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Antimicrobial susceptibility of *Escherichia coli* isolates obtained from wild mammals between 2013 and 2017 in Japan

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ABSTRACT. The emergence and prevalence of antimicrobial-resistant bacteria in wild animals are a great concern for public health. A total of 963 *Escherichia coli* isolates from 475 wild mammals (242 sika deers, 112 wild boars, 113 small mammals, 4 Japanese badger, 2 Tokara cows, and 2 Amani rabbits), collected between 2013 and 2017, were examined for antimicrobial susceptibility. Resistance to at least one antimicrobial was observed in 92 of 963 isolates (9.3%). No isolates exhibited resistance to carbapenem (meropenem). Resistance to third-generation cephalosporin (cefotaxime) and fluoroquinolone (ciprofloxacin) was observed in less than 1% of the isolates. Thus, low prevalence of bacterial antimicrobial resistance was observed in wild mammals between 2013 and 2017 in Japan.

KEY WORDS: antimicrobial resistance, *Escherichia coli*, wild animal

The emergence and prevalence of antimicrobial-resistant bacteria is closely related to the extensive use of antimicrobials and may result in the therapeutic failure of these drugs in medical and veterinary settings. Therefore, the importance of estimating the environmental prevalence of antimicrobial resistance is increasingly recognized. Since the establishment of the Global Action Plan on Antimicrobial Resistance, integrated surveillance (human, animals, and environments) is a great concern to control antimicrobial resistance. In Japan, surveillance and monitoring of antimicrobial-resistant bacteria have been annually conducted in humans since 2000 (Japan Nosocomial Infections Surveillance, JANIS) and in food-producing animals since 1999 (Japanese Veterinary Antimicrobial Resistance Monitoring System, JVARM). However, to date, antimicrobial resistance in the environment, including wild animals, has been poorly investigated.

Escherichia coli, which commonly prevails in the intestine of warm-blooded animals, is used as a bacterial indicator in humans and animals to monitor antimicrobial resistance possibly due to the selective pressure exerted by antimicrobial drugs [8]. *E. coli* is used in several countries as a bacterial indicator for national surveillance [5, 24]. *E. coli* from animal feces is considered an appropriate indicator to estimate the prevalence of antimicrobial resistance in different countries and for multiple applications.

Current data on antimicrobial resistance in wild mammals are insufficient, and One Health approaches would be required to adequately tackle this issue. Several studies on antimicrobial-resistant bacteria in wild animals have been conducted in Japan. In the late 1970s, resistance to tetracycline (TET), streptomycin, and sulfamides was frequently reported in *E. coli* from wild birds [12, 20, 26]. A low prevalence of antimicrobial-resistant bacteria was reported in wild animals, such as Japanese serow, in the 1980s [13], and in wild mice in 2006 [11]. After 2007, resistance to β-lactam antibiotics and fluoroquinolone was reported in *E. coli* isolates from crane [14]. However, a limited number of studies have addressed this issue in wild mammals. The objective of this study was to estimate the state-of-the-art antimicrobial resistance in wild mammals in Japan. To this end, the antimicrobial susceptibility of *E. coli* isolates obtained from wild mammals in various areas of Japan from 2013 to 2017 was investigated.

A total of 963 *E. coli* isolates were obtained from 475 animals, comprising 242 sika deer (C*ervus Nippon*: 525 isolates), 112 wild boars (*Sus scrofa*: 224 isolates), 113 small mammals (64 *Rattus*, *rattus,* 33 *Rattus norvegicus*, 10 *Mus musculus*, 5 *Apodemus speciosus*, 3 *Apodemus argqateus*, 2 *Urotrichus talpoides*: 199 isolates), 4 Japanese badger (*Meles anakuma*: 10 isolates), 2 Amami rabbits (*Pentalagus furnessi*: 2 isolates), and 2 Tokara cows (3 isolates), collected between 2013 and 2017 (Table 1). The sampling locations were classified as “mountain area”, “urban area”, “countryside”, and “animal facilities” (Table 1). The “urban area” group

