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Prevalence of Shiga toxin-producing *Escherichia coli* in Yezo sika deer (*Cervus nippon yesoensis*) in the Tokachi sub-prefecture of Hokkaido, Japan

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ABSTRACT. In food hygiene, the surveillance of foodborne pathogens in wild animals is indispensable because we cannot control hygienic status of them. Yezo sika deer (*Cervus nippon yesoensis*), which are found only on the island of Hokkaido, Japan, are the most common game animal in the country. In this study, we analyzed the incidence of Shiga toxin-producing *Escherichia coli* (STEC) in Yezo sika deer hunted in the Tokachi sub-prefecture, which is one of the densest zones for the sub-species. Real-time polymerase chain reaction testing detected STEC in 18.3% of fecal samples (59/323) collected from deer hunted between 2016 and 2017, whereas no *Shigella* and *Salmonella* markers were detected. No correlation was found between STEC detection from fecal samples and characteristics of carcasses, such as hunting area, age, and fascioliasis. From 59 STEC-positive fecal samples, we isolated 37 STEC strains, including 34 O- and H-genotyped strains, in which 16 different serogroups were detected. Genetic analysis revealed that our isolates included various *stx* gene types (*stx1*$^+$/*stx2*$^-$, *stx1*$^+$/*stx2*$^+$, and *stx1*$^-$/*stx2*$^+$) and carried *eae*. This study demonstrated that STEC strains with various features colonized the Yezo sika deer, similar to other subspecies of sika deer. We conclude that continuous surveillance activity is important to monitor the suitability of game animals as a food source and to assess the validity of the food safety management system for game meat production.

KEYWORDS: *Cervus nippon yesoensis*, risk assessment of game meat, Shiga toxin-producing *Escherichia coli*

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The increasing population of wild animals represents a cause of serious damage not only to agricultural and forestry products, but also to economic activities in Japan [15, 16]. In addition, the high habitat density of wild animals increases the risk of pathogens spreading in the environment. As a countermeasure, the Japanese Ministry of the Environment revised the Protection and Control of Wild Birds and Mammals and Hunting Management Law in 2015 to regulate the population of wild animals [16]. The revised measures aim to halve the population of certain animals, including deer and boar, within one decade by increasing hunting activities [16]. Based on the increasing number of animals hunted, the increased use of wild animals as food sources is anticipated. The Ministry of Health, Labor, and Welfare has published national guidelines for processing wild deer and boars to promote safe game meat consumption [17]. The guidelines provide generalized recommended practices for hunting, transporting, slaughtering, meat processing, cooking, and distribution. To increase the effectiveness of the guidelines, microbiological risk assessments are required for each game animal, which require continuous and careful refinement.

Cervus nippon (sika deer) is recognized as a major game animal in Japan. *Cervus nippon yesoensis* (Yezo sika deer), which is only found on the northernmost island of Hokkaido island, in Japan, is one of the most hunted subspecies, and accounts for more than 40% of slaughtered deer in Japan [25]. The size of the Yezo sika deer is 2- to 3-fold larger than that of other subspecies [23]. The hunting sites are usually far from slaughter houses. Based on food safety management, it is recommended that hunted animals be transported to slaughter houses within 2 hr of capture. However, it is not easy to transport Yezo sika deer to slaughter houses within the time frame due to their large body size. These difficulties attributable to the subspecies-specific features suggest the need to construct a food safety management system in consideration of subspecies-specific features [11, 30].

