The Journal of Veterinary Medical Science

FULL PAPER

Public Health

Surveillance of Shiga toxin-producing *Escherichia coli* and *Campylobacter* spp. in wild Japanese deer (*Cervus nippon*) and boar (*Sus scrofa*)

Yoshiyuki TOMINO[1)], Masako ANDOH[2)]*, Yuta HORIUCHI[1)], Jiye SHIN[1)], Ryunosuke AI[2)], Takaki NAKAMURA[2)], Mizuki TODA[2)], Kenzo YONEMITSU[3)], Ai TAKANO[3)], Hiroshi SHIMODA[3)], Ken MAEDA[3)], Yuuji KODERA[4)], Ichiro OSHIMA[5)], Koji TAKAYAMA[5)], Takayasu INADOME[6)], Katsunori SHIOYA[6)], Motoki FUKAZAWA[7)], Kanako ISHIHARA[8)] and Takehisa CHUMA[1,2)]

[1)]Laboratory of Veterinary Public Health, Department of Veterinary Medicine, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima, Kagoshima 890-0065, Japan
[2)]Laboratory of Pathological and Preventive Veterinary Science, Department of Veterinary Medicine, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima, Kagoshima 890-0065, Japan
[3)]Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yamaguchi, Yamaguchi 753-8515, Japan
[4)]Center for Weed and Wildlife Management, Utsunomiya University, 350 Mine-machi, Utsunomiya, Tochigi 321-8505, Japan
[5)]Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima, Kagoshima 890-0065, Japan
[6)]General Incorporated Foundation Kagoshima Environmental Research and Service, 1-1-5 Nanatsujima, Kagoshima, Kagoshima 891-0132, Japan
[7)]Tamagawa University Farm Kushi, Kagoshima, 880 Bonotsucho Kushi, Misamisatuma, Kagoshima 898-0211, Japan
[8)]Laboratory of Veterinary Public Health, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwaicho, Fuchu, Tokyo 183-8509, Japan

ABSTRACT. Increasing game meat consumption in Japan requires the dissemination of safety information regarding the presence of human pathogens in game animals. Health information regarding the suitability of these animals as a meat source is not widely available. In this study, we aimed to evaluate the safety of game meat and detect potential human pathogens in wild deer (*Cervus nippon*) and boar (*Sus scrofa*) in Japan. Fecal samples from 305 wild deer and 248 boars of Yamaguchi, Kagoshima, and Tochigi prefectures collected monthly for 2 years were examined for the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) and *Campylobacter* spp. STEC was isolated from 51 deer consistently throughout the year and from three boars; O-antigen genotype O146, the expression of *stx2b*, and *eaeA* absence (n=33) were the major characteristics of our STEC isolates. Other serotypes included the medically important O157, *stx2b* or *stx2c*, and *eaeA*-positive (n=4) and O26, *stx1a*, and *eaeA*-positive strains (n=1). *Campylobacter* spp. were isolated from 17 deer and 31 boars. *Campylobacter hyointestinalis* was the most common species isolated from 17 deer and 25 boars, whereas *Campylobacter lanienae* and *Campylobacter coli* were isolated from three and two boars, respectively. Seasonal trends for the isolation of these bacteria were not significant. This study demonstrates that wild game animals carry human pathogens; therefore, detailed knowledge of the safe handling of game meat is needed to prevent foodborne infections.

KEY WORDS: boar, *Campylobacter*, deer, game meat safety, Shiga toxin-producing *Escherichia coli*

J. Vet. Med. Sci.
82(9): 1287–1294, 2020
doi: 10.1292/jvms.19-0265

Received: 15 May 2019
Accepted: 27 June 2020
Advanced Epub:
13 July 2020

The marketing of game meat as a food source is a practical method to utilize wild animals as a natural resource. In addition, increasing populations and densities of wild animals, such as deer (*Cervus nippon*) and boar (*Sus scrofa*) [16], have accelerated hunting to counter mounting economic damage to agriculture and forestry from the overpopulation of these animals [17]. Thus, the Japanese Ministry of the Environment has proposed addressing the overpopulation issue through nationwide population control measures. The Ministry began by revising the Protection and Control of Wild Birds and Mammals and Hunting Management Law

*Correspondence to: Andoh, M.: masako@vet.kagoshima-u.ac.jp

in 2015, with the goal of halving the populations by 2023 [20]. The Ministry of Agriculture, Forestry, and Fisheries also promotes the use of wild animals as a food source as a way to control agricultural economic losses caused by high game animal populations [18]. As a result, the game meat market is rapidly growing [17], but appropriate hygiene for processing game meat has not been fully established in Japan. Therefore, the risk of foodborne infections from game meat is unknown.

There are national guidelines for processing wild deer and boar published by the Ministry of Health, Labor, and Welfare (MHLW) [22]; however, these comprise a modified version of the Slaughterhouse Act, addressing commercial processing, which is not easily followed by laypersons, since additional expertise is required. Both national and local governments have made efforts to improve the food safety of game meat by inspecting slaughter sites and providing advice on the best hygienic practices. However, to accomplish this task, basic information on which pathogens are carried by game animals is necessary. Previous research has reported the prevalence of foodborne bacteria in fecal samples from a number of areas in Japan between July and December [38], specifically *Campylobacter* spp. in boar (43.8%) and Shiga toxin-producing *Escherichia coli* (STEC) O157 (2.3%) and O26 (0.8%) in deer. Another research group isolated seven STEC strains (16.3%) from deer in Hokkaido, Japan in June and July [3]. These studies suggest that there is a real possibility of a health hazard in game meat. In domestic animals, foodborne bacteria surveys are performed regularly throughout the year on farms or in specific areas [14, 15, 25]. These surveillance studies show seasonal diversity and area specificity that are not available for wild game animals.

