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Bacteriology

A survey of antimicrobial-resistant *Escherichia coli* prevalence in wild mammals in Japan using antimicrobial-containing media

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ABSTRACT. The emergence and spread of antimicrobial-resistant bacteria and resistance genes pose serious human and animal health concerns. Therefore, to control antimicrobial-resistant bacteria in the environment, the status of antimicrobial resistance of *Escherichia coli* in a variety of wild mammals and their prevalence were examined using antimicrobial-containing media. In total, 750 isolates were obtained from 274/366 (74.9%) wild mammals, and antimicrobial-resistant *E. coli* was detected in 37/750 isolates (4.9%) from 7 animal species (26/366 [7.1%] individuals). Using antimicrobial-containing media, 14 cefotaxime (CTX)- and 35 nalidixic acid-resistant isolates were obtained from 5 (1.4%) and 17 (4.6%) individuals, respectively. CTX-resistant isolates carried $bla_{CTX\text{-}M\text{-}27}$, $bla_{CTX\text{-}M\text{-}55}$, $bla_{CTX\text{-}M\text{-}1}$, and $bla_{CMY\text{-}2}$, with multiple resistance genes. Fluoroquinolone-resistant isolates had multiple mutations in the quinolone-resistance determining regions of *gyrA* and *parC* or *qnrB19*. Most resistant isolates exhibited resistance to multiple antimicrobials. The prevalence of antimicrobial-resistant bacteria observed in wild mammals was low; however, it is essential to elucidate the causative factors related to the low prevalence and transmission route of antimicrobial-resistant bacteria/resistance genes released from human activities to wild animals and prevent an increase in their frequency.

KEYWORDS: antimicrobial resistance, *Escherichia coli*, human activity, medium, wild mammal

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Antimicrobial resistance (AMR) is a growing global public and animal health concern. In response to the Global Action Plan on AMR adopted in 2015 [31], the National Action Plan for AMR (2016–2020) was published by the Japanese government in 2016 [28]; multi-sectoral surveillance (human, animal, and environmental) was integrated to estimate the status of AMR in multiple sectors using the One Health approach. Therefore, the Nippon AMR One Health Report was published in 2017 to combat AMR through multi-sectoral collaboration [12].

Antimicrobials are essential for treating bacterial infections in medical and veterinary fields, although the emergence and prevalence of antimicrobial-resistant bacteria are closely related to their extensive use [8]. In addition, human activities, including livestock, release antimicrobial-resistant bacteria and AMR genes to the environment via wastewater and gavage, and then transmitted to wild animals [30]. Wild animals act as reservoirs for antimicrobial-resistant bacteria and AMR genes [30]. In this context, the transmission of antimicrobial-resistant bacteria/resistance determinants between wild and domestic animals results in the maintenance of AMR in these organisms [33].

Escherichia coli is a commensal bacterium prevalent in the intestines of many animal species and is used as a bacterial indicator of AMR [8]. Recent studies on antimicrobial-resistant bacteria in wild animals in Japan revealed a low prevalence of antimicrobial-resistant *E. coli* in Japanese serows in the 1980s [15], wild mice in 2006 [11], wild cranes in 2007–2008 [16], deer and wild boars in 2013–2017 [1], deer in 2016–2019 [27], great cormorants in 2018–2019 [20], greater white-fronted geese in 2019 [7], and Amami rabbits in 2017–2020 [18]. However, the application of antimicrobial-containing media for the isolation of antimicrobial-resistant *E. coli* indicated a high prevalence of fluoroquinolone-resistant *E. coli* in deer in urban regions [10] and extended-spectrum beta-lactamase (ESBL)-producing *E. coli* in weasels around animal facilities [33]. In a previous study by Kinjo *et al*. [15], a survey using antimicrobial-containing media effectively estimated the status of antimicrobial-resistant bacteria in wild animals.

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The aim of this study was to clarify the prevalence and spread of antimicrobial-resistant bacteria in wild mammals in Japan. Antimicrobial-containing media were used to clarify the prevalence of *E. coli* resistance to medically important antimicrobials in wild mammals. This study will provide a valuable understanding of AMR in *E. coli* in a wide range of wild mammals.

