



# Whole-genome sequence analysis of Shiga toxin-producing *Escherichia coli* O157 strains isolated from wild deer and boar in Japan

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**ABSTRACT.** The prevalence of Shiga toxin-producing *Escherichia coli* O157 (STEC O157) strains in wild deer and boar in Japan was investigated. STEC O157 strains were isolated from 1.9% (9/474) of the wild deer and 0.7% (3/426) of the wild boar examined. Pulsed-field gel electrophoresis (PFGE) analysis classified the wild deer and boar strains into five and three PFGE patterns, respectively. The PFGE pattern of one wild boar strain was similar to that of a cattle strain that had been isolated from a farm in the same area the wild boar was caught, suggesting that a STEC O157 strain may have been transmitted between wild boar and cattle. Clade analysis indicated that, although most of the strains were classified in clade 12, two strains were classified in clade 7. Whole-genome sequence (WGS) analysis indicated that all the strains carried *mdfA*, a drug resistance gene for macrolide antibiotics, and also pathogenicity-related genes similar to those in the Sakai strain. In conclusion, our study emphasized the importance of food hygiene in processing meat from Japanese wild animals for human consumption.

**KEY WORDS:** boar, deer, game meat, public health, shiga toxin-producing *Escherichia coli* (STEC)

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Shiga toxin-producing *Escherichia coli* (STEC) strains cause diarrhea and hemorrhagic uremic syndrome (HUS) in humans. STEC strains are prevalent in the feces of ruminants, especially cattle, and therefore ruminants are considered to be an important source of STEC infections in humans. Although many STEC serotypes have been found in human patients, O157:H7 (O157) is the most prevalent and important serotype associated with outbreaks and sporadic cases in many countries [40].

In recent years, there has been a significant increase in the population of wild animals in Japan, with a consequent increase in serious damage to agricultural crops and natural vegetation [26]. To control the wild animal population, the Japanese government has encouraged hunters to capture the wild animals in restricted areas. Since the Japanese government has also promoted the use of wild animals for food as game meat, wild deer and boar meat have become popular in local restaurants and retail meat shops. Venison and wild boar meat are also sold through the Internet for consumption in homes. However, only a limited number of studies have analyzed the health risk of eating wild animal meat.

The prevalence of STEC O157 in wild deer has been reported to be 0.3–2.4% in the United States [13, 43, 46], 1.5% in Spain [45], and 2.3% in Japan [47]. The prevalence of STEC strains in wild boar was also reported to be 0–3.4% in Spain [11, 36, 44]

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and 1.4% in Sweden [52]. Recently, Tomino *et al.* [51] detected potential human pathogens including STEC O157 in wild deer and boar in Japan. However, there have been few reports, thus far, on the prevalence of STEC O157 strains among wild animals in Japan [47], although sporadic cases of STEC O157 infections due to contaminated wild deer meat have been reported in Japan [34] and the United States [2, 31, 39]. Therefore, the microbiota, especially enteropathogens, in wild animal meat needs to be analyzed from the standpoint of food hygiene for human health [49].

The relationship between phylogenetic groups and the pathogenicity of STEC O157 strains has been reported in the last two decades. Manning *et al.* [33] classified STEC O157 strains associated with disease outbreaks by single nucleotide polymorphisms (SNPs) into nine clades, which were related to the clinical symptoms produced by these strains. Yang *et al.* [56] used a lineage-specific polymorphism assay (LSPA-6) to classify STEC O157 strains into two lineages (lineage I and II), each with a different pathogenicity. Zhang *et al.* [59] also reported that STEC O157 strains could be subdivided into lineages I, I/II, and II based on the biased IS629 distribution among these strains. Hirai *et al.* [22] compared the model of STEC O157 evolution with the clade and lineage analyzes and proposed a new evolution model that reclassified the STEC O157 strains into 13 clades. Several of these clades have been reported to be highly pathogenic [33], but there is currently no information on the STEC O157 clade distribution among wild animals.

Whole-genome sequencing (WGS) has been recently used for comprehensive gene analysis. Numerous studies have identified genes associated with pathogenesis using WGS data analysis of STEC O157 strains isolated from human cases [14, 20, 32, 38, 41, 42]. However, there have been only a few reports on WGS analysis of STEC strains from wild animals [4, 6].