There have been several wild game meat-related cases of pathogenic bacterial infections reported in Japan; for example, three distinct cases of enterohemorrhagic *E. coli* infection in 1997, 2001, and 2009 were linked to deer meat consumption [21, 34, 40]. Additionally, while human campylobacteriosis related to game meat has not been reported in Japan, *Campylobacter* is a common agent of bacterial foodborne illness in this country [23]. Moreover, it has, as noted previously herein, been found in wild boar, indicating that establishing the infectious potential of game meat is necessary. Recently, surveillance of bacterial contamination of commercial game meat in Japan revealed STEC contamination in deer meat (0.8% of samples) [1], indicating the potential for game meat-related foodborne infection.

This study investigated the prevalence of STEC and *Campylobacter* spp. in wild deer and boar prior to meat processing. Fecal samples were regularly collected to obtain year-round data in a fixed area by sampling every month for 2 years in Yamaguchi and Kagoshima prefectures and for several months in Tochigi prefecture. We sought to determine whether isolates were identical clones or varied between animal species and sites. Variation in STEC was evaluated by O-antigen genotype (Og), *stx* gene type, and the presence of *eaeA* (intimin). *Stx* gene typing and *eaeA* detection were conducted to provide epidemiological information of STEC in wild animals to consider the possible threat to humans. *Campylobacter* species were identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and confirmed through an assessment of 16S rRNA gene sequences. The findings of this study, the largest survey of its kind in Japan, demonstrated differences in the rates of bacterial presence, seasonal trends, and geographical locations of STEC and *Campylobacter* spp. in wild deer and boars.

MATERIALS AND METHODS

Sample collection

Fresh fecal samples were collected from freshly killed wild deer and boar hunted in Japan in Yamaguchi and Kagoshima prefectures from June 2014 to May 2016 and in Tochigi prefecture from November 2015 to February 2016. Local hunters acted as volunteer sample collectors under instructions provided by the members of this study group. Hunting procedures complied with the Protection and Control of Wild Birds and Mammals and Hunting Management Law [19]. Thirty-seven boar samples were collected in Tochigi, 90 in Yamaguchi, and 121 in Kagoshima prefectures. A total of 142 deer samples were collected in Yamaguchi and 163 in Kagoshima. In total, samples from 248 boars and 305 deer were tested. In Yamaguchi and Kagoshima prefectures, it was possible to obtain samples on a regular basis. The number of animals that were sampled every month at each area ranged from 1 to 32, based on the monthly average over a 2-year period. Outside the normal hunting season (April to October), animals were hunted for wildlife control. Rectal fecal samples were collected in sterilized 10- or 30-m*l* tubes, and then immediately placed in a cold box or refrigerator until they were sent to Kagoshima University using refrigerated transportation and stored at 4°C until testing. Samples were processed for cultivation within 3 days of collection, except for samples from Tochigi, which were processed 5 days after collection due to a longer transport time.

STEC isolation and analysis

Using sterilized cotton swabs, fecal samples were streaked directly onto CHROMagar STEC medium (Kanto Chemical Co., Inc., Tokyo, Japan) and incubated at 37°C for 18 hr. Purple colonies were selected and inoculated onto DHL agar plates (Eiken Chemical Co., Inc., Tokyo, Japan) to confirm lactose degradation and then inoculated onto a nutrient agar plate (Nissui Pharmaceutical Co., Inc., Tokyo, Japan) for further characterization. If purple colonies were numerous, multiple colonies were selected for further evaluation. For species identification of isolated bacteria, MALDI-TOF MS was performed using an autoflex speed TOF/TOF-KG (Bruker Daltonics Inc., Bremen, Germany) based on the software MBT_FC1.par method of flexControl (Burker Daltonics) and real-time classification with the Autoxecute method of the MALDI Biotyper (Burker Daltonics). Bacterial samples were prepared with the formic acid-extraction method, according to the manufacturer's instructions, and spotted onto an MTP 384 target plate polished steel BC (Burker Daltonics). A Bacterial Test Standard (Burker Daltonics) was used for the calibration of each test run. A matching score over 1.7 was considered successful identification.

Shiga toxin type 1 (*stx1*) and 2 (*stx2*) genes were detected using PCR and sequenced for subtyping as previously reported [39].

E. coli isolates were considered STEC when they carried either *stx1* or *stx2*. The intimin-coding gene *eaeA* was detected by PCR as previously reported [5].

Isolate O-antigens were determined using O-genotyping multiplex PCR, which can comprehensively identify 147 individual O serogroups [12]. When the O-genotype (Og) of an isolate was identified as one of the major serotypes for foodborne STEC infection in Japan (O26, O103, O111, O121, O145, O157, or O165) [32], a seroagglutination test was performed to confirm the O-antigen type using commercial antiserum according to the manufacturer's protocol (Denka Seiken Co., Ltd., Tokyo, Japan).