MATERIALS AND METHODS

Sample collection and processing

In total, 366 fecal samples from 243 sika deer (*Cervus Nippon*), 43 nutrias (*Myocastor coypus*), 22 masked palm civets (*Paguma larvata*), 18 wild boars (*Sus scrofa*), 8 raccoon dogs (*Nyctereutes procyonoides*), 6 Japanese badgers (*Meles anakuma*), 5 small Japanese field mice, and 21 others (8 species) were obtained between 2018 and 2021 for the isolation of *E. coli* (Table 1). All mammals were tracked for harmful wildlife control, hunting, and academic capture, which were conducted with the approval of the prefectural government as shown in Table 1.

Isolation and identification of E. coli from fecal samples

Isolation and identification of *E. coli* from fecal samples were performed as previously described [1]. Briefly, *E. coli* was isolated from fecal samples using deoxycholate-hydrogen sulfide-lactose (DHL) agar (Eiken Chemical Co., Ltd., Tokyo, Japan) or CHROMagar™ STEC plates (CHROMagar, Paris, France). Antimicrobial-resistant *E. coli* was screened using DHL medium containing cephalosporins (50 µg/mL of cefazolin [CFZ], 50 µg/mL of cephalexin, or 1 µg/mL of cefotaxime [CTX]) or quinolone (25 µg/mL of nalidixic acid [NAL]). Isolates were stored in stock media (20% glycerin buffer or 10% skim milk solution) at −80°C.

Minimum inhibitory concentration (MIC) analysis of antimicrobial agents

Broth microdilution tests (GDB7; Eiken Chemical, Tokyo, Japan) were performed to determine the MICs of the antimicrobial agents, as previously described [1]. Twelve antimicrobials were tested, namely ampicillin (AMP), CFZ, CTX, meropenem (MEM), gentamicin (GEN), kanamycin (KAN), tetracycline (TET), NAL, ciprofloxacin (CIP), colistin (CST), chloramphenicol (CHL), and trimethoprim/sulfamethoxazole (SXT). The resistance breakpoints of all antimicrobial agents were defined according to the guidelines of the Clinical and Laboratory Standards Institute [4].

Table 1. Isolation of *Escherichia coli* from wild mammals

Animal species	Sampling location	No. of Animals tested	No. of animals positive for isolation	%	No. of isolates	No. of Animals positive for antimicrobial resistant bacteria	%	No. of isolates exhibitting antimicrobial resistance	%	No. of Animals positive for cefotaxime (CTX) resistant bacteria	%	No. of isolates exhibitting CTX resistance	No. of Animals positive for quinolone resistant bacteria	%	No. of isolates exhibitting quinolone resistance
Sika deer	Gifu, Wakayama, and Kagoshima (Yaku island) prefectures	243	189	77.8	517	19	7.8	28	5.4	2	0.8	6	10	4.1	17
Nutria	Yamaguchi prefecture	43	12	27.9	33	0	0	0	0						
Masked palm civet	Gifu prefecture	22	22	100	61	1	4.5	1	1.6				3	13.6	7
Wild boar	Gifu, Wakayama prefectures	18	18	100	54	2	11.1	4	7.4						
Raccoon dog	Nagano, Gifu, Kagoshima prefectures	8	8	100	24	0	0	0	0				1	12.5	2
Japanese badger	Gifu and Yamaguchi prefectures	6	5	83.3	9	1	16.7	1	11	1	16.7	3			
Small Japanese field mouse	Gifu prefecture	5	0	0.0	0										
Large Japanese field mouse	Gifu prefecture	4	4	100	7	0	0	0	0						
Siberian weasel	Gifu prefecture	4	4	100	11	0	0	0	0						
Fox	Gifu prefecture	4	4	100	11	1	25	1	9.1	1	25	2	2	50	6
Japanese monkey	Gifu prefecture	3	3	100	9	1	33.3	1	11.1						
Raccoon	Gifu prefecture	2	1	50	2	1	50	1	50	1	50	3	1	50	3
Cat	Gifu prefecture	2	2	100	6	0	0	0	0						
Bear	Akita prefecture	1	1	100	3	0	0	0	0						
Japanese marten	Gifu prefecture	1	1	100	3	0	0	0	0						
Total		366	274	74.9	750	26	7.1	37	4.9	5	1.4	14	17	4.6	35