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Assessment of the hazards of each animal is necessary for improving the effectiveness of food safety management. Previous studies have indicated that various foodborne pathogens can be detected in sika deer [21, 30]. Epidemiological studies have reported the prevalence of foodborne pathogens including *Toxoplasma gondii*, *Sarcocystis* spp., and hepatitis E virus in Yezo sika deer [12, 14, 24, 29, 30, 35]. In addition, Shiga toxin-producing *Escherichia coli* (STEC) has been reported to be isolated from Yezo sika deer [2, 3, 30]. STEC is considered one of the major causes of foodborne disease, and approximately 3 incidences per 100,000 population have been reported annually in Japan [31]. A surveillance program revealed that the STEC O157 serogroup is the predominant serotype; however, other serogroups such as O26 and O111 have also been reported as causative agents of foodborne outbreaks in Japan. Shiga toxin (Stx) is a major virulence determinant of STEC. Two antigenically different Stxs, Stx1 and Stx2, are known to exist, and STEC produces either one or both of them. Although it is known that Stx2 shows higher toxicity in mice than that of Stx1 [32], the virulence of bacteria containing these toxins is not determined by the Stx profile alone. A chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE), which carries genes contributing to the formation of attaching and effacing (A/E) lesions in the host intestinal mucosa, is considered an additional virulence marker in STEC. Intimin, the protein encoded by the *eae* gene in the LEE pathogenicity island, mediates the intimate attachment of the pathogen to the host cell. Although intimin is not indispensable to the virulence of STEC strains, it has been demonstrated that intimin is associated with severe symptoms caused by STEC [6]. In addition, it was previously reported that Stx contributes to adherence of bacteria to the host cell via the initial binding of intimin to the host cell [26].

In addition to animal meat representing a cause of food poisoning, wild animals, including sika deer, are considered natural reservoirs and transmitters of STEC [19]. Increasing trends in the population of Yezo sika deer and concomitant increases in numbers of hunted and consumed animals have made it necessary to elucidate the prevalence of pathogens in Yezo sika deer. In this study, we collected fecal samples from Yezo sika deer, which were hunted in the Tokachi sub-prefecture, located in the southeastern area of Hokkaido Island (Fig. 1) and known as a principal habitat of Yezo sika deer, and analyzed the prevalence of STEC via real-time polymerase chain reaction (PCR) testing. Concomitantly with fecal sample collection, we recorded the features of each carcass. In addition, we isolated STEC organisms from fecal samples and analyzed the O- and H-genotype, *stx* gene profile, and distribution of *eae* in these isolates.

MATERIALS AND METHODS

Animals and fecal samples

From September 2016 to December 2017, a total of 323 fecal samples were collected from Yezo sika deer hunted commercially in the Tokachi subprefecture and its surrounding area for human consumption (Fig. 1). The hunters recorded the sex and age of the deer, and date and location of the hunt. The age of the deer was determined based on horn morphology for males and body constitution in maternal line clusters for females. To evaluate the prevalence of fascioliasis, the presence of parasites was