Campylobacter isolation and analysis

One gram of a fecal sample was added to 9 m*l* of Preston enrichment broth (Oxoid Ltd., Hampshire, UK) and incubated under microaerobic conditions (7.5% CO_2, 5% O_2, 7.5% H_2, and 80% N_2) at 42°C for 48 hr. The enriched cultures were then streaked on Butzler agar plates (Oxoid Ltd.) and again incubated under microaerobic conditions at 42°C for 48 hr. Colonies were observed for the typical screw movement indicative of *Campylobacter* using phase-contrast microscopy (Nikon Corp., Tokyo, Japan) and then tested for catalase and oxidase as previously described [14]. Bacterial species of suspected colonies were identified by MALDI-TOF MS as described previously herein. For species confirmation, 16S rRNA gene sequences of the isolates were analyzed using primers CG12F (TTGATCCTGGCTCAGAGT) and CG1507R (TTCACCCCAGTCGCTGAT) [27, 30] to amplify a 1,495-bp product and the additional primer C412F (GGATGACACTTTTCGGAGC) for sequence analyses as reported elsewhere [33, 37]. The resulting sequences were searched for homologous sequences using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiplex PCR specific for the *Campylobacter jejuni hipO* gene and *Campylobacter coli ceuE* gene was conducted to confirm these two species as previously reported [42].

Statistical analyses

Statistics were performed using the statistical program file ystat2013 (Igaku Tosho Shuppan, Tokyo, Japan). Isolation rate comparisons between animal species, prefectures, and seasons were calculated by the 2 × 2 or m × n χ^2 test or 2 × 2 or m × n Yate's χ^2 test when the dataset had fewer than 10 entries. $P<0.05$ was considered significant. Seasonal differences were compared over four seasons as defined by the Japan Meteorological Agency as follows: winter (December to February), spring (March to May), summer (June to August), and autumn (September to November).

RESULTS

STEC prevalence

STEC was isolated from wild deer (n=51, 16.7%) and boar (n=3, 1.2%) in Yamaguchi and Kagoshima prefectures. STEC presence in deer was significantly higher than that in boar ($P<0.05$, Yate's χ^2 test; Table 1), and multiple STEC isolates were obtained from some individual animals; thus, the number of isolates (n=66) exceeded the number of positive animals (Table 2). Total numbers of STEC-carrying animals were not significantly different between prefectures. STEC was regularly isolated from wild deer throughout the year in both prefectures and no significant difference was observed.

Table 1. Prevalence of Shiga toxin-producing *Escherichia coli* (STEC) and *Campylobacter* species in wild deer and boars and their prefecture of origin

	Origin prefecture	Number of positive animals (%)				
		All season	Winter	Spring	Summer	Autumn
STEC		54 / 553 (9.8)	10 / 179 (5.6)	18 / 132 (13.6)	13 / 111 (11.7)	13 / 131 (9.9)
Deer	Total	51 / 305 (16.7)[a)]	10 / 82 (12.2)	17 / 83 (20.5)	12 / 77 (15.6)	12 / 63 (19.0)
	Yamaguchi	22 / 142 (15.5)	0 / 19 (0)	9 / 43 (20.9)	9 / 56 (16.1)	4 / 24 (16.7)
	Kagoshima	29 / 163 (17.8)	10 / 63 (15.9)	8 / 40 (20.0)	3 / 21 (14.3)	8 / 39 (20.5)
Boar	Total	3 / 248 (1.2)[a)]	0 / 97 (0)	1 / 49 (2.0)	1 / 34 (2.9)	1 / 68 (1.5)
	Yamaguchi	2 / 90 (2.2)	0 / 23 (0)	0 / 28 (0)	1 / 16 (6.3)	1 / 23 (4.3)
	Kagoshima	1 / 121 (0.8)	0 / 63 (0)	1 / 16 (6.3)	0 / 8 (0)	0 / 24 (0)
	Tochigi	0 / 37 (0.0)	0 / 11 (0)	0 / 5 (0)	NT	0 / 21 (0)
Campylobacter spp.		48 / 553 (8.7)	21 / 179 (11.7)	9 / 132 (6.8)	10 / 111 (9.0)	8 / 131 (6.1)
Deer	Total	17 / 305 (5.6)[b)]	7 / 82 (8.5)	4 / 83 (4.8)	2 / 77 (2.6)	4 / 63 (6.3)
	Yamaguchi	1 / 142 (0.7)[c)]	0 / 19 (0)	0 / 43 (0)	1 / 56 (1.8)	0 / 24 (0)
	Kagoshima	16 / 163 (9.8)[c)]	7 / 63 (11.1)	4 / 40 (10.0)	1 / 21 (4.8)	4 / 39 (10.3)
Boar	Total	31 / 248 (12.5)[b)]	14 / 97 (14.4)	5 / 49 (10.2)	8 / 34 (23.5)	4 / 68 (5.9)
	Yamaguchi	12 / 90 (13.3)	7 / 23 (30.4)	1 / 28 (3.6)	2 / 16 (12.5)	2 / 23 (8.7)
	Kagoshima	18 / 121 (14.9)	7 / 63 (11.1)	4 / 16 (25.0)	6 / 18 (33.3)	1 / 24 (4.2)
	Tochigi	1 / 37 (2.7)	0 / 11 (0)	0 / 5 (0)	NT	1 / 21 (4.8)[§]

NT, Not tested. § Only October and November. a), b), c): Significant difference between same alphabet ($P<0.05$).

STEC isolate characteristics

O-antigen genotyping, *stx* gene identification, and *eaeA* presence revealed the diversity of STEC in wild deer and boar. Ten O antigens were identified from wild deer and two from boar (Table 2). The *stx* gene identification was determined by sequence analyses and sequences were deposited in GenBank as follows: *stx1*, LC388483–LC388499; *stx2*, LC388500–LC388568. By combining the three identifiers (Og, *stx*, and *eaeA*), we were able to characterize our STEC isolates. The major subtype of STEC was Og146, *stx2b*, and *eaeA*-negative (n=33) from deer and boar, followed by Og11, *stx2b*, and *eaeA*-negative (n=11) and Og54, *stx2a* or *stx2b*, and *eaeA*-negative (n=9) from deer.