β-lactamase gene identification and whole-genome sequencing (WGS) analysis

CTX- and CIP-resistant *E. coli* were subjected to β-lactamase gene identification [5] and WGS analysis, as previously described [21]. After *de novo* assembly using the CLC Genomics Workbench pipeline, the obtained contigs were analyzed to determine multilocus sequence typing (MLST 2.0), bacterial serotyping (SeroTypeFinder 2.0), and FimH typing (FimTyper 1.0), as well as to detect AMR genes (ResFinder 4.1) in the Center for Genomic Epidemiology (http://www.genomicepidemiology.org). To identify the location of the resistance genes, the contig sequences including the ESBL genes and *qnrB19* were screened against the nucleotide database of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/).

Ethics approval statement

This study was approved by the Ethics Committee for Animal Research and Welfare of Gifu University, Japan (approval number 17055) and the Ethics Committee for Academic Research of Captured Animals in Gifu Prefecture (approval number 269). Other approvals were not applicable, as feces from wild mammals were collected from dead or slaughtered animals and used in this study.

RESULTS

E. coli strains isolated from wild mammals

A total of 750 *E. coli* strains were isolated from 274/366 (74.9%) fecal samples, including 517 isolates from 189/243 (77.8%) deer, 33 from 12/43 (27.9%) nutrias, 61 from 22/22 (100%) masked palm civets, 54 from 18/18 (100%) wild boars, and 24 isolates from 8/8 (100%) raccoon dogs (Table 1).

Antimicrobial susceptibility tests detected antimicrobial-resistant E. coli isolates

Of the 750 isolates, 37 were antimicrobial-resistant. These isolates were collected from 26 samples (7.1%) from 7 animal species, as follows: deer (19/243, 7.8%), masked palm civet (1/22, 4.5%), wild boar (2/18, 11.1%), Japanese badger (1/6, 16.7%), fox (1/4, 25%), Japanese monkeys (1/3, 33.3%), and raccoons (1/2, 50%) (Table 1). In antimicrobial susceptibility tests, resistance to TET was the highest at 3.5%, followed by resistance to CST (0.9%) and AMP (0.5%) (Table 2). The percentage of resistance to CFZ, NAL, CIP, and CHL was 0.1%; however, that to CTX, MEM, GEN, KAN, and SXT was zero. One fox isolate was resistant to AMP, TET, NAL, CIP, and CHL.

Using DHL medium containing cephalosporins, 14 CTX-resistant isolates were obtained from 5 wild mammals (1.4%): 2/243 sika deer (0.8%, 6 isolates), 1/6 Japanese badgers (16.7%, 3 isolates), 1/4 foxes (25%, 2 isolates), and 1/2 raccoons (50%, 3 isolates) (Table 1). Based on the resistance profile and β-lactamase types, identical isolates were obtained from four of the five animals (two sika deer, one Japanese badger, and one raccoon); however, two different isolates were obtained from the same fox. The sika deer isolates exhibited resistance to AMP, CFZ, CTX, TET, and NAL (WLCEX47-49 [isolate ID]), as well as AMP, CFZ, and CTX (WLCEX65-67) with CTX-M groups 1 and 9 β-lactamase, respectively (Table 3). The raccoon isolates exhibited resistance to AMP, CFZ, CTX, TET, NAL, and CIP (WLCTX8-10) with CTX-M group 9 β-lactamase. Japanese badger isolates (WLCTX11-13) exhibited resistance to AMP, CFZ, and CTX with CTX-M group 1 β-lactamase. One of the two fox isolates exhibited resistance to AMP, CFZ, CTX, TET, NAL, CIP, and CHL (WLCTX4) with CTX-M group 1 β-lactamase, and the other exhibited resistance to AMP, CFZ, CTX, TET,

