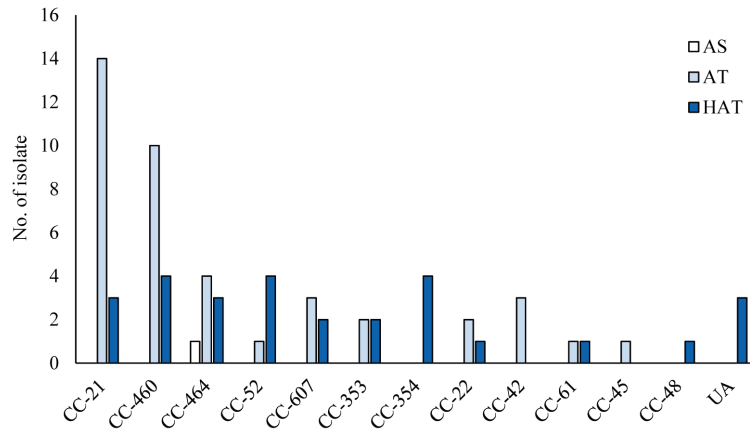


Fig. 2. Frequency distribution of *Campylobacter jejuni* clonal complexes (CCs) among strains with different levels of aerotolerance. AS, aero-sensitive; AT, aerotolerant; HAT, hyper-aerotolerant. UA represents the sequence types unassigned to any CC.

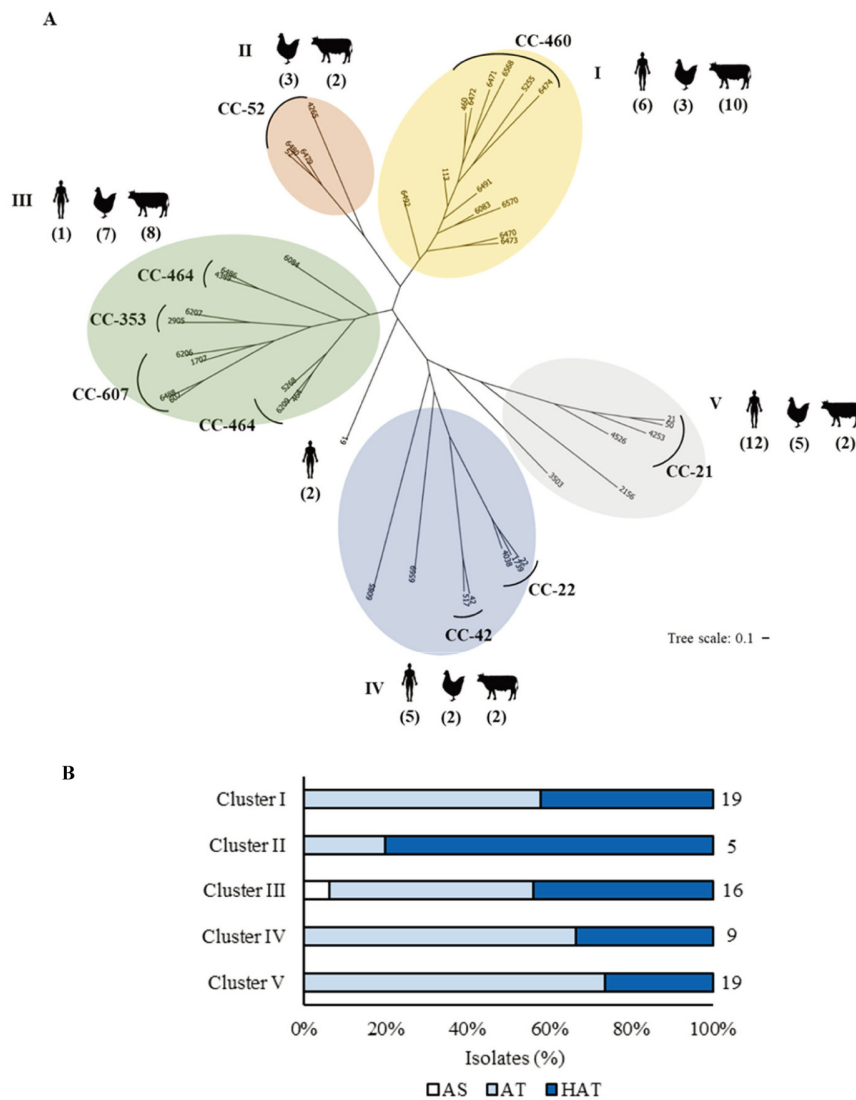


Fig. 3. Phylogenetic analysis of *Campylobacter jejuni* strains isolated from humans, broilers, and cattle in Miyazaki, Japan. (A) A neighbor-joining tree of *Campylobacter jejuni* generated from the multilocus sequence typing data showing relationships among the 70 *Campylobacter jejuni* strains examined. The sequence type numbers are shown, and the numbers of isolates from different sources are indicated in parentheses. (B) The percentage distribution of each cluster based on aerotolerance. AS, aero-sensitive; AT, aerotolerant; HAT, hyper-aerotolerant. The numbers indicate the number of isolates in each group.