Two major serotypes from severe enterohemorrhagic *E. coli* infection in humans [32] were found in deer and confirmed serologically, specifically O26 (n=1) and O157 (n=4). The O157 isolates, one with *stx2b* and one with *stx2c,* were obtained from the same animal, demonstrating that a single animal can carry different subtypes of STEC. Of the *stx* gene subtypes tested, *stx2b* was dominant (n=55) in both deer and boar, but *stx1a*, *stx1*c, *stx2a*, *stx2b*, *stx2c*, and *stx2d* were also found (Table 2). Furthermore, *eaeA* was present in certain Og types, though not in the major ones noted previously herein.

Campylobacter prevalence

Campylobacter species were also isolated from both animals in all prefectures (Table 1). Wild boar (n=31, 12.5%) had a significantly higher *Campylobacter* spp. isolation rate than deer (n=17, 5.6%; $P<0.05$, χ^2 test). Deer *Campylobacter* spp. isolation rates were higher in Kagoshima prefecture than in Yamaguchi ($P<0.05$, Yate's χ^2 test), but boar *Campylobacter* spp. isolation rates did not differ significantly between prefectures. Seasonal trends were not significant for any animal or prefecture. Isolation rates peaked in winter for deer and in summer for boar; however, in boar, the season with the highest isolation rate differed by prefecture (Table 1). The dominant species for all conditions was *Campylobacter hyointestinalis,* and results for this species showed statistical significance for the same factors as those for all *Campylobacter* spp.; specifically, boar had higher isolation rates than deer ($P<0.05$, χ^2 test) and deer in Kagoshima had higher isolation rates than those in Yamaguchi ($P<0.05$, Yate's χ^2 test; Table 3).

Table 2. Characteristics of Shiga toxin-producing *Escherichia coli* isolates

O antigen genotype	*Stx* gene	*eaeA* gene	Animal	Origin[b)]	Number of isolates
Og 11	*stx2b*	-	Deer	K	11
Og 26	*stx2a*	+	Deer	Y	1
Og 54	*stx2a*	-	Deer	K	1
	stx2b	-	Deer	K	8
Og 84	*stx1a*	+	Deer	K	3
Og 113	*stx1c* + *stx2d*	-	Deer	K	1
Og 128	*stx2b*	-	Deer	Y	1
Og 131	*stx2b*	-	Deer	Y	1
Og 146	*stx1c* + *stx2b*	-	Deer, Boar	Y	6, 1
	stx2b	-	Deer	K, Y	12, 14
	stx2c	-	Deer	Y	1
Og 156	*stx1* + *stx2*[a)]	+	Deer	K	1
Og 157	*stx2b*	+	Boar	Y	1
	stx2c	+	Deer	K	1
		+	Boar	Y, K	1, 1

a) Subtype undetermined. b) K, Kagoshima; Y, Yamaguchi.

Table 3. Number of animals carrying *Campylobacter* species and the species of isolated *Campylobacter*

	Origin prefecture	Number of animals	Number of positive animals (%)				
			Total	*C. hyointestinalis*	*C. lanienae*	*C. coli*	*Campylobacter* sp.
Deer	Total	305	17 (5.6)[a)]	17 (5.6)[b)]	0 (0)	0 (0)	0 (0)
	Yamaguchi	142	1 (0.7)[c)]	1 (0.7)[d)]	0 (0)	0 (0)	0 (0)
	Kagoshima	163	16 (9.8)[c)]	16 (9.8)[d)]	0 (0)	0 (0)	0 (0)
Boar	Total	248	31 (12.5)[a)]	25 (10.1)[b)]	3 (1.2)	2 (0.8)	1 (0.4)
	Yamaguchi	90	12 (13.3)	12 (13.3)	0 (0)	0 (0)	0 (0)
	Kagoshima	121	18 (14.9)	12 (9.9)	3 (2.5)	2 (1.7)	1* (0.8)
	Tochigi	37	1 (2.7)	1 (2.7)	0 (0)	0 (0)	0 (0)
Total		553	48 (8.7)	42 (7.6)	3 (0.5)	2 (0.4)	1 (0.2)

a), b), c), d): Significant difference between same alphabet ($P<0.05$). *Isolate C147, identified as *C. jejuni* via time of flight mass spectrometry, *C. coli* from 16S rRNA sequence, and negative for both *C. jejuni* and *C. coli* via multiplex PCR.

Species identification of Campylobacter isolates

A total of 48 isolates of *Campylobacter* were initially identified by TOF-MS. The major species was *C. hyointestinalis* (n=42, 87.5% of all *Campylobacter* isolates), which was found in both deer and boar in all areas studied. *C. coli* (two isolates in winter), *C. jejuni* (one isolate in winter), and *Campylobacter lanienae* (three isolates, in winter, spring, and summer) were isolated only from wild boar in Kagoshima.

The MALDI TOF-MS Biotyper database was constructed primarily for medical use, but the isolates in the current study were field samples. Thus, 16S rRNA gene sequence analysis was conducted to confirm bacterial species identification. The resulting sequences were deposited in GenBank (16S rRNA, LC388439–LC388476). Eight isolates were not sequenced owing to inadequate bacterial storage. Using BLAST analyses, the subspecies of all *C. hyointestinalis* isolates was identified as *C. hyointestinalis* subspecies *hyointestinalis* with 100% identity (1,303 bp; Accession numbers AB301960.1 and AB301956.1). Isolates C139 and C198, however, had only 99.8% and 99.9% identity to the same sequences, respectively. C439, however, had 100% identity to another unspecified *C. hyointestinalis* subspecies sequence (Accession numbers LR698977.1, NR_118518.1, and NR_037052.1).