Table 2. Distribution of antimicrobial resistance in *Escherichia coli* isolates from wild mammals

Animal species	No. of isolates	%Resistance (break point: μg/mL)											
		AMP (32)	CFZ (32)	CTX (4)	MEM (2)	GEN (16)	KAN (64)	TET (16)	NAL (32)	CIP (2)	CST (4)	CHL (32)	SXT (76/4)
Sika deer	517	0.4	0.2	0	0	0	0	4.1	0	0	1.0	0	0
Masked palm civet	61	1.6	0	0	0	0	0	0	0	0	0	0	0
Wild boar	54	0	0	0	0	0	0	7.4	0	0	0	0	0
Nutria	33	0	0	0	0	0	0	0	0	0	0	0	0
Raccoon dog	24	0	0	0	0	0	0	0	0	0	0	0	0
Fox	11	9.1	0	0	0	0	0	9.1	9.1	9.1	0	9.1	0
Siberian weasel	11	0	0	0	0	0	0	0	0	0	0	0	0
Japanese badger	9	0	0	0	0	0	0	0	0	0	0	0	0
Japanese monkey	9	0	0	0	0	0	0	0	0	0	11.1	0	0
Large Japanese field mouse	7	0	0	0	0	0	0	0	0	0	0	0	0
Cat	6	0	0	0	0	0	0	0	0	0	0	0	0
Bear	3	0	0	0	0	0	0	0	0	0	0	0	0
Japanese marten	3	0	0	0	0	0	0	0	0	0	0	0	0
Raccoon	2	0	0	0	0	0	0	0	0	0	50	0	0
Total	750	0.5	0.1	0	0	0	0	3.5	0.1	0.1	0.9	0.1	0

AMP, ampicillin; CFZ, cefazolin; CTX, cefotaxime; MEM, meropenem; GEN, gentamicin; KAN, kanamycin; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; CST, colistin; CHL, chloramphenicol; SXT trimethoprim/sulfamethoxazole.

Table 3. Resistance profile of cefotaxime-resistant *Escherichia coli* from wild mammals using cephalosporin-containing deoxycholate-hydrogen sulfide-lactose (DHL) medium

Animal species	Sample ID	Isolate ID	Location	Isolation year	Resistance profile	β-lactamase gene
Sika deer	WL33	WLCEX-47	Gifu	2019	AMP-CFZ-CTX-TET-NAL	CTX-M group 1
		WLCEX-48	Gifu	2019	AMP-CFZ-CTX-TET-NAL	CTX-M group 1
		WLCEX-49	Gifu	2019	AMP-CFZ-CTX-TET-NAL	CTX-M group 1
	WL62	WLCEX-65	Gifu	2019	AMP-CFZ-CTX	CTX-M group 9
		WLCEX-66	Gifu	2019	AMP-CFZ-CTX	CTX-M group 9
		WLCEX-67	Gifu	2019	AMP-CFZ-CTX	CTX-M group 9
Fox	WL174	WLCTX4	Gifu	2021	AMP-CFZ-CTX-TET-NAL-CIP-CHL	CTX-M group 1
		WLCTX5	Gifu	2021	AMP-CFZ-CTX-TET-NAL-CHL	CIT group
Raccoon	WL176	WLCTX8	Gifu	2021	AMP-CFZ-CTX-TET-NAL-CIP	CTX-M group 9
		WLCTX9	Gifu	2021	AMP-CFZ-CTX-TET-NAL-CIP	CTX-M group 9
		WLCTX10	Gifu	2021	AMP-CFZ-CTX-TET-NAL-CIP	CTX-M group 9
Japanese badger	WL177	WLCTX11	Gifu	2021	AMP-CFZ-CTX	CTX-M group 1
		WLCTX12	Gifu	2021	AMP-CFZ-CTX	CTX-M group 1
		WLCTX13	Gifu	2021	AMP-CFZ-CTX	CTX-M group 1

AMP, ampicillin; CFZ, cefazolin; CTX, cefotaxime; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; CHL, chloramphenicol.