In this study, we investigated the prevalence of STEC O157 strains in wild deer and boar in Japan and analyzed the possible transmission of STEC O157 strains between wild animals and livestock by pulse-field gel electrophoresis (PFGE). In addition, we evaluated the possible pathogenicity of these strains by clade and WGS analysis.

## MATERIALS AND METHODS

### Sample collection

From September 2012 to August 2019, we collected the rectal stools of 474 wild deer in 21 prefectures (8 districts), 426 wild boars in 16 prefectures (7 districts) in Japan. In addition, a cattle strain was included in this study as reference. We examined for STEC O157 among 20 cattle, which were raised in a farm near the area where wild boar had been caught in prefecture U, Kyushu district. The 17C15-2 was the only cattle strain, isolated from the 20 cattle. The fecal samples were kept below 4°C and sent to the Laboratory of Veterinary Food Hygiene, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University and cultured within 72 hr after the samples were collected.

### Isolation and identification of STEC O157 strains

Fecal samples (0.5 g) were inoculated into 4.5 ml mEC medium supplemented with novobiocin (Eiken Chemical Co., Ltd., Tokyo, Japan) and cultured at 42°C for 18 hr. STEC O157 strains were concentrated using immunomagnetic beads O157 “Seiken” (Denka Seiken Co., Ltd., Tokyo, Japan), and then cultured on CHROMagar™ O157 plates (CHROMagar, Paris, France) and on cefiximetellurite-sorbitol-MacConkey agar plates (CT-SMAC), which were Sorbitol MacConkey Agar (Eiken) with a CT-supplement (0.05 µg cefixime/ml and 2.5 µg tellurite/ml; Merck, Darmstadt, Germany). Colonies showing the STEC O157 phenotype were subcultured on agar plates at 37°C for 24 hr. Bacterial DNA was extracted by a commercial kit InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) and then the presence of Shiga toxin genes (*stx1*, *stx2*) was examined by PCR [7]. Strains that were *stx*-positive were examined for the O antigen gene (*rfbE*<sub>O157</sub>) by PCR [53]. A slide latex aggregation test was also carried out on strains carrying the *rfbE*<sub>O157</sub> gene using *E. coli* O157-F “Seiken” (Denka Seiken) to confirm the strains as STEC O157.

### PFGE analysis of STEC O157 strains

Following the PulseNet method (available at <https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf>), PFGE analysis of the STEC O157 strains was carried out using the *Xba* I restriction enzyme. The resulting band patterns were analyzed using Bionumerics software version 5.10 (Applied Maths, Sint-Martem, Belgium). Strains with ≥90% homology were assumed to be identical in this study.

### Classification of STEC O157 strains by a combination of the clade and lineage analyzes

Clade analysis of the STEC O157 strains was carried out as previously reported [58]. Briefly, we analyzed the genomes of the STEC O157 strains for the presence of SNPs at 7 loci (i.e., ECs2521, ECs3881, ECs4130, ECs3942, ECs0517, ECs2357, and ECs0654) by amplification refractory mutation system PCR (ARMS-PCR). The strains then were classified into 8 clades (i.e., clade 1, 2, 3, 4/5, 6, 7, 8, and 9). Lineage analysis was carried out by LSPA-6 [57]. Briefly, a combination of 4 genes and 2 intergenic regions (i.e., *fold-sfmA*, *Z5935*, *yhcg*, *rbsB*, *rtcB*, and *arp-iclR*) were amplified by PCR. The amplicons were designated alleles based on their size. The strains then were classified in 3 lineages (i.e., lineage I, I/II, and II) based on a combination of the results for 6 alleles (i.e., the LSPA-6 profile). The strains were classified into a total of 13 clades based on a combination of the clade and lineage analysis [22].