Isolates identified as *C. lanienae* by TOF-MS corresponded with the *C. lanienae* strains with identity up to 99.9% (1301 bp; Accession number AF550664.1). However, the identification of *C. jejuni* and *C. coli* isolates (C116, C211, and C147) was confusing. Isolate C147 was initially identified as *C. jejuni* via TOF-MS; however, the 16S rRNA gene sequence (1315 bp) had 100% identity with that of *C. coli* (Accession number HG326877.1). Conventional duplex PCR was negative for both *C. jejuni* and *C. coli*, and thus, we could not confirm species identification for this isolate. The *C. coli* isolates C116 and C211 were first identified by TOF-MS, and the sequences of the 16S rRNA genes, 1,335 bp of C116 and 1,318 bp of C211, had 100% identity with those of *C. coli* strains (multiple sequences, including Accession number AF550621.1 for C116 and CP046317.1 for C211). Interestingly, C211 also had 100% identity with many *C. jejuni* stains (i.e. Accession numbers CP048769.1, CP048767.1, CP048765.1, and others). Moreover, C116 and C211 were positive for *C. coli* and negative for *C. jejuni* by duplex PCR. To clarify the identification of these three isolates, we added a biochemical characterization test using API Campy (bioMerieux SA, Marcy l'Etoile, France); C116 and C211 were confirmed as *C. coli* but C147 was nonspecific (data not shown). From these results, we concluded that *Campylobacter* species identification by TOF-MS and gene sequences was consistent for all isolates, except for the one isolate identified as *C. jejuni*. Final species identification results are summarized in Table 3.

DISCUSSION

Foodborne disease-causing bacteria were isolated from wild deer (*C. nippon*) and boar (*S. scrofa*), both hunted and processed locally for consumption in Japan. The study was conducted over 2 years and at three locations. Regular, year-round sampling resulted in the largest investigation ever into STEC and *Campylobacter* occurrence in these two major game animal species. STEC O157, which is well-known to cause severe disease in humans [6], was found in both animals. Previously wild deer in Japan have been reported to carry O157 [38], but it has not been reported in boar. While STEC prevalence was low in this study, particularly in wild boar, both deer and boar meat could still be a source of foodborne infection. Characterization of isolates revealed heterogeneity of the pathogens in both animals.

To date, STEC is one of the most serious bacterial hazards from game meat. In Japan, STEC outbreaks in humans from the consumption of game meat were reported in 1997, 2001, and 2009 [21, 34, 40]. However, the official foodborne STEC-infection record is limited, but STEC from retail deer meat was recently isolated in Japan [1] and has been previously identified in deer game meat in Belgium (16 to 22%) and Spain (5.6%) [7, 36]. Here, we isolated many serotypes of STEC with the ability to infect humans, as shown in the official records in Japan [32]. We were able to identify a variety of STEC O-antigens using genotyping PCR [12], and we recommend this method for serotyping *E. coli* isolated from miscellaneous sources, since the seroagglutination test using commercial antiserum did not work in our case. In our hands, isolates from our field samples agglutinated with multiple O-antigen antisera, even though control STEC strains reacted as expected (data not shown). Therefore, we suggest PCR for the initial identification of O antigens.

The relationship between O antigens and *stx* genes and their subtypes is unclear. However, Og146 and *stx2b* were widely detected in the present study, particularly in deer. This might indicate some unique characteristics of wild deer, but further work would be required. Recent studies have suggested the potential adaptation of specific STEC strains to deer, as they frequently detected *stx2d* from deer fecal samples from Hokkaido and commercially available deer meat in Japan [1–3]. Hokkaido is the northernmost prefecture and is geographically separated from other major islands of Japan. It is possible that this has caused some regional specificity in STEC characteristics.

The actual pathogenicity of the STEC isolates associated with *stx* genes and subtypes was not examined in our study, since none of our fecal samples appeared to include diarrhea (data not shown), suggesting that these do not function as apparent enteropathogenic factors in deer and boar. Some of our isolates contained *stx1a* or *stx2a,* both of which have been shown to correlate with severe infections in humans [4, 28]. We considered the presence of the *eaeA* gene, an additional STEC virulence criterion that was found in certain O-antigen types, suggesting that game animals in Japan have the potential to carry highly virulent bacteria.

Previous studies have suggested that high air temperature and humidity (summer) can promote *Campylobacter* (particularly *C. jejuni* and *C. coli*) colonization in broiler chicken flocks [14, 24, 35]. Interestingly, the season associated with the highest isolation rate for *Campylobacter* spp. in this study was not summer and varied according to animal species and sampling location, which suggests that there are no seasonal trends for *Campylobacter* contamination of game meat. We speculate that the difference between

wild and domestic animals with respect to *Campylobacter* colonization season was due to the dominant *Campylobacter* species (*C. hyointestinalis* in wild animals as opposed to *C. jejuni* and *C. coli,* as noted previously herein, in domestic flocks). Nevertheless, *C. coli* was found in samples in the present study but were obtained during winter, suggesting a difference between domesticated animals and wild game meat animals. Since the usual hunting season in Japan is from November to February, seasonal information for *Campylobacter* is critical to evaluate human health hazards, especially to hunters, abattoir personnel, and consumers. A previous study on STEC in dairy cattle revealed that bacteria are significantly more likely to be shed at higher temperatures [43]. Again, in our study, there was no seasonal variation in STEC colonization among wild deer and boars. This demonstrates the importance of our year-round survey, which enables a more complete collection of data that accurately reflects possible seasonal variations within each population. Our study also showed that wild animals have a different bacterial presence rate compared to domestic animals and that the distribution of bacteria in wild animals differs by geographical location.

