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NOTE

Bacteriology

A survey of antimicrobial resistance in *Escherichia coli* isolated from wild sika deer (*Cervus nippon*) in Japan

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ABSTRACT. We examined the antimicrobial susceptibility of 848 *Escherichia coli* isolates from 237 feces samples of wild sika deer (*Cervus nippon*) captured between 2016 and 2019 in 39 of the 47 prefectures of Japan. Five of the 237 wild sika deer (2.1%) carried *E. coli* with resistance to at least one antimicrobial, and all the resistant isolates showed resistance to tetracycline. The resistant isolates contained antimicrobial resistance genes that were similar to those in *E. coli* derived from humans and farm animals. Although wild sika deer are not currently likely to be a source for the transmission of antimicrobial resistance in Japan, they can potentially mediate antimicrobial resistance spread by coming into contact with humans, animals, and their surroundings.

KEY WORDS: antimicrobial resistance, *Escherichia coli*, wild sika deer

The prevalence of antimicrobial-resistant bacteria is one of the most important concerns related to both public health and animal health worldwide. Because the wide use of antimicrobials in human and veterinary medicine is one of the risk factors in the selection of antimicrobial-resistant bacteria, reducing the usage of antimicrobial agents is an important approach for preventing the spread of antimicrobial resistance [10, 14]. Environments contaminated with human and animal wastes have become reservoirs of antimicrobial-resistant bacteria or resistance genes, and wild animals play a certain role in the spread of antimicrobial resistance [2, 7, 9]. Therefore, the surveillance of antimicrobial-resistant bacteria in wild animals is also important in terms of the One Health approach [18]. *Escherichia coli* is used as one of the representative species for the national surveillance of antimicrobial-resistant bacteria in farm animals [13], and antimicrobial-resistant *E. coli* has been found in various wild animals [7, 26]. In Japan, antimicrobial-resistant *E. coli* strains have been derived from wild mouse, boar, and sika deer [3, 12]. However, the survey areas used in previous studies were limited, and nationwide surveillance is required. In addition, there is little information about the molecular characteristics of wild animal-derived antimicrobial-resistant *E. coli* in Japan.

The sika deer *Cervus nippon* is widespread throughout Japan and can be divided based on mitochondrial DNA sequences into six subspecies, and each of these subspecies lives in a different area: *C. n. yesoensis* (Hokkaido Island), *C. n. centralis* (Honshu Mainland other than Yamaguchi Prefecture), *C. n. nippon* (Shikoku and Kyushu Islands and Yamaguchi Prefecture), *C. n. mageshimae* (Mageshima and Tanegashima Remote Islands), *C. n. yakushimae* (Yakushima and Kuchinoerabu Remote Islands), and *C. n. keramae* (Ryukyu Islands) [21]. To estimate the impacts of wild animals on the emergence and prevalence of antimicrobial-resistant bacteria, we captured wild sika deer in various areas of Japan (39 of the 47 prefectures) in 2016 to 2019 during the hunting season (from October to February), and the antimicrobial susceptibility and molecular characteristics of *E. coli* isolates from rectal feces were investigated.

A total of 848 *E. coli* isolates were obtained from 237 wild sika deer: 132 and 105 were captured in various areas, which were mountain areas rather than sightseeing areas, of Hokkaido Island and 38 other prefectures throughout Honshu, Shikoku, and Kyushu Islands, respectively (Fig. 1 and Supplementary Fig. 1). The fecal samples were chilled and transported to our laboratory.