### Whole-genome sequence (WGS) analysis

DNA was extracted from each strain using NucleoBond Buffer Set III (MACHEREY-NAGEL, Düren, Germany) and NucleoBond AXG 20 (MACHEREY-NAGEL) following the manufacturer's instructions. Each extracted DNA was fragmented using NEBNext dsDNA Fragmentase (Bio-Rad Laboratories) with incubation at 37°C for 20 min. The fragmented DNA was purified using AM Pure XP (Beckman Coulter, Brea, CA, USA) and the length of the fragmented DNA was checked by the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA) using Agilent D1000 ScreenTape & Reagents (Agilent Technologies). The DNA library was prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA, USA) and the NEBNext Multiplex Oligos for Illumina (New England BioLabs) following the manufacturer's instructions, and the DNA concentrations were measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Whole-genome sequencing was performed using an Illumina MiSeq platform (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. MiSeq paired-end 300-nucleotide read MiSeq V2 chemistry runs were carried out. The raw reads were trimmed (minimum length 35 bp, quality score 0.05), assembled and mapped against the genome sequence of STEC O157 Sakai strain (accession No. NC\_002695.2) as a reference strain using the CLC Genomics Workbench 8.5.1 (QIAGEN, Venlo, The Netherlands). The whole genome sequences were annotated by the DDBJ Fast Annotation and Submission Tool (DFAST).

### Identification of drug resistance genes and virulence-associated genes in STEC O157 strains

Using the Center for Genomic Epidemiology (CGE) website, we analyzed the STEC O157 genome sequences for the presence of drug resistance-related genes by ResFinder 4.1 and for virulence-associated genes by VirulenceFinder 2.0.

### Drug susceptibility tests

The drug susceptibilities of the strains were determined by the disk-diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) recommendations [9]. Briefly, the STEC O157 strains were cultured on Nutrient agar (Eiken), that was then adjusted to the McFarland 0.5 concentration using sterile physiological saline. Using a sterile cotton swab, each bacterial solution was streaked on Mueller-Hinton agar (Eiken) and antimicrobial susceptibility test discs containing ampicillin (ABPC), cefotaxime (CTX), cefazolin (CEZ), gentamicin (GM), kanamycin (KM), oxytetracycline (OTC), nalidixic acid (NA), chloramphenicol (CP), sulfamethoxazole-trimethoprim (SXT) or erythromycin (EM) (BD BBLTM Sensi-Disc™, Franklin Lakes, NJ, USA) were placed on the plates. After incubation at 37°C for 16 to 18 hr, the diameter of each inhibition ring was measured. The determination of resistance or sensitivity for each antibiotic was in accordance with the CLSI standards [37].

Point mutations in 23S rRNA gene among the STEC O157 strains were examined by screening genome sequences for the presences of point mutations which had been reported to be involved in showing resistance to macrolide drugs [54, 55].

## RESULTS

### Prevalence of STEC O157 among wild deer and boar in Japan

STEC O157 was isolated from 1.9% (9/474) of the wild deer and 0.7% (3/426) of the wild boar examined in this study (Table 1). The STEC O157-positive wild deer were found only in 2 (H and M) out of 21 prefectures, and those of wild boar were found only in prefecture U. Properties of the positive samples and their strains were summarized in Table 2.

### PFGE analysis of the STEC O157 strains isolated from deer, boar, and cattle

PFGE analysis of the nine wild deer STEC O157 strains and the three wild boar STEC O157 strains showed that these strains produced a number of different PFGE patterns (Fig. 1).

The five wild deer strains isolated in prefecture M (i.e., strains 15D98-2, 15D124-1, 15D128-2, 15D129-1, and 15D131-1) had a similar PFGE pattern. The one remaining wild deer strain (15D133-2) isolated in prefecture M and the three wild deer strains isolated in prefecture H (i.e., strains 12D102-4, 15D8-1, and 15D138-1) each had a different PFGE pattern. Each of the three wild boar strains isolated in prefecture U had a distinct three PFGE pattern, different from each other and from the wild deer strains. The reference strain isolated from cattle in prefecture U had a PFGE pattern similar to that of one of the wild boar strains (18B50-1) in the same prefecture.

### Clade analysis of the STEC O157 strains isolated from deer, boar and cattle

Clade analysis classified all nine wild deer strains, two of the three wild boar strains (i.e., 17B15-1 and 18B50-1) and the cattle strain as clade 7 with an ARMS-PCR profile of GCCAGCT (Table 3). The remaining wild boar strain (17B60-3) was classified as clade 9 with an ARMS-PCR profile of GCCAGCC (Table 3). In addition, lineage analysis classified one wild deer strain (15D8-1) and one wild boar strain (17B15-1) as lineage I/II (2N111N), with the remaining strains classified as lineage II. The clade and lineage analyzes suggested a reclassification of the wild deer strains, except strain 15D8-1 and 17B15-1, wild boar 17B60-3 strain, and the cattle 17C15-2 strain to clade 12, putative clade 10 and clade 12, respectively.