We showed that wild boars carried *C. coli*, *C. lanienae*, and *C. hyointestinalis*, but deer carried only *C. hyointestinalis. Campylobacter* was more common in boars than deer. The low prevalence that we found for *C. jejuni* and *C. coli*, which are the major species associated with worldwide foodborne bacterial contamination [41], is consistent with previous reports for wild deer and boar. Inoue *et al*. reported *C. jejuni* in deer (4.5%) and boar (1.9%) and *C. coli* only in boar (6.5%) in Tokushima prefecture [13], whereas Sasaki *et al.* reported *C. jejuni* only in boar (0.8%) and no *Campylobacter* species in deer in the selected areas in Japan [38]. Different isolation rates could occur due to different populations, sample quantities, and/or culture media and conditions, but worldwide epidemiology tends to have similar issues. Since our study and previous studies [13, 38] used high culture temperature for thermotolerant *C. jejuni* and *C. coli,* the prevalence of non-thermotolerant species might be higher in wild deer and boars. *C. hyointestinalis* was originally found in domestic swine and ruminants [33] and later in humans with gastric diseases, including a case wherein the agent was identified as *C. hyointestinalis* subsp. *hyointestinalis* [9, 26]. *C. hyointestinalis* subsp. *hyointestinalis* was reported as the most common *Campylobacter* species in semi-domestic reindeer in Finland [10], which suggests that it might be common to the family Cervidae. *C. lanienae* was first isolated from a routine hygiene survey in abattoir workers [31] and was later identified in healthy domestic porcine populations [33, 37]. Recently, evidence of *C. lanienae* as a human pathogen has been reported [29]. Although *C. hyointestinalis* and *C. lanienae* might not be foodborne agents for humans, people who have frequent contact with these animals should understand the risk of infection.

There was one *Campylobacter* isolate from a boar that could not identified to the species level by the methods that we used. It was not a major species in this study. However, this demonstrates the difficulty in identifying microorganisms from wild animals that might carry unidentified or novel species.

The data from this study emphasize the importance of proper handling of game animals after hunting, which is upstream of game meat processing. The guidelines established by MHLW for the hygienic slaughtering process of wildlife, based on the Slaughterhouse Act of 2013 and the HACCP-based hygienic control program of 2018, remind us of the risk of zoonotic infection during wild animal handling and that the risk is not limited to foodborne transmission. Human pathogenic bacteria commonly exist in different areas throughout the year, including during the hunting season, as confirmed in our study. The prevalence of pathogenic bacteria in game animals, mainly deer and boars, has been surveyed in many countries [7, 8, 10, 11, 36]. These studies show that the presence of human pathogenic bacteria is common in game animals. Therefore, epidemiological data of these pathogens is important to evaluate the risk associated with game meat in Japan and to properly convey that risk to consumers and hunters. The necessity of public education based on scientific data to prevent foodborne illness is of particular importance with the recent increase in game meat consumption. Future studies will continue the surveillance for human pathogens, both foodborne and otherwise, in game animals to provide further information for zoonotic disease control.

ACKNOWLEDGMENTS. We are thankful to the hunters of Yamaguchi, Kagoshima, and Tochigi prefectures for collecting samples with such care and dedication. We appreciate the input of Ms. Nodoka Hozumi, Ms. Mutsuyo Gokuden, Dr. Ken-ichi Lee, and Dr. Tadasuke Ooka for their technical advice. This study was supported by Health and Labor Science Research Grants (grant number H27-Syokuhin-Ippan-011); the Ito Foundation, Tokyo, Japan (2014 and 2016); and Kagoshima Prefectural Livestock Hygiene Guidance Association.

REFERENCES

1. Asakura, H., Kawase, J., Ikeda, T., Honda, M., Sasaki, Y., Uema, M., Kabeya, H., Sugiyama, H., Igimi, S. and Takai, S. 2017. Microbiological quality assessment of game meats at retail in Japan. *J. Food Prot.* **80**: 2119–2126. [Medline] [CrossRef]
2. Asakura, H., Makino, S., Kobori, H., Watarai, M., Shirahata, T., Ikeda, T. and Takeshi, K. 2001. Phylogenetic diversity and similarity of active sites of Shiga toxin (*stx*) in Shiga toxin-producing *Escherichia coli* (STEC) isolates from humans and animals. *Epidemiol. Infect.* **127**: 27–36. [Medline] [CrossRef]
3. Asakura, H., Makino, S., Shirahata, T., Tsukamoto, T., Kurazono, H., Ikeda, T. and Takeshi, K. 1998. Detection and genetical characterization of Shiga toxin-producing *Escherichia coli* from wild deer. *Microbiol. Immunol.* **42**: 815–822. [Medline] [CrossRef]
4. Casanova, N. A., Redondo, L. M., Dailoff, G. C., Arenas, D. and Fernández Miyakawa, M. E. 2018. Overview of the role of Shiga toxins in porcine edema disease pathogenesis. *Toxicon* **148**: 149–154. [Medline] [CrossRef]
5. Chapman, T. A., Wu, X. Y., Barchia, I., Bettelheim, K. A., Driesen, S., Trott, D., Wilson, M. and Chin, J. J. 2006. Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. *Appl. Environ. Microbiol.* **72**: 4782–4795. [Medline] [CrossRef]
6. Croxen, M. A., Law, R. J., Scholz, R., Keeney, K. M., Wlodarska, M. and Finlay, B. B. 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clin. Microbiol. Rev.* **26**: 822–880. [Medline] [CrossRef]