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

Animals	Sampling place	Number of				%Resistance											
		Animals	Isolates	Resistance	(%)	AMP (32)[a)]	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																	
	Mountain area in Hokkaido, Shizuoka, Gifu, Yamaguchi, Kagoshima	191	327	15	(4.6)	0.6	0	0	0	0.3	0.9	3.1	0.9	0.3	1.2	0	0.6
	Miyajima	37	102	5	(4.9)	2.0	0	0	0	0	0	2.0	0	0	2.9	0	2.0
	Nara park	36	96	11	(11.5)	0.0	0	0	0	0	0	11.5	0	0	1.0	0	0
	Subtotal	242	525	31	(5.9)	0.8	0	0	0	0.2	0.6	4.4	0.6	0.2	1.5	0	0.8
Wild boars																	
	Mountain area in Tochigi, Ishikawa, Gifu, Yamaguchi, Kagoshima	112	224	18	(8.0)	3.6	0	0	0	0.4	1.3	4.0	0.9	0	1.3	1.8	0.9
Small mammals																	
	Animal facilities in Gifu and Hokkaido	58	106	30	(28.3)	23.6	2.8	1.9	0	2.8	5.7	17.9	11.3	0.0	3.8	1.9	18.9
	Urban area	33	43	6	(14.0)	0	0	0	0	0	0	14.0	0	0	0	0	6.4
	Mountain area in Gifu, Nagano and Kagoshima	26	50	0	(0)	0	0	0	0	0	0	0	0	0	0	0	0
	Subtotal	113	199	36	(18.1)	12.6	1.5	1.0	0	1.5	3.0	12.6	6.0	0	2.0	1.0	11.6
Japanese badger																	
	Countryside[b)] in Kagoshima	4	10	4	(40.0)	10.0	0	0	0	0	20.0	20.0	0	0	10.0	0	0
Tokara cows																	
	Mountain area in Kuchinoshima island, Kagoshima	2	3	2	(66.7)	0	0	0	0	0	0	33.3	0	0	33.3	0	0
Amami rabbits																	
	Mountain area in Amami-Oshima island, Kagoshima	2	2	1	(50.0)	0	0	0	0	0	0	0	0	0	50.0	0	0
Total		475	963	92	(9.6)	17.8	1.4	0.9	0	2.3	6.5	28.0	7.9	0.5	8.4	2.8	13.6

AMP, ampicillin; CFZ, cefazolin; CTX, cefotaxime; MEM, meropenem; GEN, gentamycin; KAN, kanamycin; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; CST, colistin; CHL, chloramphenicol; SXT, sulfamethoxazole/trimethoprim. a) Breakpoints. b) The area combines house, field, and forest animals.

contained the rats captured in buildings in the cities. The "animal facilities" group included poultry houses, a daily cow house, an experimental animal house, and poultry slaughterhouses. *E. coli* was isolated from fecal samples using deoxycholate-hydrogen sulfide-lactose (DHL) agar plates (Eiken Chemical Co., Ltd., Tokyo, Japan) or CHROMagar STEC (CHROMagar Microbiology, Paris, France) media, and identified using a commercial system (API 20E, bioMérieux Japan Ltd., Tokyo, Japan) or matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) with a MALDI Biotyper (Bruker Daltonics Inc., Bremen, Germany) according to the manufacturer's instructions. The bacteria were stored in stock media (20% glycerin buffer or 10% skimmed milk solution) at −80°C.

The minimum inhibitory concentrations (MICs) of antimicrobials were determined by broth microdilution methods using custom-made plates (Eiken Chemical, Co., Ltd., Tokyo, Japan) according to the manufacturer's instruction. The following antimicrobials were tested: ampicillin (AMP, range of antimicrobial dilution: 1–128 μg/m*l*), cefazolin (CFZ, 1–128 μg/m*l*), cefotaxime (CTX, 0.5–64 μg/m*l*), meropenem (MEM, 0.25–32 μg/m*l*), gentamicin (GEN, 0.5–64 μg/m*l*), kanamycin (KAN, 1–128 μg/m*l*), TET (0.5–64 μg/m*l*), nalidixic acid (NAL, 1–128 μg/m*l*), ciprofloxacin (CIP, 0.03–4 μg/m*l*), colistin (CST, 0.12–16 μg/m*l*), chloramphenicol (CHL, 1–128 μg/m*l*), and trimethoprim/sulfamethoxazole (SXT, 0.12–2.38/8–152 μg/m*l*). *Staphylococcus aureus* ATCC29213 and *E. coli* ATCC25922 were used for quality control according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [2]. The MICs of antimicrobials except for CST were analyzed on the basis of CLSI resistance breakpoints [3]. The resistance breakpoint of CST was based on the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [25].

The β-lactamase genes in CTX-resistant *E. coli* were analyzed by multiplex PCR [4], and the subtype of CTX-M-1 group β-lactamases was determined by sequencing of the PCR products using previously reported primer pairs [17] at the Life Science Research Center of Gifu University.