### WGS analysis of the STEC O157 strains isolated from deer, boar and cattle

WGS analysis was carried out on five of the wild deer strains (12D102-4, 15D8-1, 15B98-2, 15D133-2 and 15D138-1), the three wild boar strains (17B15-1, 17B60-3 and 18B50-1) i.e., the strains that each had a PFGE pattern different from the others. The

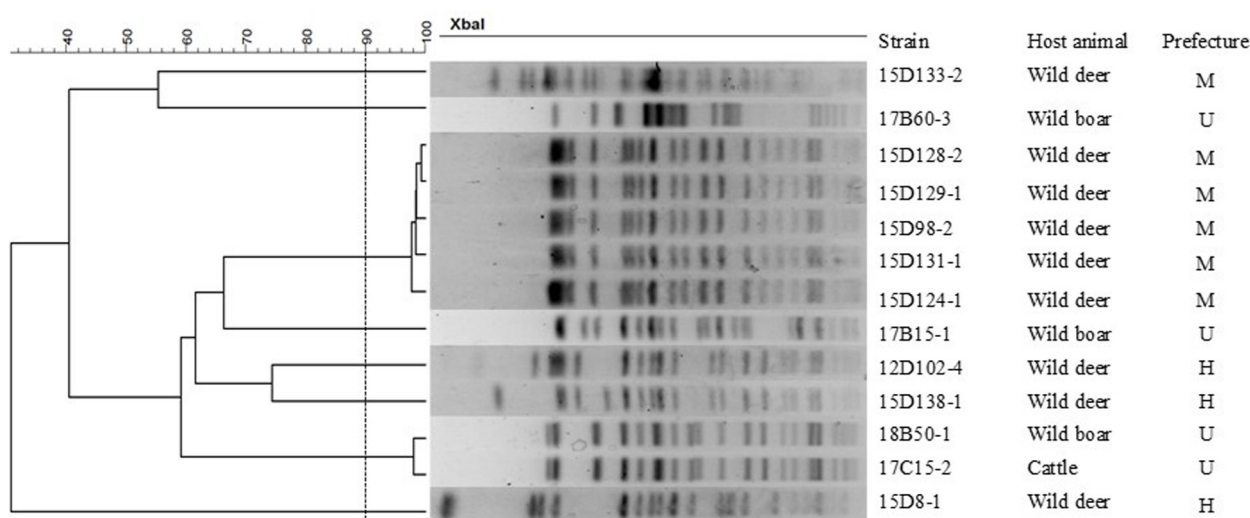
**Table 1.** Prevalence of Shiga toxin-producing *Escherichia coli* O157 in wild deer and boars by prefectures

District	Prefecture	Wild deer		Wild boar	
		No. of samples	No. of positive samples (%)	No. of samples	No. of positive samples (%)
Hokkaido	A	13	0	n.a.	
Kita	B	1	0	4	0
Kanto/Koushin	C	33	0	n.a.	
	F	9	0	n.a.	
Minami Kanto	D	22	0	22	0
	E	20	0	4	0
Hokuriku	F	n.a.		9	0
	G	n.a.		3	0
Tokai	H	53	3 (5.7)	8	0
	I	6	0	2	0
	J	4	0	2	0
	K	18	0	n.a.	
Kinki	L	30	0	n.a.	
	M	39	6 (15.4%)	n.a.	
	N	2	0	n.a.	
Chugoku	O	22	0	8	0
	P	9	0	2	0
	Q	n.a.		2	0
Shikoku	R	87	0	92	0
	S	n.a.		6	0
Kyushu	T	51	0	n.a.	
	U	31	0	243	3 (1.2%)
	V	7	0	17	0
	W	9	0	n.a.	
X	8	0	2	0	
Total	474	9 (1.9%)	426	3 (0.7%)	

n.a.: not applicable.