7. Díaz-Sánchez, S., Sánchez, S., Sánchez, M., Herrera-León, S., Hanning, I. and Vidal, D. 2012. Detection and characterization of Shiga toxin-producing *Escherichia coli* in game meat and ready-to-eat meat products. *Int. J. Food Microbiol.* **160**: 179–182. [Medline] [CrossRef]
8. Favier, G. I., Lucero Estrada, C., Cortiñas, T. I. and Escudero, M. E. 2014. Detection and characterization of Shiga toxin-producing *Escherichia coli, Salmonella* spp., and *Yersinia* strains from human, animal, and food samples in San Luis, Argentina. *Int. J. Microbiol.* **2014**: 284649. [Medline] [CrossRef]
9. Gorkiewicz, G., Feierl, G., Zechner, R. and Zechner, E. L. 2002. Transmission of *Campylobacter hyointestinalis* from a pig to a human. *J. Clin. Microbiol.* **40**: 2601–2605. [Medline] [CrossRef]
10. Hänninen, M. L., Sarelli, L., Sukura, A., On, S. L., Harrington, C. S., Matero, P. and Hirvelä-Koski, V. 2002. *Campylobacter hyointestinalis* subsp. *hyointestinalis*, a common *Campylobacter* species in reindeer. *J. Appl. Microbiol.* **92**: 717–723. [Medline] [CrossRef]
11. Hofer, E., Cernela, N. and Stephan, R. 2012. Shiga toxin subtypes associated with Shiga toxin-producing *Escherichia coli* strains isolated from red deer, roe deer, chamois, and ibex. *Foodborne Pathog. Dis.* **9**: 792–795. [Medline] [CrossRef]
12. Iguchi, A., Iyoda, S., Seto, K., Morita-Ishihara, T., Scheutz, F., Ohnishi M., Pathogenic E. coli Working Group in Japan. 2015. *Escherichia coli* O-Genotyping PCR: a comprehensive and practical platform for molecular O serogrouping. *J. Clin. Microbiol.* **53**: 2427–2432. [Medline] [CrossRef]
13. Inoue, K., Sato, G., Tobiume, M. and Akamatsu, S. 2016. Prevalence of food borne pathogen in game meat produced in Tokushima prefecture. *J. Jap. Assoc. Public Health Vet.* **19**: 59–61.
14. Ishihara, K., Chuma, T., Andoh, M., Yamashita, M., Asakura, H. and Yamamoto, S. 2017. Effect of climatic elements on *Campylobacter* colonization in broiler flocks reared in southern Japan from 2008 to 2012. *Poult. Sci.* **96**: 931–937. [Medline] [CrossRef]
15. Ishihara, K., Takahashi, R., Andoh, M., Ueno, H., Muramatsu, Y. and Tamura, Y. 2012. Seasonal variation in *Campylobacter*-contaminated retail chicken products: a year-round investigation in Japan. *J. Vet. Med. Sci.* **74**: 117–120. [Medline] [CrossRef]
16. Japan Ministry of Agriculture. 2017. Annual transition of economic loss situation in agriculture by wild animals. http://www.maff.go.jp/j/seisan/tyozyu/higai/h_zyokyo2/h29/attach/pdf/181026–1.pdf [accessed on March 28, 2019].
17. Japan Ministry of Agriculture. 2017. Fact-finding for use of wild animal resource. http://www.maff.go.jp/j/tokei/kouhyou/jibie/index.html [accessed on March 28, 2019].
18. Japan Ministry of Agriculture Fishery and Forest. 2018. Provision session for Damagae control by animals and birds. http://www.maff.go.jp/j/seisan/tyozyu/higai/index.html [accessed on December 3, 2018].
19. Japan Ministry of Environments. 2002. Protection and control of wild birds and mammals and hunting management law. Act No. 88. https://www.env.go.jp/nature/choju/law/pdf/H26_hogohou.pdf (in Japanese) [accessed on March 28, 2019].
20. Japan Ministry of Environments. 2015. Statistical predection model for the populaiton of wild deer and boar in Japan. http://www.env.go.jp/press/files/jp/26914.pdf [accessed on March 28, 2019].
21. Japan Ministry of Health Labour and Walfare. 2002. List of foodborne disease cases 2001. https://www.mhlw.go.jp/stf/seisakunitsuite/bunya/kenkou_iryou/shokuhin/syokuchu/04.html [accessed on March 28, 2019].
22. Japan Ministry of Health Labour and Walfare. 2014. Guideline for hygiene control of food meat proceeded from wild animals. http://www.mhlw.go.jp/file/06-Seisakujouhou-11130500-Shokuhinanzenbu/GLhonbun_1.pdf [accessed on March 28, 2019].
23. Japan Ministry of Health Labour and Walfare. 2017. Foodborne disease incidence 2017. https://www.mhlw.go.jp/file/05-Shingikai-11121000-Iyakushokuhinkyoku-Soumuka/0000197212.pdf [accessed on March 28, 2019].
24. Jore, S., Viljugrein, H., Brun, E., Heier, B. T., Borck, B., Ethelberg, S., Hakkinen, M., Kuusi, M., Reiersen, J., Hansson, I., Engvall, E. O., Løfdahl, M., Wagenaar, J. A., van Pelt, W. and Hofshagen, M. 2010. Trends in *Campylobacter* incidence in broilers and humans in six European countries, 1997–2007. *Prev. Vet. Med.* **93**: 33–41. [Medline] [CrossRef]