Table 4. Resistance profile of ciprofloxacin-resistant *Escherichia coli* from wild mammals using nalidixic acid-containing deoxycholate-hydrogen sulfide-lactose (DHL) medium

Animal species	Sample ID	Isolate ID	Location	Isolation year	Resistance profile
Masked palm civet	WL-30	WLNA12	Gifu	2019	AMP-GEN-TET-NAL-CIP
		WLNA13	Gifu	2019	AMP-GEN-TET-NAL-CIP
		WLNA14	Gifu	2019	AMP-GEN-TET-NAL-CIP
Sika deer	WL-33	WLNA15	Gifu	2019	AMP-GEN-NAL-CIP-CHL-SXT
		WLNA16	Gifu	2019	AMP-GEN-NAL-CIP-CHL-SXT
		WLNA17	Gifu	2019	AMP-GEN-NAL-CIP-CHL-SXT
	WL-64	WLNA21	Gifu	2019	AMP-GEN-TET-NAL-CIP
		WLNA22	Gifu	2019	AMP-GEN-TET-NAL-CIP
		WLNA23	Gifu	2019	AMP-GEN-TET-NAL-CIP
Fox	WL174	WLNA50	Gifu	2021	AMP-TET-NAL-CIP-CHL
	WL175	WLNA52	Gifu	2021	AMP-TET-NAL-CIP-CHL
		WLNA53	Gifu	2021	AMP-TET-NAL-CIP-CHL
		WLNA54	Gifu	2021	AMP-TET-NAL-CIP-CHL
Raccoon	WL176	WLNA55	Gifu	2021	AMP-NAL-CIP
		WLNA56	Gifu	2021	AMP-TET-NAL-CIP-CHL-SXT
		WLNA57	Gifu	2021	AMP-KAN-TET-NAL-CIP-CHL-SXT

AMP, ampicillin; GEN, gentamicin; KAN, kanamycin; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; CST, colistin; CHL, chloramphenicol; SXT trimethoprim/sulfamethoxazole.

NAL, and CHL (WLCTX5) with CIT group β-lactamase. Additionally, 35 NAL-resistant *E. coli* strains were isolated from 17 (4.6%) of the 366 wild mammals, including deer (10/243, 4.1%), palm civets (3/22, 13.6%), raccoon dogs (1/8, 12.5%), fox (2/4, 50%), and raccoons (1/2, 50%) (Table 1). Based on the MIC analysis, 16 (45.7%) of the 35 NAL-resistant isolates showed CIP resistance with resistance to AMP (100%), TET (12/16, 75%), GEN (9/16, 56.3%), CHL (9/16, 56.3%), and/or SXT (5/16, 31.3%) (Table 4). The sika deer isolates exhibited resistance to AMP, GEN, NAL, CIP, CHL, and SXT (WLNA15-17) and AMP, GEN, TET, NAL, and CIP (WLNA21-23). The palm civet isolates exhibited resistance to AMP, GEN, TET, NAL, and CIP (WLNA12-14). The isolates from the two foxes exhibited resistance to AMP, TET, NAL, CIP, and CHL (WLNA50, 52–54). Among the three raccoon isolates, one exhibited resistance to AMP, NAL, and CIP (WLNA55); another to AMP, TET, NAL, CIP, CHL, and SXT (WLNA56); and the other to AMP, KAN, TET, NAL, CIP, CHL, and SXT (WLNA57) (Table 4).

Next-generation sequencing (NGS)-detected AMR genes

Fourteen CTX- and CIP-resistant *E. coli* isolates (4 CTX-resistant isolates, 8 CIP-resistant isolates, and 2 both resistant isolates) were subjected to NGS (Table 5). The datasets of the 14 *E. coli* isolates (Table 5) are available in the GenBank database (accession number: DRA014645; SRA-run numbers: DRR397947–DRR397960) (https://ddbj.nig.ac.jp/resource/sra-submission/DRA014645).