**Table 2.** Properties of Shiga toxin-producing *Escherichia coli* O157 strains obtained in this study

Host animal	Strains	Isolation Year Month	Prefecture
Wild deer	12D102-4	Oct., 2012	H
	15D8-1	Feb., 2015	H
	15D98-2	Jun., 2015	M
	15D124-1	Jun., 2015	M
	15D128-2	Jul., 2015	M
	15D129-1	Jul., 2015	M
	15D131-1	Jul., 2015	M
	15D133-2	Jul., 2015	M
15D138-1	Aug., 2015	H	
Wild boar	17B15-1	Mar., 2017	U
	17B60-3	Nov., 2017	U
	18B50-1	Jun., 2018	U
Cattle	17C15-2	Dec., 2017	U



**Fig. 1.** Pulsed-field gel electrophoresis (PFGE) analysis of 13 STEC O157 strains: nine wild deer strains, four wild boar strains, and one cattle strain. The dendrogram of the *Xba* I restriction enzyme PFGE patterns was constructed by the unweighted-pair group method with arithmetic averages. Strains showing  $\geq 90\%$  similarity were assumed to be in the same cluster. The Strain, Host animal, and Prefecture columns show the strain designation, animal host, and prefecture where the strain was isolated, respectively.

**Table 3.** Clade analysis, lineage analysis and clade reclassification of the Shiga toxin-producing *Escherichia coli* O157 strains in this study

Host animal	Strains	Clade classification		Lineage classification		Clade reclassification
		ARMS-PCR profile	Clade	LSPA-6 profile <sup>a)</sup>	Lineage	
Wild deer	12D102-4	GCCAGCT	Clade 7	222122	Lineage II	Clade 12
	15D8-1	GCCAGCT	Clade 7	211111	Lineage I/II	Clade 7
	15D98-2	GCCAGCT	Clade 7	221122	Lineage II	Clade 12
	15D124-1	GCCAGCT	Clade 7	221122	Lineage II	Clade 12
	15D128-2	GCCAGCT	Clade 7	221122	Lineage II	Clade 12
	15D129-1	GCCAGCT	Clade 7	221122	Lineage II	Clade 12
	15D131-1	GCCAGCT	Clade 7	221122	Lineage II	Clade 12
	15D133-2	GCCAGCT	Clade 7	221122	Lineage II	Clade 12
	15D138-1	GCCAGCT	Clade 7	222122	Lineage II	Clade 12
Wild boar	17B15-1	GCCAGCT	Clade 7	211111	Lineage I/II	Clade 7
	17B60-3	GCCAGCC	Clade 9	222111	Lineage II	Putative clade 10
	18B50-1	GCCAGCT	Clade 7	222123	Lineage II	Clade 12
Cattle	17C15-2	GCCAGCT	Clade 7	222121	Lineage II	Clade 12

ARMS-PCR: amplification refractory mutation system–polymerase chain reaction. LSPA-6: lineage-specific polymorphism assay-6. <sup>a)</sup> The LSPA-6 profile was classified into three lineages according to the consensus code: Lineage I, 1N111N; Lineage I/II, 2N111N; Lineage II: Other.

**Table 4.** Summary of the whole-genome sequence analysis of the Shiga toxin-producing *Escherichia coli* O157 strains in this study

Host animal	Strain	Sequence size (bp)	G+C content (%)	No. of CDSs (with protein)	No. of rRNAs	No. of tRNAs	DRA or Accession numbers
Wild deer	12D102-4	5,364,531	50.6	4,994	22	102	DRA012110
	15D8-1	5,367,102	50.5	4,904	22	104	DRA012107
	15D98-2	5,498,470	50.5	5,150	22	105	DRA012109
	15D133-2	5,498,471	50.5	5,146	22	105	DRA012074
	15D138-1	5,330,816	50.6	4,666	22	98	DRA012108
Wild boar	17B15-1	5,290,931	50.6	4,677	22	102	DRA012073
	17B60-3	5,356,686	50.6	5,033	22	101	DRA012072
	18B50-1	5,360,487	50.6	4,679	22	101	DRA012071
Cattle	17C15-2	5,351,671	50.6	4,633	22	102	DRA012106
Human	Sakai	5,498,578	50.5	5,047	22	103	NC_002695.2

CDSs: coding sequences.