25. Kijima-Tanaka, M., Ishihara, K., Kojima, A., Morioka, A., Nagata, R., Kawanishi, M., Nakazawa, M., Tamura, Y. and Takahashi, T. 2005. A national surveillance of Shiga toxin-producing *Escherichia coli* in food-producing animals in Japan. *J. Vet. Med. B Infect. Dis. Vet. Public Health* **52**: 230–237. [Medline] [CrossRef]
26. Kim, D. K., Hong, S. K., Kim, M., Ahn, J. Y., Yong, D. and Lee, K. 2015. *Campylobacter hyointestinalis* isolated from a human stool specimen. *Ann. Lab. Med.* **35**: 657–659. [Medline] [CrossRef]
27. Lawson, A. J., Shafi, M. S., Pathak, K. and Stanley, J. 1998. Detection of *campylobacter* in gastroenteritis: comparison of direct PCR assay of faecal samples with selective culture. *Epidemiol. Infect.* **121**: 547–553. [Medline] [CrossRef]
28. Lee, M. S. and Tesh, V. L. 2019. Roles of Shiga toxins in immunopathology. *Toxins (Basel)* **11**: 212. [Medline] [CrossRef]
29. Lévesque, S., Lemay, F., Bekal, S., Frost, E. H. and Michaud, S. 2016. First reported case of *Campylobacter lanienae* enteritis in a human. *JMM Case Rep.* **3**: e005045. [Medline] [CrossRef]
30. Linton, D., Owen, R. J. and Stanley, J. 1996. Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. *Res. Microbiol.* **147**: 707–718. [Medline] [CrossRef]
31. Logan, J. M., Burnens, A., Linton, D., Lawson, A. J. and Stanley, J. 2000. *Campylobacter lanienae* sp. nov., a new species isolated from workers in an abattoir. *Int. J. Syst. Evol. Microbiol.* **50**: 865–872. [Medline] [CrossRef]
32. National Institute of Infectious Diseases. 2018. Enterohemorrhagic *Escherichia coli* infection. *I.A.S.R.* **39**: 71–72.
33. Oporto, B. and Hurtado, A. 2011. Emerging thermotolerant *Campylobacter* species in healthy ruminants and swine. *Foodborne Pathog. Dis.* **8**: 807–813. [Medline] [CrossRef]
34. Otani, K. 1997. Case of enterrohaemoradic *Escherichia coli* O157:H7 infection caused by raw deer meat consumption in Yamagata prefecture. *I.A.S.R.* **18**.
35. Patrick, M. E., Christiansen, L. E., Wainø, M., Ethelberg, S., Madsen, H. and Wegener, H. C. 2004. Effects of climate on incidence of *Campylobacter* spp. in humans and prevalence in broiler flocks in Denmark. *Appl. Environ. Microbiol.* **70**: 7474–7480. [Medline] [CrossRef]
36. Piérard, D., Van Damme, L., Moriau, L., Stevens, D. and Lauwers, S. 1997. Virulence factors of verocytotoxin-producing *Escherichia coli* isolated from raw meats. *Appl. Environ. Microbiol.* **63**: 4585–4587. [Medline] [CrossRef]
37. Sasaki, Y., Fujisawa, T., Ogikubo, K., Ohzono, T., Ishihara, K. and Takahashi, T. 2003. Characterization of *Campylobacter lanienae* from pig feces. *J. Vet. Med. Sci.* **65**: 129–131. [Medline] [CrossRef]
38. Sasaki, Y., Goshima, T., Mori, T., Murakami, M., Haruna, M., Ito, K. and Yamada, Y. 2013. Prevalence and antimicrobial susceptibility of foodborne bacteria in wild boars (*Sus scrofa*) and wild deer (*Cervus nippon*) in Japan. *Foodborne Pathog. Dis.* **10**: 985–991. [Medline] [CrossRef]
39. Scheutz, F., Teel, L. D., Beutin, L., Piérard, D., Buvens, G., Karch, H., Mellmann, A., Caprioli, A., Tozzoli, R., Morabito, S., Strockbine, N. A., Melton-Celsa, A. R., Sanchez, M., Persson, S. and O'Brien, A. D. 2012. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *J. Clin. Microbiol.* **50**: 2951–2963. [Medline] [CrossRef]
40. Shigeta, M., Moro, K., Ishikawa, K., Sugai, R., Shimizu, K., Yoshikawa, M., Tsujikawa, T. and Okabe, H. 2009. A case of enterohaemorrhagic

Escherichia coli infection developing after the intake of raw meat of wild bear and deer. *Jap. J. Med. Tech.* **58**: 1333–1336.
41. Suzuki, H. and Yamamoto, S. 2009. *Campylobacter* contamination in retail poultry meats and by-products in the world: a literature survey. *J. Vet. Med. Sci.* **71**: 255–261. [Medline] [CrossRef]
42. Ugarte-Ruiz, M., Gómez-Barrero, S., Porrero, M. C., Alvarez, J., García, M., Comerón, M. C., Wassenaar, T. M. and Domínguez, L. 2012. Evaluation of four protocols for the detection and isolation of thermophilic *Campylobacter* from different matrices. *J. Appl. Microbiol.* **113**: 200–208. [Medline] [CrossRef]
43. Venegas-Vargas, C., Henderson, S., Khare, A., Mosci, R. E., Lehnert, J. D., Singh, P., Ouellette, L. M., Norby, B., Funk, J. A., Rust, S., Bartlett, P. C., Grooms, D. and Manning, S. D. 2016. Factors associated with Shiga toxin-producing *Escherichia coli* shedding by dairy and beef cattle. *Appl. Environ. Microbiol.* **82**: 5049–5056. [Medline] [CrossRef]