Table 5. Whole genome analysis of selected cefotaxim/ciprofloxacin-resistant *Escherichia coli* isolates obtained using antimicrobial-containing DHL medium

Host	Sample ID	Isolates ID	Putative Serotype	MLST	HimH type	β-lactamase type	NAL MIC	CIP MIC	Quinolone-resistance determining-regions (QRDR) mutation		
									gyrA	*parC*	*parE*
Dear	WL33	WLCEX48	H14	ST2144	fimH31	$bla_{CTX-M-1}$, bla_{TEM-1}	32 (R)	1 (S)	Wild type	Wild type	
		WLNA15	O9:H19	ST162	fimH32	bla_{TEM-1}	>128 (R)	>4 (R)	S83L, D87N	S80I	
	WL62	WLCEX65	O11:H6	ST457	fimH145	$bla_{CTX-M-27}$	2 (S)	≤0.03 (S)	Wild type	Wild type	
	WL-64	WLNA22	O184:H51	ST155	fimH32	bla_{TEM-1}	>128 (R)	>4 (R)	S83L, D87N	S80I	
Fox	WL174	WLCTX4	O89:O162:H10	ST744	fimH 54	$bla_{CTX-M-55}$	>128 (R)	>4 (R)	S83L, D87N	A56T, S80I	
		WLCTX5	O88:H39	ST1140	fimH 221	bla_{CMY-2}	>128 (R)	0.5 (S)	S83L	Wild type	
		WLNA50	H8	ST164	fimH1109	bla_{TEM-1}	>128 (R)	4 (R)	S83L	S80I	
	WL175	WLNA54	H8	ST164	fimH1109	bla_{TEM-1}	>128 (R)	4 (R)	S83L	S80I	
Raccoon	WL176	WLCTX8	O25:H4	ST131	fimH 30	$bla_{CTX-M-27}$	>128 (R)	>4 (R)	S83L, D87N	S80I, E84V	I529L
		WLNA55	O75:H5	ST1193	fimH64	bla_{TEM-1}	>128 (R)	>4 (R)	S83L, D87N	S80I	L416F
		WLNA56	O65:H14	ST533	fimH31	bla_{TEM-1}	>128 (R)	>4 (R)	S83L, D87N	S80I	
		WLNA57	O65:H14	ST533	fimH31	bla_{TEM-1}	>128 (R)	>4 (R)	S83L, D87N	S80I	
Badger	WL177	WLCTX11	O24:H18	ST657	fimH 97	$bla_{CTX-M-55}$	2 (S)	≤0.03 (S)	Wild type	Wild type	
Masked palm civet	WL30	WLNA12	O184:H51	ST155	fimH32	bla_{TEM-1}	>128 (R)	>4 (R)	S83L, D87N	S80I	

Host	Isolates ID	Aminogycoside resistance					Tetracycline resistance			Penicol resistance			Methoprim resistance			Sulfonamide resistance			Others						
		aac(3)-IId	*aadA2*	*aph(3")-Ib*	*aph(3')-Ia*	*aph(6)-Id*	*tet(A)*	*tet(B)*	*tet(M)*	*catA1*	*cmlA1*	*floR*	*dfrA1*	*dfrA14*	*dfrA17*	*sul1*	*sul2*	*sul3*	*mdf(A)*	*mef(B)*	*mph(A)*	*qacG2*	*qacEdelta1*	*qnrB19*	*sitABCD*
Dear	WLCEX48			+		+	+					+					+		+		+			+	
	WLNA15	+		+		+						+		+			+								+
	WLCEX65																		+						
	WLNA22	+					+								+										+
Fox	WLCTX4							+				+						+	+						+
	WLCTX5		+	+		+	+		+		+	+					+	+	+	+		+			
	WLNA50			+		+		+				+					+							+	
	WLNA54			+		+		+				+					+							+	
Raccoon	WLCTX8			+		+	+										+		+						+
	WLNA55																								+
	WLNA56			+		+		+		+			+			+	+						+		+
	WLNA57			+	+	+		+		+			+			+	+						+		+
Badger	WLCTX11																		+						+
Masked palm civet	WLNA12	+					+																		+
CTX resistance	(n=6)	0	1	3	0	3	3	1	1	0	1	3	0	0	0	0	3	2	6	1	1	1	0	1	3
CIP resistance	(n=10)	3	0	6	1	6	3	5	0	2	0	4	2	1	1	2	6	1	2	0	0	0	2	2	8
Total	(n=14)*	3	1	8	1	8	5	5	1	2	1	6	2	1	1	2	8	2	6	1	1	1	2	3	9

CTX, cefotaxime; NAL, nalidixic acid; CIP, ciprofloxacin. *Two isolates exhibited resistance to both CTX and CIP.