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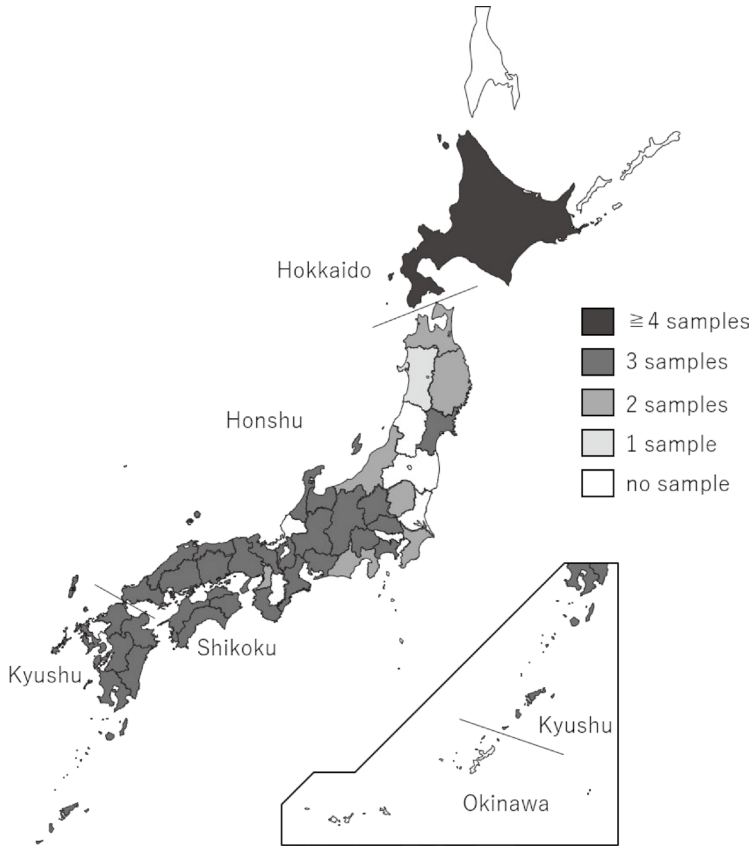

Fig. 1. Map showing the sampling locations. Rectal feces samples were obtained from wild sika deer captured in 39 prefectures of Japan. The number of samples in each prefecture is indicated by grayscale.

E. coli was isolated from a 10% (w/v) suspension of feces in phosphate-buffered saline using Chromocult Coliform Agar (Merck KGaA, Darmstadt, Germany). One to five violet colonies, which were positive for both β-galactosidase and β-glucuronidase, were selected on each agar plate and tested for indole positivity using Kovac's reagent (Merck KGaA). The indole-positive isolates were subcultured on DHL agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and the red colonies, which were positive for lactose dissociation, were identified as *E. coli*. Although the subspecies of the captured sika deer were not determined, the *E. coli* isolates were summarized according to the capture area where the predicted subspecies live (Table 1).

The antimicrobial susceptibilities of all the isolates were tested by the Kirby-Bauer disk diffusion test using Sensi-Disc Susceptibility Disks (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI) [4]. The following antimicrobials were tested: ampicillin (AMP, 10 µg), cefazolin (CFZ, 30 µg), cefotaxime (CTX, 30 µg), cefoxitin (FOX, 30 µg), gentamicin (GEN, 10 µg), kanamycin (KAN, 30 µg), streptomycin (STR, 10 µg), tetracycline (TET, 30 µg), chloramphenicol (CHL, 30 µg), nalidixic acid (NAL, 30 µg), ciprofloxacin (CIP, 5 µg), and sulfamethoxazole-trimethoprim (SXT, 23.75/1.25 µg). An *E. coli* ATCC 25922 strain was used for quality control. The prevalence of *E. coli* isolates with resistance to at least one antimicrobial in Hokkaido Island (habitat of *C. n. yesoensis*) or in Shikoku and Kyushu Islands and Yamaguchi Prefecture (habitat of *C. n. nippon*) was 1.5% (8 of 531) or 1.0% (1 of 99), respectively (Table 1). TET resistance was found most frequently on Hokkaido Island (1.5%) and exhibited the same tendency as that found in a previous study conducted in Japan by Asai *et al.* [3]. No antimicrobial-resistant *E. coli* isolates were detected in the Honshu Mainland outside of Yamaguchi Prefecture (habitat of *C. n. centralis*). Notably, no *E. coli* isolate showed resistance to third-generation cephalosporins or fluoroquinolones, which are important antimicrobials in human and veterinary medicine.