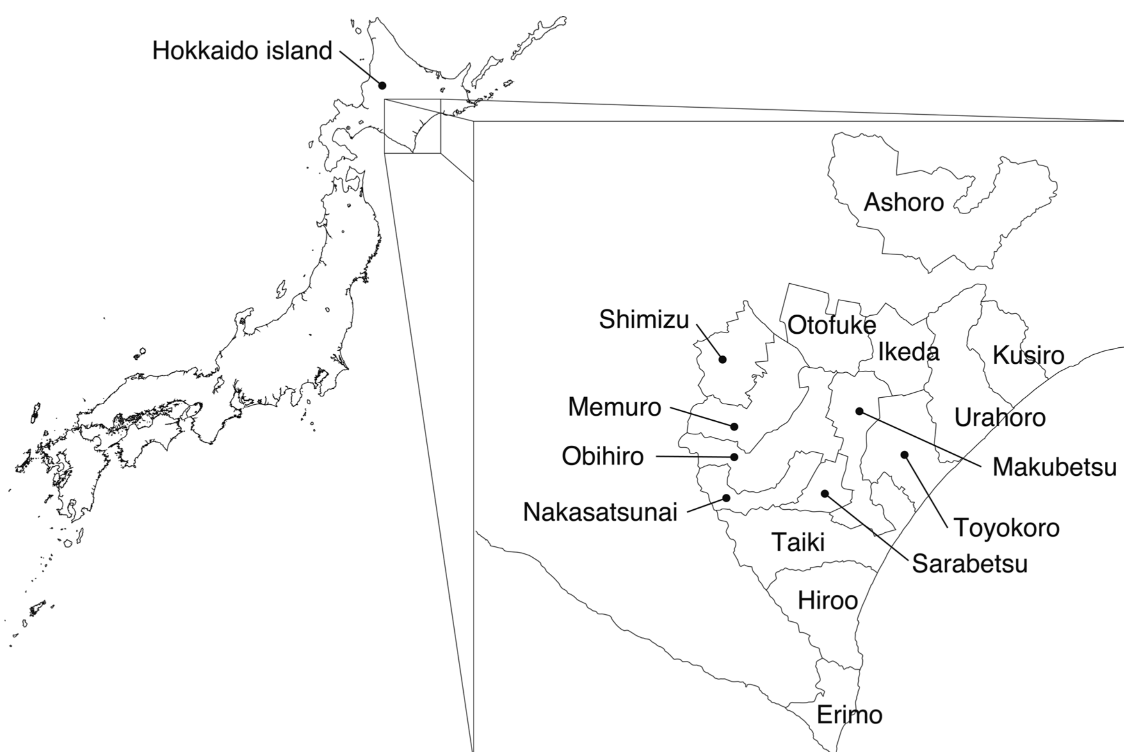

Fig. 1. A schematic map of hunting sites for Yezo sika deer in this study.

screened for via the whole liver examination method [1]. The fecal samples were collected from the rectum, suspended in 20% glycerol solution, and stored at −20°C until DNA extraction and STEC isolation. This study was performed in accordance with the recommendations of the Guide for Laboratory Animals of Obihiro University of Agriculture and Veterinary Medicine. The Committee of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine approved the protocol (Submission no. 29-39 and 29-75).

Detection of pathogenic bacteria in fecal samples

Total DNA was extracted from approximately 0.2 g of fecal samples using the PureLink™ Microbiome DNA purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. All extracted DNA samples were adjusted to 5 ng/µl for subsequent analyses. The real-time PCR-based enteric pathogenetic bacterial gene screening kit (catalog No. RR139A; TaKaRa Bio Inc., Kusatsu, Japan) was used to detect STEC together with *Shigella* and *Salmonella* species. Real-time PCR analysis was performed using the StepOne™ Real-Time PCR System (Thermo Fisher Scientific) according to the instructions provided by TaKaRa Bio Inc. (https://catalog.takara-bio.co.jp/PDFS/rr139a_j.pdf).

Isolation of STEC from fecal samples

Fecal samples were directly inoculated onto CHROMagar™ STEC (CHROMagar, Paris, France) and incubated at 37°C for 24 hr. Alternatively, approximately 0.5 g of fecal samples were cultured in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA) overnight at 37°C, and enriched cultures were inoculated onto CHROMagar™ STEC, followed by incubation at 37°C for 24 hr. Mauve-colored colonies on CHROMagar™ STEC were subsequently cultured in Luria-Bertani broth (Becton Dickinson and Co., Franklin Lakes, NJ, USA) overnight at 37°C. The bacterial cell precipitates obtained from the cultures were resuspended in distilled water, heated at 100°C for 5 min, and centrifuged at 20,000 × *g* for 5 min to obtain the DNA extract as the supernatant. To identify the *stx* gene, the O-157 (Verocytotoxin Genes) PCR Typing Set (catalog No. PR105A; TaKaRa Bio Inc.) was used according to the manufacturer's instructions. Subtypes of the *stx* genes were determined, as described by Scheutz *et al.* [28]. Two strains (STEC isolate ID 10 and 16, see Table 3) were isolated by direct fecal sample inoculation onto CHROMagar™ STEC, whereas other isolates were required to be pre-cultured in brain heart infusion broth before inoculation onto CHROMagar™ STEC.