In *E. coli* isolates from sika deers (n=525), resistance to the following antimicrobials was detected: TET (4.4%), CST (1.5%), AMP (0.8%), SXT (0.8%), KAN (0.6%), NAL (0.6%), GEN (0.2%), and CIP (0.2%) (Table 1). On the other hand, resistance to TET (4.0%), AMP (3.6%), CHL (1.8%), KAN (1.3%), CST (1.3%), NAL (0.9%), SXT (0.9%), and GEN (0.4%) was found in *E. coli* isolates from wild boars (n=224) (Table 1). The prevalence of antimicrobial-resistant bacteria in wild animals depends on the dietary habits and/or living place of their hosts [12, 13, 20]. Several studies reported a high prevalence of antimicrobial-resistant *E. coli* in omnivorous wild animals and wild birds, compared to their herbivorous counterparts [16, 23, 28]. Dias *et al.* showed a higher prevalence of antimicrobial-resistant *E. coli* in wild boars (25%) compared to red deers (11%) in Portugal, and *E. coli* resistance to AMP and SXT was frequently found in wild boars [6]. In this study, the proportion of bacteria that were resistant to at least one antimicrobial was comparable between sika deers (5.9%, 31/525) and wild boars (8.0%, 18/224). In addition, the rate of bacterial resistance to at least one antimicrobial was 4.6% (15/327) in sika deers captured in mountain areas, while for the same species living in urban areas this rate was 4.9% (5/102) and 11.5% (11/96) in Miyajima and Nara park, respectively. Regarding Miyajima deers, feeding and garbage dumping were prohibited by the Miyajima deer management plan established by the Hatsukaichi city government in 2000. In the late 1980s, although AMR bacteria were rarely found in Japanese serows captured in the mountain area (2.5%, 7/283), these animals rapidly acquired AMR bacteria when reared in anthropized areas [13]. The low resistance rates observed in sika deers in Miyajima could result from the above-mentioned city government measures.

In *E. coli* isolates (n=199) from small mammals, resistance was found against all antimicrobials tested, except for MEM and CPFX: AMP (12.6%), TET (12.6%), SXT (11.6%), NAL (6.0%), KAN (3.0%), CST (2.0%), CFZ (1.5%), GEN (1.5%), CTX (1.0%), and CHL (1.0%) (Table 1). TET and SXT resistance was frequently found in mammals from urban areas, while no isolates from small mammals captured in mountain areas exhibited resistance to any of the tested antimicrobials. On the other hand, in the isolates from mammals of animal facilities, high resistance rates were observed for AMP (23.6%), SXT (18.9%), TET (17.9%), and NAL (11.3%) (Table 1). Along the Okinawa rail between farm and forest areas, the birds in the farm area were more frequent carriers of AMR *E. coli* (69%, 11/16) compared to those in the forest area (20%, 3/15) [10]. Usui *et al.* suggested that flies in animal farms may carry and spread antimicrobial-resistant bacteria of reared animals [27]. These results suggested that the prevalence of antimicrobial-resistant *E. coli* isolates in wild small mammals may be affected by factors of the surrounding environments, such as animals to live there. As number of facilities and species of animals are limited in this study, further study is required to clarify the situation of the resistant bacteria prevalence in small mammals.

As for the remaining animal species, resistance to AMP and CST (one isolate each) and KAN-TET (2 isolates) was detected among ten isolates from Japanese badgers. Among three isolates from Tokara cows, two exhibited TET and CST resistance, respectively, and one of 2 isolates from Amami rabbits exhibited CST resistance. In animals other than sika deers, wild boars, and small mammals, for which a limited number of isolates were tested, the antimicrobial prevalence was difficult to estimate. In European wild rabbits captured in Azorean Islands, Portugal, a low prevalence of resistance to TET, SXT, amikacin, and NAL was observed, but resistance to streptomycin and AMP was frequently found (42% and 17%, respectively) [16]. In Spain, 5 of 122 red deers and one of 95 small mammals carried bacteria resistant to at least one of the tested antimicrobials [1]. Thus, species and regional differences were observed in the prevalence of antimicrobial resistance between Spain and Portugal. A previous study in Okinawa Prefecture showed that, differently from small Asian mongooses captured on the main island of Okinawa, Japanese weasels captured on Zamami Island, a small remote island of Okinawa, frequently carried antimicrobial-resistant *E. coli* (e.g., resistance to AMP, TET, and NAL) [19]. The evaluation of a higher number of isolates is required to establish the most appropriate animal species for the surveillance of antimicrobial resistance in each country.

Multidrug-resistant (MDR: resistance to two or more antimicrobial classes) *E. coli* was found in five (2.2%), eight (1.5%), and two isolates (20%) from wild boars, sika deers, and Japanese badgers, respectively (Table 2). All MDR isolates from Japanese badgers showed KAN-TET resistance. Most of the MDR isolates also showed resistance to TET and other antimicrobials in wild boar (4 of five isolates) and sika deers (7 of 8 isolates). High prevalence of TET resistance was found in *E. coli* from food-producing animals in Japan, i.e., cattle (36.8%), pigs (70.2%), broiler chickens (68.6%), and layer chickens (40.8%) [15]. On the other hand, of the *E. coli* isolates from wild small mammals, 30 showed MDR in farm environments (28.3%), but only 3 (6.9%) in urban areas. The veterinary use of antimicrobials in food-producing animals is associated with pollution and prevalence of antimicrobial-resistant bacteria in the animals and in farm environments. Therefore, wild small mammals commonly found in farms may easily be exposed to MDR bacteria derived from reared animals.