The O genotypes of the nine antimicrobial-resistant *E. coli* isolates were analyzed by O-genotyping PCR [11]. Minimum inhibitory concentrations (MICs) of the nine antimicrobial resistant isolates against AMP, CEZ, STR, TET, CHL, and SXT were determined by the agar dilution method according to the CLSI recommendations [4]. The antimicrobial resistance profile, MICs, and molecular characteristics of each *E. coli* isolate are summarized in Table 2. Five antimicrobial-resistant *E. coli* isolates obtained from one fecal sample (B22) collected in Hokkaido showed the same O genotype and antimicrobial resistance profile based on the MICs and were considered to be the same clone. Therefore, five antimicrobial-resistant *E. coli* isolates were found in five of the 237 wild sika deer samples investigated in this study. The prevalence rate (2.1%, 5 of 237) was slightly lower than that obtained in a previous study conducted in Japan: 7.9% (15 of 191) of wild sika deer in the Hokkaido, Shizuoka, Gifu, Yamaguchi, and Kagoshima Prefectures carried antimicrobial-resistant *E. coli* [3]. The difference in the prevalence rate is considered to result from

Table 1. *Escherichia coli* isolates from wild sika deer and their susceptibility to antimicrobials

Sampling place	Number of				% Resistance of isolates											
	Animals	Isolates	Resistance	(%)	AMP	CFZ	CTX	FOX	GEN	KAN	STR	TET	SXT	CHL	NAL	CIP
Hokkaido island	132	531	8	(1.5)	0.2	0.2	0	0	0	0	0.2	1.5	0.9	0.2	0	0
Honshu mainland other than Yamaguchi prefecture	72	218	0	(0)	0	0	0	0	0	0	0	0	0	0	0	0
Shikoku and Kyushu islands and Yamaguchi prefecture	33	99	1	(1.0)	0	0	0	0	0	0	1.0	1.0	0	0	0	0
Total	237	848	9	(1.1)	0.1	0.1	0	0	0	0	0.2	1.1	0.6	0.1	0	0

AMP, ampicillin; CFZ, cefazolin; CTX, cefotaxime; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; TET, tetracycline; SXT, sulfamethoxazole-trimethoprim; CHL, chloramphenicol; NAL, nalidixic acid; CIP, ciprofloxacin.

Table 2. Properties of antimicrobial resistant *Escherichia coli* isolates

Isolate No.	Sampling place	Sample No.	O-genotype[a)]	Sequence type[b)]	Resistance profile	Resistance gene[c)]	Plasmid replicon[d)]	MIC (μg/ml)						Virulence factor gene
								AMP	CFZ	CHL	STR	TET	SXT (TMP/SMX)	
D1133	Hokkaido	A40	Og145	ST137	AMP-CFZ-CHL-TET	*bla*$_{TEM-1A}$, *floR*, *sul2*, *tetA*, *strA*, *strB*	Inc.FIB, Inc.R	512	2	256	32	128	0.03/0.59	*astA*, *chuA*, *cif*, *eae*, *efa1*, *ehxA*, *espA*, *espB*, *espJ*, *etpD*, *gad*, *iha*, *iutA*, *neuC*, *nleA*, *nleB*, *nleC*, *ompT*, *terC*, *tir*, *traT*
D1205	Hokkaido	A61	UT	ST6488	TET	*tetA*	Inc.FIB	4	2	8	4	64	0.13/2.38	*lpfA*, *terC*, *traT*
D1399	Hokkaido	B22	Og120	ST711	TET-SXT	*sul1*, *dfrA12*, *tetA*, *aadA2*	Inc.FIB, Inc.FII, Inc.I1	4	2	2	16	64	>16/304	*cib*, *iss*, *lpfA*, *ompT*, *terC*, *traT*
D1400	Hokkaido	B22	Og120	NT	TET-SXT	NT	NT	4	2	4	8	64	>16/304	
D1401	Hokkaido	B22	Og120	NT	TET-SXT	NT	NT	4	2	4	16	64	>16/304	
D1402	Hokkaido	B22	Og120	NT	TET-SXT	NT	NT	4	2	4	16	64	>16/304	
D1403	Hokkaido	B22	Og120	NT	TET-SXT	NT	NT	4	2	4	16	64	>16/304	
D1477	Hokkaido	B57	Og142	ST154	STR-TET	*sul2*, *tetB*, *strA*, *strB*	Inc.FIB, Inc.FII	4	2	8	32	128	0.5/9.5	*astA*, *gad*, *hra*, *iss*, *lpfA*, *ompT*, *papC*, *terC*, *traT*
D2028	Shikoku	H28-10	UT	NA	STR-TET	*tetA*, *strA*, *strB*	Inc.FII	4	1	8	64	128	0.13/2.38	*chuA*, *gad*, *iss*, *lpfA*, *ompT*, *terC*, *traT*, *usp*