Genoserotyping of STEC strains and detection of eae

The O- and H-genotypes of each isolate were determined using the O- and H-genotyping PCR method described by Iguchi *et al.* and Banjo *et al.* [4, 13]. The *eae* gene in each isolate was screened via PCR using the primers SK1 (5′-CCCGAATTCGGCACAAGCATAAGC-3′) and SK2 (5′-CCCGGATCCGTCTCGCCAGTATTCG-3′) [5], and KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan). The PCR cycle included 40 cycles at 98°C for 10 sec, 63°C for 60 sec, and 68°C for 10 sec. PCR products were analyzed using agarose gel electrophoresis.

Statistical analysis

Differences in the proportion of STEC-positive cases were analyzed using the z-test, and correlations between STEC-positive cases and fascioliasis were analyzed using the χ^2 test. All hypothetical tests were performed using Bell Curve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). A P value of <0.05 was considered significant for all comparisons.

RESULTS

Prevalence of STEC in Yezo sika deer

Fecal samples were obtained from animals hunted in the southeastern area of Hokkaido island, Tokachi subprefecture and its surrounding area, which is one of the most dense zones inhabited by the Yezo sika deer (Fig. 1). Fecal samples were obtained from 323 hunted animals aged 1–8 years (average age, 2.8 years). All fecal samples were examined for the presence of STEC together with *Shigella* and *Salmonella* species using a pathogen-specific real-time PCR system (Table 1). Fifty-nine cases (18.3%) were positive for STEC, whereas no positive cases were determined for *Shigella* and *Salmonella* species. STEC-positive animals were spread throughout the hunting area. No statistical differences between age groups were observed among the STEC-positive cases.

In addition to STEC detection in fecal samples, we evaluated the prevalence of fascioliasis in each animal during slaughter. *Fasciola* was detected in 22 cases (6.8%). No correlation was found between fascioliasis in carcasses and STEC detection in fecal samples (χ^2=1.28, P=0.26) (Table 2).

Features of STEC isolated from Yezo sika deer

Using the 59 fecal samples that tested positive for STEC based on real-time PCR, we attempted to isolate STEC using selective agar. Consequently, 37 isolates were successfully obtained from 36 individual fecal samples (Table 3). O- and H-genotyping based on the methodology reported by Iguchi *et al.* and Banjo *et al.* [4, 13] revealed that various genoserotypes could be isolated from Yezo sika deer. In addition, distribution of the pathogenic factor-encoding *eae* gene was analyzed in these STEC isolates. Of the 37 isolates, 15 (40.5%) were positive for *eae* gene. Og83:Hg14 was the most prevalent genoserotype (11 out of 37 isolates), followed by genoserotypes Og22:Hg16 and Og10:Hg45. All isolates in genoserotype Og83:Hg14 harbored the *stx2c* gene, and nine out of 11 isolates harbored the *eae* gene. In genoserotype Og22:Hg16, all isolates harbored the *stx2b* gene and did not harbor the *eae* gene even though the dates and seasons of the hunts were different. These data indicate the possibility that certain STEC strains

Table 1. Prevalence of Shiga toxin-producing *Escherichia coli* (STEC) in fecal samples obtained from Yezo sika deer

		n	Age					STEC	
			1	2	3	>3	Unknown	Positive	Prevalence rate (%)
Region	Ashoro	1	0	0	1	0	0	0	0.0
	Erimo	2	0	0	2	0	0	0	0.0
	Hiroo	45	4	14	24	3	0	8	17.8
	Ikeda	1	0	0	1	0	0	0	0.0
	Kushiro	15	3	5	6	1	0	2	13.3
	Makubetsu	31	7	9	12	3	0	7	25.0
	Memuro	1	1	0	0	0	0	0	0.0
	Nakasatsunai	1	0	0	1	0	0	0	0.0
	Obihiro	8	3	2	2	1	0	1	12.5
	Otofuke	1	0	0	1	0	0	0	0.0
	Sarabetsu	1	1	0	0	0	0	0	0.0
	Shimizu	6	0	1	0	5	0	1	16.7
	Taiki	20	4	6	8	2	0	4	20.0
	Toyokoro	168	23	46	85	12	2	31	18.5
	Urahoro	14	2	4	7	1	0	2	14.3
	Unknown	8	0	1	3	0	4	3	37.5
	Total	323	48	88	153	28	6	59	18.3
STEC	Positive	59	7	15	29	5	3		
	Prevalence rate (%)		14.6	17.1	19.0	17.9	50.0		

STEC, Shiga toxin-producing *Escherichia coli*.