DISCUSSION

The results of the MLST analysis of 70 *C. jejuni* strains from various sources in Miyazaki, Japan, revealed CC-21 as the major CC (Table 1). Previous reports from Japan have also indicated CC-21 as the most common CC [2, 3, 28, 29], possibly owing to its association with strains from various sources [14, 37]. CC-21 is widely spread in Japan, even in Kyushu, which is separated from the main island of Honshu. According to a previous study by Asakura *et al.* (2012), ST-4526 within CC-21 is unique to Japan, and the strains in this ST express filamentous hemagglutination domain protein (FHA) and increased antibiotic resistance [3]. In the present study, two *C. jejuni* strains belonging to ST-4526 were isolated from broilers (Table 1), indicative of the presence of ST-4526 in Miyazaki. ST-4253 was predominant in human isolates, consistent with the finding of a recent report that indicated the wide spread of ST-4253 in Japan. The strains in ST-4253 have superior ability to colonize chicken intestine [2]; however, we were unable to identify any isolates from broilers that belonged to ST-4253 (Table 1). In Japan, CC-21 and CC-48 are the major CCs in *C. jejuni* isolates from broilers [29]. Interestingly, we found that only one isolate belonged to CC-48, and broiler isolates were widely distributed in various CCs without any major ones (Table 1). While CC-460 and CC-464 were the major CCs associated with cattle isolates in the present study (Table 1), a previous study reported CC-21 as the major clone for cattle isolates [3]. Another study conducted in Japan found CC-460 and CC-464 as the major CCs in human and chicken isolates, followed by CC-21 [28]. Our data and a previous report suggest that CC-460 and CC-464 are expanding in Japan.

The phylogenetic analysis conducted herein divided *C. jejuni* isolates in Miyazaki into five clusters (Fig. 3A). Each cluster showed variations in the source of isolation and level of *C. jejuni* aerotolerance. More than 50% of all isolates (n=38) belonged to clusters I and V. In cluster I, cattle isolates accounted for the major proportion, suggesting that *C. jejuni* strains from cattle in this cluster may be responsible for human infection and that broilers appear to be the primary source for human infection in cluster V (Fig. 3A). Unlike the strong association between clusters IV and V and human clinical isolates, clusters II and III mainly comprised animal isolates (Fig. 3A), indicative of the association of different phylogenetic groups with preferred hosts for *C. jejuni*. The CC-52 assigned in cluster II in this study mainly comprised animal isolates, while other reports showed that the strains in CC-52 were mostly isolated from humans. These differences may be influenced by the regions studied [24, 32, 33].

In the present study, we also determined the level of aerotolerance in the 70 strains of *C. jejuni* from various sources. Interestingly, high prevalence of HAT strains was observed among *C. jejuni* isolated from broilers and cattle, whereas AT strains were predominant in human clinical isolates (Table 2). A previous study from Canada showed that 35.7% of *C. jejuni* isolates from retail raw chicken were HAT. In the present study, we similarly observed 40% HAT strains among *C. jejuni* isolates from various sources (Table 2), suggesting that the overall proportion of HAT strains would be similar between the two countries [25]. However, further experiments are warranted to compare the prevalence using *C. jejuni* strains isolated from the same type of sample (i.e., retail chicken meat).

The CCs that had more than three HAT strains of *C. jejuni* included CC-460, CC-464, and CC-354 (Fig. 2). These results are quite different from the Canadian study, wherein HAT *C. jejuni* strains from retail raw chicken predominantly belonged to CC-21 [25]. In this study, CC-21 was the dominant clone but contained relatively low numbers of HAT *C. jejuni* (n=3 from 17 isolates in CC-21) (Fig. 2). Although the reason underlying this difference is unclear, the prevalence of HAT strains may be affected by the source and perhaps the region of origin. We performed aerotolerance testing at 37°C unlike 42°C by Oh *et al.* [25], owing to the use of strains from humans, cattle, and chickens. As the levels of aerotolerance were evaluated based on the growth ability of the bacteria, not by proliferation under aerobic conditions, the most appropriate incubation temperature for the testing is still debatable.

Our results confirmed that HAT *C. jejuni* were highly prevalent among the strains isolated from animals such as broilers and cattle (Table 2), suggesting that hyper-aerotolerance may play a role in the colonization of *C. jejuni* in the gastrointestinal tracts of animals. HAT *C. jejuni* strains harbor more virulence genes than AS strains [26]. In the same study, most of the tested virulence genes were involved in *C. jejuni* adhesion to epithelial cells and colonization of chicken intestine; thus, HAT *C. jejuni* may effectively colonize chicken intestine [7, 25]. In addition, aerotolerance was closely related to oxidative stress defense in *C. jejuni*, and the genes involved affected *C. jejuni* colonization in the chicken intestine [30]. HAT *C. jejuni* strains presumably possess greater ability to resist oxidative stress and exhibit colonization; however, this hypothesis requires future experimental validation.

Oh *et al.* (2018) observed high prevalence (53.7%) of HAT among human clinical strains of *C. jejuni* in Canada. These authors also investigated the tolerance of *C. jejuni* to other stress conditions, and the multi-stress-tolerant strains were the main cause of human infection [9, 21, 27]. The high prevalence of HAT *C. jejuni* in cattle and broiler isolates may potentially be a public health concern. As *C. jejuni* could survive the oxidative stress in atmospheric environment during transmission to humans [20], the high prevalence of HAT *C. jejuni* in broilers and cattle may pose a high risk of food contamination and human infection. However, aerotolerance is not the sole prominent determinant affecting human transmission. In addition, our study showed that the cluster with the highest proportion of HAT strains was mainly associated with animals (Fig. 3A and 3B), and that the proportion of HAT *C. jejuni* in human clinical samples was relatively low (Table 2). Whether the discrepancy was related to the geographical differences between Canada and Japan or if the infection routes for *C. jejuni* differ between the two countries is questionable. More information is also needed about the tolerance of human clinical strains to other stress conditions that might affect their transmission to humans via food. To our knowledge, this is the first reported study on the aerotolerance of *C. jejuni* isolates in Japan. Further studies on stress tolerance are needed to clarify how this stress-sensitive bacterial pathogen survives and is zoonotically transmitted to humans.

ACKNOWLEDGMENT. The work was supported by grants for a project on Core-to-Core Program, Asia-Africa Science Platforms by the Japan Society for the Promotion of Science (JSPS).

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