[a)]UT, untypable. [b)]NT, not tested; NA, not assigned. [c)]NT, not tested. [d)]NT, not tested; ND, not detected. AMP, ampicillin; CFZ, cefazolin; CHL, chloramphenicol; STR, streptomycin; TET, tetracycline; SXT, sulfamethoxazole-trimethoprim.

the differences between the two study designs, such as the capture location of each wild sika deer and the spatial sampling density. However, both studies suggested that the prevalence of antimicrobial-resistant *E. coli* in wild sika deer is currently low.

Draft genome sequences of the five independent isolates, D1133, D1205, D1399, D1477, and D2028, were determined using a NovaSeq 6000 sequencer (Illumina, Inc., San Diego, CA, USA). The raw sequencing reads were deposited in the DDBJ Sequence Read Archive under the accession numbers DRX247858 to DRX247862. The antimicrobial resistance gene, sequence type (ST), and plasmid replicon type of the sequenced isolates were analyzed using open-access bioinformatic tools, i.e., MLST, ResFinder, PlasmidFinder, and VirulenceFinder supplied by the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) [28]. As shown in Table 2, four types of antimicrobial resistance profiles were observed, and all antimicrobial-resistant *E. coli* isolates showed resistance to TET. Among the five independent isolates, four (67%) showed resistance to multiple antimicrobials: D1133, AMP-CEZ-CHL-TET; D1399, SXT-TET; and D1477 and D2028, STR-TET (Table 2). Resistance of *E. coli* to TET, STR, and SXT has frequently been found in farm animals in Japan [13]. Furthermore, the antimicrobial resistance genes detected in this study, i.e., *bla*$_{TEM-1A}$, *aadA2*, *strA*, *strB*, *tetA*, *tetB*, *floR*, *sul1*, *sul2*, and *dfrA12* (Table 2), are widely distributed among *E. coli* found in humans and animals [17, 23–25]. Inc. F-type replicons including Inc.FIB and Inc.FII, which were detected in most antimicrobial-resistant isolates (Table 2), are also widely distributed among *E. coli* found in humans and animals and contribute to the transfer of antimicrobial resistance genes such as *bla*$_{CTX-M}$ [1, 15]. Therefore, wild sika deer is considered a potential risk for the transmission of antimicrobial resistance, even with the currently low prevalence of carriers.

We found 30 virulence factor genes identified in the five antimicrobial resistant isolates (Table 2). The D1133 strain could be categorized as atypical enteropathogenic *E. coli* (aEPEC) based on the profile of virulence factor genes, i.e., the presence of *eae* and the absence of *stx* and EPEC adherence factor plasmid (pEAF) [8]. Clonal relationships among aEPEC strains isolated from humans, farm animals, and wild animals have been reported [5, 20]. Moreover, various STs were found in the antimicrobial-resistant isolates, i.e., ST137, ST154, ST711, and ST6488, but the D2028 isolate could not be assigned to any existing ST (Table 2). A combination of ST137, serogroup O145, and the Inc.FIB replicon type has been reported in human clinical cases [16, 19]. Although the serogroups and replicon types were different or have not been analyzed, ST154, ST711, and ST6488 were isolated from farm animals and wastewater in previous studies [6, 22, 27]. Taken together, these results showed that most of the molecular

characteristics of antimicrobial-resistant *E. coli* isolates derived from wild sika deer in this study are similar to those of *E. coli* derived from humans, farm animals, and the environment. Notably, all five antimicrobial-resistant *E. coli* isolates were isolated from sika deer captured within 10 km from hospitals, farms, or river, and these sika deer might have contacted human and farm animals.

The low prevalence of antimicrobial-resistant *E. coli* in wild sika deer suggests that wild sika deer are not currently likely to be a source for the emergence and transmission of antimicrobial resistance in the natural environments of Japan. However, most of the repertoires of antimicrobial resistance genes and the majority of the combinations of molecular characteristics in the *E. coli* isolates obtained in this study were common with those in *E. coli* derived from humans, farm animals, and the environment. Therefore, wild sika deer can potentially mediate antimicrobial resistance by coming into contact with humans, animals, and their surroundings.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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