Table 2. Relationship between fascioliasis in carcasses and STEC detection in fecal samples of Yezo sika deer

		STEC	
		Positive	Negative
Fascioliasis	Positive	6	16
	Negative	53	248

STEC, Shiga toxin-producing *Escherichia coli*.

may be prevalent in the habitat of Yezo sika deer. For genoserotype Og10:Hg45, strains possessing the *stx2b* and *stx2c* genes were isolated. In addition to these three major genoserotypes, we also isolated 13 other genoserotypes. The diversity in genoserotype and virulence genes of the isolates indicate the possibility that Yezo sika deer were infected with STEC from various sources.

DISCUSSION

The Yezo sika deer subspecies is the largest in size among sika deer and is found only on the island of Hokkaido, which is geographically separated from other islands in Japan. This influences characteristic subspecies-specific ecological features, including feeding habits. For these reasons, it is recommended that subspecies-specific risk assessments be conducted to evaluate the suitability of Yezo sika deer as a food source. Previous studies have reported the isolation of certain food-borne pathogens such as *Campylobacter* and *Yersinia* species, but not *Shigella* and *Salmonella* species from fecal samples of sika deer [21, 30]. In this study, we attempted to detect *Shigella* and *Salmonella* species, but did not detect any marker genes of these bacteria in fecal samples of Yezo sika deer, similar to previous studies. Regarding STEC, we detected the marker gene of the bacterium in 59 out of 323 fecal samples (18.3%). The prevalence of STEC in our study was almost the same as the prevalence in Yezo sika deer and other subspecies of sika deer reported in previous studies [3, 21, 30, 34]. The prevalence of pathogenic bacteria in sika deer is considered common regardless of the subspecies and their habitat area. Asakura *et al*. reported the isolation of STEC from fecal samples collected from the habitat of Yezo sika deer [3]; the sampling spots mentioned in their study were included in the hunting sites in the present study. In their study, Asakura *et al*. isolated seven STEC strains with four different serotypes (O111:H45, Out:H45, O93:H$^-$, and O96:H$^-$) comprising *stx1*$^-$/*stx2*$^+$ strains. In the present study, we isolated 16 different serogroups that were not determined previously in Yezo sika deer, and isolated *stx1*$^+$/*stx2*$^-$ and *stx1*$^+$/*stx2*$^+$ strains in addition to *stx1*$^-$/*stx2*$^+$ strains. We conclude that various STEC strains with different features colonize the Yezo sika deer, similar to other subspecies of sika deer [18, 34]. When comparing serogroups isolated from Yezo sika deer with serogroups from other subspecies, only two serogroups, namely O26 and O156, were common to the species [34]. In previous studies, serogroup O146 were isolated in common not only from sika deer but also from other genus or species of deer in European countries, whereas serogroup O146 was not isolated from

Table 3. Characteristics of Shiga toxin-producing *Escherichia coli* (STEC) isolated from stool samples of Yezo Sika deer