Resistance to medically important antimicrobials, such as third-generation cephalosporins and fluoroquinolones, remains low in wild animals in Japan. All isolates, except for one obtained from rat, proved susceptible to CTX. In addition, only one isolate from deer exhibited resistance to CIP. Regarding the resistance to β-lactam antibiotics in wild animals in Japan, penicillin antibiotics were tested before 2000 [12, 13, 20, 26], whereas, from then on, both penicillin and cephalosporin antibiotics were tested [11, 22]. Resistance to penicillin antibiotics was rarely found, and resistance to cephalosporins was never detected, except for *E. coli* resistance to cephalothin in 6.5% of Japanese monkeys from the Shimokita Peninsula of Aomori Prefecture [22]. In the present study, one isolate from a rat captured in a poultry house exhibited resistance to CTX, a third-generation cephalosporin, and carried $bla_{\text{CTX-M-1}}$, an extended-spectrum beta-lactamase (ESBL) gene, which is frequently found in *E. coli* from food animals [9]. In addition, $bla_{\text{CTX-M-1}}$ was detected in *E. coli* isolates from healthy humans in Japan [18, 21]. Notably, CTX-M-1-producing *E. coli* was isolated from poultry in the same house in which the rat carrying CTX-M-1-producing *E. coli* was captured (unpublished data). In the present study, antimicrobial-containing agar was not used for the isolation of *E. coli*, as it could overestimate antimicrobial resistance. Antimicrobial-resistant bacteria can be isolated from fecal samples of animals, including wild animals, using antimicrobial-containing agar. Notably, several studies have reported the prevalence of ESBL producers among wild animals in Europe [7].

The present study showed that, in spite of the low rate of antimicrobial resistance, wild animals carried antimicrobial-resistant bacteria in their intestines. JVARM reported that *E. coli* isolates from food-producing animals, especially pigs or broilers, frequently exhibit resistance to TET, streptomycin, and AMP, in Japan [15]. Specifically, the prevalence is approximately 50% for TET resistance and 40% for streptomycin and AMP resistance. In animal husbandries, domestic animals are treated with antimicrobials for bacterial infections, which results in selective pressure favoring resistant bacteria. On the contrary, wild animals are exclusively treated when rescued and, therefore, rarely exposed to antimicrobials. The present results strongly suggested that wild mammals were not subjected to antimicrobial selective force in natural environments.

Table 2. Multi-drug resistance profile of *Escherichia coli* isolates from wild manmals

Animals	Multi-drug resistance profiles	Total	Mountain area	Miyajima	Nara park	Animal houses	Urban area
Sika deer							
	AMP-GEN-KAN-TET-NAL-CIP-SXT	1	1	0	0	-	-
	AMP-TET-SXT	3	1	2	0	-	-
	KAN-TET	2	2	0	0	-	-
	NAL-CST	1	1	0	0	-	-
	TET-CST	1	1	0	1	-	-
	Subtotal	8	6	2	1	-	-
Wild boars							
	AMP-GEN-TET-NAL-CST-CHL-SXT	1	-	-	-	-	-
	AMP-KAN-TET-CHL	1	-	-	-	-	-
	KAN-TET-CHL-SXT	1	-	-	-	-	-
	KAN-TET-CHL	1	-	-	-	-	-
	AMP-CHL	1	-	-	-	-	-
	Subtotal	5	-	-	-	-	-
Small manmals							
	AMP-GEN-KAN-TET-NAL-CST-CHL-SXT	2	-	-	-	2	0
	AMP-GEN-KAN-TET-CST-SXT	1	-	-	-	1	0
	AMP-CFZ-CTX-TET	1	-	-	-	1	0
	AMP-TET-NAL-SXT	7	-	-	-	7	0
	AMP-CFZ-CST	1	-	-	-	1	0
	AMP-CFZ-CTX	1	-	-	-	1	0
	AMP-KAN-SXT	1	-	-	-	1	0
	AMP-TET-SXT	3	-	-	-	3	0
	TET-NAL-SXT	1	-	-	-	1	0
	AMP-KAN	2	-	-	-	2	0
	AMP-SXT	5	-	-	-	5	0
	TET-NAL	2	-	-	-	2	0
	TET-SXT	3	-	-	-	0	3
	Subtotal	30	-	-	-	27	3

AMP, ampicillin; CFZ, cefazolin; CTX, cefotaxime; GEN, gentamycin; KAN, kanamycin; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; CST, colistin; CHL, chloramphenicol; SXT, sulfamethoxazole/trimethoprim; -, Not applicable.

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