cattle strain (17C15-2) was also included in the analysis. The data showed that the genome sizes of these nine animal STEC O157 strains were 5,290,931 to 5,498,471bp, with an average G+C content of 50.6% (Table 4). Annotation of these genome sequences identified 4,633 to 5,150 coding sequences (CDSs), 22 rRNAs, and 98 to 105 tRNAs. These data were similar to those of the Sakai strain, with a genome size of 5,498,578 bp, G+C content of 50.5%, 5047 CDSs, 22 rRNAs and 103 tRNAs. Analysis of the WGS data by ResFinder 4.1 showed the presence of *mdfA*, a macrolide antibiotic drug resistance gene, in all the strains (data not shown). VirulenceFinder 2.0 identified 20 types of pathogenicity-related genes in these strains, including Shiga toxin genes (*stx1a*, *stx2a*, *stx2c*), adhesion factors (*ea*e, *iha*), LEE encoded proteins (*espA*, *espB*, *espF*, *tir*), non-LEE encoded effector proteins (*nleA*, *nleB*, *nleC*), prophage encoded Type III secretory system proteins (*espJ*), outer membrane proteins (*chuA*, *ompT*, *traT*) and other pathogenicity-related factors (*astA*, *gad*, *iss*, *tccp*, *terC*) (Table 5).

The number of different pathogenicity-related genes identified in each of the strains in this study ranged from 27 to 29. The number of copies of each pathogenicity-related gene in these strains also varied (Table 5). For example, strains 15D98-2, 15D133-2 and 17B15-1 have 2 copies of *astA*, while the other strains only have 1 copy of this gene. The number of copies of the *iss* and *traT* genes varied from 1 to 3 and 1 or 2, respectively, in different strains. The human Sakai strain had 20 different pathogenicity-related genes, with a total of 29 copies of these genes, which was similar to the other STEC O157 strains in this study, except the Sakai strain that carried a copy of the *stx2a* gene.

#### Drug sensitivity of the STEC O157 strains

All the nine strains obtained in this study showed resistance to macrolide drug EM, but sensitivity to nine other drugs; i.e., ABPC, CTX, CEZ, GM, KM, OTC, NA, CP, and ST (data not shown).

#### Point mutations in 23SrRNA genes

Point mutations, including G2032A and G2057A were observed in 6 strains (12D102-4, 15D8-1, 15D98-2, 15D133-2, 15D138-1, and 17B15-1).

**Table 5.** Number of copies of each pathogenicity-related gene in the Shiga toxin-producing *Escherichia coli* O157 strains in this study

Genes	Strain									
	12D102-4	15D8-1	15D98-2	15D133-2	15D138-1	17B15-1	17B60-3	18B50-1	17C15-2	Sakai
<i>astA</i>	1	1	2	2	1	2	1	1	1	2
<i>chuA</i>	1	1	1	1	1	1	1	1	1	1
<i>eae</i>	1	1	1	1	1	1	1	1	1	1
<i>espA</i>	1	1	1	1	1	1	1	1	1	1
<i>espB</i>	1	1	1	1	1	1	1	1	1	1
<i>espF</i>	1	1	1	1	1	1	1	1	1	1
<i>espJ</i>	1	1	1	1	1	1	1	1	1	1
<i>gad</i>	2	2	2	2	2	2	2	2	2	2
<i>iha</i>	1	1	1	1	1	1	1	1	1	1
<i>iss</i>	3	1	2	2	3	1	3	3	3	2
<i>nleA</i>	1	1	1	1	1	1	1	1	1	1
<i>nleB</i>	2	2	2	2	2	2	2	2	2	2
<i>nleC</i>	1	1	1	1	1	1	1	1	1	1
<i>ompT</i>	1	1	1	1	1	1	1	1	1	1
<i>tccp</i>	3	3	3	3	3	3	3	3	3	3
<i>terC</i>	3	3	3	3	3	3	3	3	3	3
<i>tir</i>	1	1	1	1	1	1	1	1	1	1
<i>traT</i>	1	2	1	2	2	1	2	2	2	2
<i>stx1a</i>	-	1	1	1	-	1	-	-	-	1
<i>stx2a</i>	-	-	-	-	-	-	-	-	-	1
<i>stx2c</i>	1	1	1	1	1	1	1	1	1	-

## DISCUSSION

In this study, we found that 1.9% (9/474) of the wild deer and 0.7% (3/426) of the wild boar in Japan carried STEC O157. A similar prevalence in other wild animals has been reported in many studies in Europe and America [11, 13, 36, 43, 45, 46], suggesting that the prevalence of STEC O157 among wild deer and boar in Japan was similar to prevalence in other countries.