STEC isolate ID	Features of animals			Features of STEC isolates		
	Hunted date	Hunted region	Age	O/H genotype	*stx* gene	*eae* gene
1	7/6/2017	Toyokoro	3	n.i. [a]:Hg49	*stx1a*	-
2	7/6/2017	Toyokoro	3	n.i. [a]:Hg25	*stx2a*	-
3	9/16/2017	Toyokoro	2	n.i. [a]:Hg25	*stx2a*	-
4	9/5/2017	Toyokoro	3	Og7:Hg15	*stx1* [b]	-
5	10/29/2016	Taiki	2	Og10:Hg45 [c]	*stx2b*	-
6	4/26/2017	Makubetsu	5	Og10:Hg45	*stx2b*	-
7	8/30/2017	Unknown	Unknown	Og10:Hg45	*stx2b*	-
8	10/11/2017	Toyokoro	3	Og10:Hg45	*stx2c*	-
9	4/18/2017	Toyokoro	3	Og21:Hg21	*stx2c*	-
10	11/14/2016	Toyokoro	3	Og22:Hg16	*stx2b*	-
11	1/24/2017	Toyokoro	3	Og22:Hg16	*stx2b*	-
12	1/31/2017	Toyokoro	3	Og22:Hg16	*stx2b*	-
13	5/15/2017	Toyokoro	1	Og22:Hg16	*stx2b*	-
14	6/3/2017	Makubetsu	3	Og22:Hg16	*stx2b*	-
15	11/2/2017	Taiki	2	Og22:Hg16	*stx2b*	-
16	10/29/2016	Taiki	2	Og26:Hg11 [c]	*stx1c* + *stx2a*	+
17	11/11/2017	Hiroo	3	Og28ac:Hg25	*stx2c*	-
18	10/23/2017	Toyokoro	2	Og75:Hg31	*stx2b*	-
19	10/28/2016	Taiki	3	Og76:Hg7	*stx2a*	+
20	9/12/2017	Hiroo	2	Og79:Hg1/12	*stx2* [b]	+
21	10/17/2016	Toyokoro	3	Og83:Hg14	*stx2c*	-
22	10/29/2016	Toyokoro	2	Og83:Hg14	*stx2c*	+
23	2/3/2017	Toyokoro	3	Og83:Hg14	*stx2c*	+
24	3/12/2017	Toyokoro	3	Og83:Hg14	*stx2c*	+
25	4/19/2017	Toyokoro	2	Og83:Hg14	*stx2c*	+
26	4/29/2017	Toyokoro	2	Og83:Hg14	*stx2c*	+
27	5/10/2017	Toyokoro	3	Og83:Hg14	*stx2c*	+
28	7/20/2017	Toyokoro	3	Og83:Hg14	*stx2c*	-
29	8/14/2017	Toyokoro	3	Og83:Hg14	*stx2c*	+
30	11/6/2017	Unknown	Unknown	Og83:Hg14	*stx2c*	+
31	12/8/2017	Toyokoro	3	Og83:Hg14	*stx2c*	+
32	10/9/2016	Toyokoro	2	Og98:Hg21	*stx1a*	+
33	9/1/2017	Makubetsu	2	Og117:Hg4	*stx2a*	-
34	10/21/2017	Toyokoro	2	Og149:Hg20	*stx1a*	+
35	10/3/2016	Kushiro	1	Og156:Hg25	*stx1a*	+
36	11/19/2016	Hiroo	3	Og159:Hg19	*stx2a*	-
37	9/13/2017	Makubetsu	3	Og181:Hg49	*stx2a*	-

[a] O genotype could not be identified. [b] Subtype of *stx* gene could not be identified. [c] Isolates were obtained from the same fecal sample.

Yezo sika deer [3, 9, 18, 20, 34]. This might be either a feature of the STEC strain that settled down in the habitat of Yezo sika deer or a feature of the subspecies, but future analysis would be required to determine this.

Although O157:H7 has been considered as the most common STEC serotype that causes diseases in humans, there is growing evidence that non-O157 serotypes represent causative agents of outbreaks and/or sporadic cases, several of which are associated with severe symptoms such as hemolytic-uremic syndrome (HUS) [7]. In our study, we isolated 34 non-O157 STEC strains that could be classified via O-genotyping, whereas serogroup O157 was not isolated. Among the isolates obtained in the present study, 11 out of 37 isolates (29.7%) belonged to serogroup O83. Although there have been no reports on the prevalence of O83 STEC among humans and any animals in Japan, the extensive screening performed by Xia *et al.* reported serotype O83:H8 as the most common serotype isolated from retail meats in the USA [37]. In their study, 7 of 11 STEC isolates isolated from ground beef or pork chops belong to serotype O83:H8, and genetic analysis revealed that these serotype O83:H8 isolates nonclonally spread among retail meats. In addition, isolation of serogroup O83 from a patient with STEC infection was reported in 1990 [36]. Serogroups O22 and O10 were the second- and third-most prevalent O-serogroups, respectively, in the present study. The National Shiga Toxin-producing *Escherichia coli* (STEC) Surveillance conducted by Centers for Disease Control and Prevention (CDC) reported that only a few cases (10 and two cases for serogroup O22 and O10, respectively) were isolated from patients in the United States during 2003–2015 [8]. In the case of serogroup O83, no isolate was detected in samples from patients in the surveillance during 2003–2015. These data indicate that O-serogroups that were frequently isolated from Yezo sika deer are rare in human infections. However, as we detected the *eae* gene in some O83 isolates, we consider that the detailed features of these O83