Apart from β-lactamase genes, 24 resistance genes for aminoglycosides, TET, phenicol, methoprim, sulfonamide and others were detected in the 14 isolates (Table 5). Most of the isolates carried multiple resistance genes. The *sitABCD* gene (9/14) was the most frequently observed, followed by *aph(3")-Ib, aph(6)-Id* and *sul2* (8/14), and *floR* and *mdf*(A) (6/14). Either *tet*(A) or *tet*(B) were found in 10/14 isolates. Diversity of serotypes, sequence types (STs), and FimH types was also observed (Table 5). Of the six CTX-resistant isolates, two from different deer samples (WL62 and WL33 [sample ID]) were O11:H6-ST457-fimH145 CTX-M-27 (WLCEX65) and OUT:H14-ST2144-fimH31 CTX-M-1 producers (WLCEX48), respectively. In addition, WLCEX48 exhibited NAL resistance carrying *qnrB19* without the QRDR mutation. One isolate each from a raccoon and a badger were O25:H4-ST131-fimH30 CTX-M-27 (WLCTX8) and O24:H18-ST657-fimH97 CTX-M-55 producers (WLCTX11), respectively. Two fox isolates (WL174) were O89:O162:H10-ST744-fimH54 CTX-M-55 producer with CIP resistance (WLCTX4) and O88:H39-ST1140-fimH221 CMY-2 producer with NAL resistance (WLCTX5). Moreover, multiple mutations in the QRDR of DNA gyrase (*gyrA*) (S83L, D87N) and topoisomerase IV (*parC*) (S80I) were observed in all CIP-resistant isolates, except for two isolates (WLNA50 and WLNA54) from two foxes (WL174 and WL175), which carried *qnrB19* with QRDR mutations in *gyrA* (S83L) and *parC* (S80I). Although the location of $bla_{\text{CTX-M-1}}$ in WLCEX48 was unclear owing to the presence of gene in a short contig, $bla_{\text{CTX-M-27}}$, $bla_{\text{CTX-M-55}}$, and *qnrB19* were detected in the plasmids.

DISCUSSION

Several studies have demonstrated a low prevalence of antimicrobial-resistant *E. coli,* isolated using non-antimicrobial agar, in wild mammals with limited contact with human activities, which are rarely treated with antimicrobials in Japan [1, 11, 15, 27]. In this study, similar results were obtained using a non-antimicrobial medium. These results indicate that wild mammals may live in environments with limited selective antimicrobial forces. Recently, a high prevalence of NAL-and/or CIP-resistant *E. coli* was reported in wild deer in urban areas [10]. Although Kinjo *et al*. [15] demonstrated a low prevalence of resistant bacteria in Japanese serows using antimicrobial-containing agar in the 1980s, recent information regarding antimicrobial-resistant *E. coli* in wild animals using antimicrobial-containing agar is limited. Therefore, in this study, *E. coli* resistance to medically important antimicrobials, such as broad-spectrum cephalosporin and fluoroquinolone, was analyzed using an antimicrobial-containing medium in wild mammals. Results showed that most antimicrobial-resistant *E. coli* strains exhibited resistance to multiple antimicrobials. Our previous studies revealed that wild animals acquire resistance genes via plasmids encoding multiple resistance genes [33] and interspecies transmission of plasmids among Enterobacterales [32]. In this study, most of the ESBL genes and *qnrB19* were detected in the plasmids of CTX- and CIP-resistant isolates. These results suggest that, despite the low prevalence of antimicrobial-resistant bacteria in wild mammals in Japan, caution is required regarding the potential transmission of AMR genes to pathogenic bacteria.