Nagano *et al.* [34] reported the isolation of STEC O157 showing atypical character, such as  $\beta$ -glucuronidase-positive from deer in Hokkaido prefecture, Japan. Although no strains used in this study were derived from any deer in Hokkaido prefecture, all the STEC O157 strains obtained in this study were negative for  $\beta$ -glucuronidase (data not shown).

A variety of pathogenicity-related genes were identified in the STEC O157 strains in this study by WGS data analysis. These included intimin, which is encoded by the bacterial *eae* gene, acts as a ligand for bacterial attachment to host cells through the Tir receptor, and is transferred into host cells by the type III secretion system (T3SS) [27, 35]. All the STEC O157 strains in this study were shown to have T3SS proteins (e.g., *espA*, *espB*) and effector proteins (e.g., *espF*, *espJ*, *tir*) encoded in the locus of enterocyte effacement (LEE) region. In addition, the *nleA*, *nleB*, and *nleC* effector protein genes are located outside the LEE region (non-LEE) [15, 19]. Almost all STEC O157 strains isolated from humans have been reported to carry the *eae*, *nleA*, *nleB*, and *nleC* genes [12, 16]. In addition, non-LEE effector proteins have been reported to play an important role in the formation of A/E lesions in the intestinal mucosa [8]. These findings support the possibility that the STEC O157 strains in this study may be human pathogens. In the present study, we searched pathogenicity-related genes in STEC O157 strains by using VirulenceFinder 2.0. program [17, 23, 28]. Although some genes on non-LEE encoding T3SS effectors could be monitored by using VirulenceFinder 2.0. program, some additional effectors or virulence genes on plasmid have also been reported [20, 50]. Effector sequences of STEC O157 Sakai strain were reported to fall into >20 families, including NleG family, functional homologs of effectors from plant pathogens and from *Shigella*, and two additional members of the Map/lpgB family. Actually, DFAST detected genes such as *nleG*, *nleF*, *sodC* and *katG* in Sakai strain and also in some, but not all strains obtained in this study (data not shown). Properties of the presence of pathogenicity-related genes which are not essential genes for survival, would be expected to differ by strains. It is required to determine the complete genome sequences of the strains obtained in this study and to analyze the presences and their functional expressions of these pathogenicity-related genes.

In addition to differences in the properties of the pathogenicity-related genes in the STEC O157 strains in this study, differences were also found in the copy number of the *astA*, *iss* and *traT* genes. The *astA* gene encodes an EAST-1 heat-stable toxin. Although the *astA* function is still unclear, it is considered to be a toxin carried by enteroaggregative *E. coli* (EAEC) and may be involved in causing diarrhea in humans [21]. The *iss* gene encodes a serum resistance factor involved in bacterial resistance to host complement and has been detected at high prevalence in chickens infected with pathogenic *E. coli* [29, 43]. The *traT* gene is carried by plasmid R6-5, encodes an outer membrane protein that contributes to resistance to host complement, and plays a role in *E. coli* resistance to phagocytosis by macrophages [1]. Future studies will be necessary to investigate differences in these gene functions among STEC O157 strains with different *astA*, *iss*, and *traT* copy numbers and differences in the pathogenicity of these strains.

A number of recent studies have used WGS data analysis for the genetic characterization of STEC O157 strains. WGS data of STEC O157 strains isolated from a variety of animals and environments is necessary to elucidate the ecology and epidemiology of these strains. However, only a limited amount of data on STEC O157 strains isolated from wild animal has been reported [4, 6]. The WGS data analysis in this study, of five STEC O157 strains from wild deer and three STEC O157 strains from wild boar in Japan, may contribute to an understanding of the ecology and epidemiology of STEC O157 bacteria in nature.

It has been reported that STEC O157 strains in clades 1 to 9 tend to be isolated from patients with severe symptoms, such as hemorrhagic diarrhea, and strains in clades 10 to 13 tend to be isolated from patients with subclinical or relatively mild symptoms, such as watery diarrhea [57]. In this study, most (8/9) of the wild deer strains, one of the wild boar strains, and the cattle strain were classified in clade 12. The remaining wild deer strain was classified as clade 7. Of the other two wild boar strains, one was classified as clade 7 and the other was classified as putative clade 10. Regarding *stx* subtype, no strains showed positive for *stx2a* which have been thought to cause more severe symptoms in patients than *stx2a*-negative strains [30]. These results indicated that the majority of STEC O157 strains in wild animals may show relatively low pathogenicity in human infections [33].