strains should be carefully analyzed to understand the risk of human consumption of Yezo sika meat.

LEE is a chromosomal 35 kb locus present in certain STEC isolates, and they can induce A/E lesions of intestinal epithelia similar to enteropathogenic *E. coli*. LEE-positive STEC strains are more commonly associated with HUS and epidemic diseases than LEE-negative STEC strains [31]. The LEE consists of five major operons, and the proteins encoded by these operons play a key role in the initial attachment of the bacterium to the host cell. *eae* is a representative gene of LEE and is recognized as one of the markers for highly virulent STEC strains. We detected *eae* in 15 out of 37 STEC isolates (40.5%). Notably, *eae* distribution was confirmed in the Og26:Hg11 isolate in our study. O26 has been identified as one of the most prevalent serogroups of STEC isolated from a previous outbreak in Japan [31]. These data suggest that Yezo sika deer may represent a significant food safety hazard. In contrast, since *eae*-negative STEC have also been reported to cause severe diseases in humans, *eae*-negative isolates should also be considered serious. Certain adherence factors produced by *eae*-negative STEC have been reported [33]. In addition, various virulence factors have been reported to be involved in the pathogenesis of STEC. For example, subtilase cytotoxins enterohemolysin and urease encoded by *ehxA* and *ureC* are known major virulence factors associated with highly virulent *eae*-negative/non-O157 STEC strains, and some of them could be isolated from deer [10, 27]. In future studies, additional virulence factors present in STEC strains isolated from Yezo sika deer should be evaluated to understand food safety hazards regarding Yezo sika deer meat consumption.

In Japan, laws regarding slaughter are not applied to game animals; therefore, inspection systems in slaughterhouses for game animals are usually not sufficiently improved. The guidelines for game animals do not require the inspection of the microbiological status [11, 17]. However, visual inspection of offal is recommended for post-dressing inspection. Based on the current system, we consider that identification of factor (s) which is correlated with the risk of STEC carriage and is detected without large-scale facilities will support slaughterhouses with poor inspection facilities to provide safe game meat. Fascioliasis represents one of the most frequently observed lesions during visual inspection, even in the Yezo sika deer [22]. There was no correlation between *Fasciola* detection in offal and STEC detection from fecal samples in the present study. In a previous study, it was reported that diarrhea does not occur in STEC-positive sika deer [34]. These data indicate that fascioliasis and diarrhea, which can be detected via visual inspection, may not represent factors for estimating the risk of STEC carriage. We expect to identify carcass characteristics which have a correlation to microbiological profile. For this purpose, further studies are required to determine the correlation between the microbiological profile and background of carcass characteristics.

There is no established method to control pathogen infection in wild animals, unlike in the case of domestic animals. Therefore, continuous surveillance of human pathogens in game animals is required to fully understand the safety status of wild animal meat. A comparison between the results obtained in the present study and previous reports indicates that the prevalence of pathogens in Yezo sika deer was mostly constant over the past decade [3, 30]. It is suggested that future surveillance studies should be conducted to promptly detect changes in the microbiological status of Yezo sika deer, and the food safety management systems should be improved depending on the trends in microbiological status.

CONFLICTS OF INTEREST. The authors declare that they have no competing interests.

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