The acquisition of antimicrobial-resistant bacteria in some communities of wild animals depends on the habitats of their hosts [30]. AMR was mostly observed in single animals of each species, although TET resistance was observed in *E. coli* isolates from multiple sika deer and wild boars using non-antimicrobial agar (Table 1). Using antimicrobial-containing agar, ESBL-producing or NAL-resistant *E. coli* was isolated from multiple animals (sika deer [herbivores]; wild boar, masked palm civet, and fox [omnivores]). Several studies have indicated that the prevalence of antimicrobial-resistant bacteria is higher in omnivores than in herbivores [1, 6]. In this study, ESBL-producing *E. coli* was isolated from sika deer, fox, raccoon, and badger using antimicrobial-containing agar, suggesting that frequent prevalence of ESBL producer in omnivores was observed relatively. According to a study conducted in Switzerland, of the 84 red deer and 64 roe deer tested, ESBL-producing *E. coli* ($bla_{\text{CTX-M-1}}$) were isolated from only one roe deer [26]. $bla_{\text{CTX-M-14}}$- and $bla_{\text{CTX-M-15}}$-producing *E. coli* were isolated from red deer (1/62, 1.6%) and fallow deer (1/29, 3.4%) [29]. ESBL-producing *E. coli* was also isolated from roe deer (13/573, 2.3%) [23]. In contrast, some studies have isolated ESBL producers from foxes (37/321, 11.5%) and badgers (13/146, 8.9%) in Northern Ireland [19] and red foxes (2/52, 3.8%) in Portugal [24]. Given that a small number of animals were examined in the present study, except for sika deer, further studies are required to clarify the prevalence of ESBL producers among omnivores.

QRDR mutations of *gyrA* and *parC* cause quinolone resistance in bacteria, and multiple QRDR mutations cause fluoroquinolone resistance [22]. In this study, most CIP-resistant isolates carried both double point mutations in *gyrA* (S83L, D87N) and single or more point mutations in *parC* (S80I). However, two fox isolates (WLNA50 and WLNA54) carrying *qnrB19* with QRDR mutations in *gyrA* (S83L) and *parC* (S80I) exhibited fluoroquinolone resistance. Previous studies have demonstrated a low prevalence of *qnrS* and *qnrB* in Enterobacterales isolated from domestic animals and humans in Japan [2, 9, 14]. Thus, in addition to ESBL genes, the presence of *qnrB19* in plasmids of *E. coli* of wildlife origin may cause a significant problem in medical and veterinary settings, wherein fluoroquinolones are available for treating various bacterial diseases.

In this study, most *E. coli* strains from wild mammals exhibiting resistance to either CTX or CIP were resistant to multiple antimicrobials and carried multiple resistance genes, such as aminoglycoside, TET, phenicol, and/or methoprim antimicrobials. High frequencies of TET resistance may be associated with food-producing animals [8]. Given that various serotypes, fimH types, and MLST types were observed in this study, the relatedness of their properties to colonization in wild animals was considered obscure. In addition, most *E. coli* resistant to either CTX or CIP carried *sitABCD*. The SitABCD system mediates the transport of iron and manganese and contributes to resistance to oxidative stress and protection against hydrogen peroxide [13, 25]. A high prevalence of *sitABCD* has been reported in *E. coli* isolated from broiler chicken farms [17]. This SitABCD system mechanism may contribute to the effective survival of *E. coli* in the environment.

Moreover, in cases with an increased prevalence of antimicrobial-resistant bacteria in wild animals, regulatory management may be essential to control AMR in food-producing animals [3]. Although a low prevalence of antimicrobial-resistant bacteria

was observed, risk assessment is required to elucidate the causative factors related to the low prevalence and transmission route of antimicrobial-resistant bacteria/resistance genes released from human activities to wild animals. Such approaches may also contribute to reducing the inter- and intraspecific dissemination of antimicrobial-resistant bacteria/resistance genes in wild animals. Given that limited information is available for estimating the risk to human societies, establishing scenarios of risk assessments and continuous multifactorial studies are required.

CONFLICT OF INTERESTS. The authors declare no conflicts of interest.

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