ResFinder 4.1 analysis showed that all the STEC O157 strains in this study carried *mdfA*, but not the other antibiotic resistance genes investigated in this study. However, antibiotic sensitivity tests by the disc method (data not shown) showed that all the wild deer, wild boar and a cattle strains obtained were resistant to macrolide antibiotics (e.g., EM), although they were sensitive to the other antibiotics in this study, including ABPC, GM, OTC, CP, and NA. Sasaki *et al.* [47] reported similar results; i.e., that some STEC O157 strains from wild deer in Japan were sensitive to ABPC, GM, OTC, CP, and NA. EM-resistant STEC O157 has been commonly found in patients and animals [5, 10]. In addition to the acquisition of *mdfA* gene, mutations in 23S rDNA may contribute to result in being EM-resistance of the STEC O157 strains obtained in this study. In fact, two kinds of mutations, namely G2032A and G2057A were observed in 6 strains [54, 55]. Further studies are needed to examine if these mutations were significant for the strains showing resistance to EM in this study. Wild animals, such as deer and boar, are not thought to be exposed to antibiotics in natural environment and may, therefore, be less likely to carry drug-resistant bacteria. Asai *et al.* [3] examined the rate of bacteria showing resistant to at least one antibiotics among wild animals including deer, wild boar and small mammals captured in mountain area and urban area. They found that increasing populations of deer carrying drug-resistant bacteria in mountain area was observed in the study in recent years (2013–2017) than those in late 1980s. Contrary, deer captured in urban area, where they prohibited for feeding deer and garbage dumping showed lower rate of drug-resistant bacteria than those in deer captured in other urban area. Tamamura-Andoh *et al.* [48] reported that although the prevalence of antimicrobial-resistant *E. coli* in wild deer was low (1.1%), properties, including the repertoires of antimicrobial resistance genes and other molecular characteristics in the *E. coli* strains were common with those in *E. coli* derived from humans and farm animals. Actually, some of the STEC O157-positive deer in prefectures H and U were caught in an area located close to farms in this study. Increasing opportunities of wild animals to contact with human and farm animals could cause the spread of drug-resistant bacteria among wild animals. The distribution of drug-resistant bacteria in wild animals, such as deer and boar, needs to be monitored to follow the transmission of drug-resistant bacteria between livestock and wild animals.

Five of the wild deer strains in this study (i.e., strains 15D98-2, 15D124-1, 15D128-2, 15D129-1, 15D131-1) were isolated in prefecture M, Kinki-district and all had a similar PFGE pattern. For comparison, three of the wild deer strains in prefecture H, Tokai-district (i.e., strains 12D102-4, 15D8-1 and 15D138-1) had different PFGE pattern each other. Similarly, the three wild boar strains in prefecture U, Kyushu-district (i.e., strains 17B15-1, 16B60-3 and 18B50-1) showed different PFGE pattern each other. These results indicated that, although a variety of STEC O157 strains have been prevalent among wild deer and boar, some specific or closely-related strains may be transmitted among these animals in the wild.

Interestingly, the PFGE pattern of the wild boar 18B50-1 strain was similar to that of the cattle strain (17C15-2). Furthermore, the results of average nucleotide identity analysis also supported the closest relationship between strains of 18B50-1 and 17C15-2 by resulting the highest homology (data not shown) [18, 24]. The wild boar with the STEC O157 18B50-1 strain had been caught in an area about 2.0 km from the farm where the cattle strain had been isolated. These results indicated that a closely-related STEC O157 strains may have been transmitted between a wild boar and cattle. Since a wild boar's habitat ranges from 60.3 to 112.5 ha [25], it is possible that a wild boar entered the farm and there may have been contacted with either the animals or their feces.

In conclusion, our study showed that both wild deer and boar in Japan carried pathogenic STEC O157. In addition, the data indicated that STEC O157 strains with the same PFGE pattern were commonly isolated from wild boar and cattle. These data emphasize the importance of food hygiene in the processing of meat from wild animals for human consumption.

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

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