

The Prevalence of Antimicrobial-Resistant *Escherichia coli* in Two Species of Invasive Alien Mammals in Japan

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ABSTRACT. The prevalence of antimicrobial resistance in 128 *Escherichia coli* isolates was investigated in two species of invasive alien mammals (IAMs): the small Asian mongoose (SAM) and Japanese weasel (JW). The SAM is found on the main island of Okinawa, Japan, where a large number of livestock is available, and the JW is present on a small island, where is isolated from the main island, and have a small number of livestock. We focused on the two IAMs, inhabiting under the different environments, and compared their prevalence of antimicrobial-resistant *E. coli*. In the comparison of the frequencies of antimicrobial-resistant *E. coli* isolates between the SAM and JW, JW showed significantly higher prevalence of resistance against three drugs, ampicillin, chlortetracycline and nalidixic acid, compared with SAM's test results ($P < 0.05$). The *bla*_{TEM} gene and the *aph*1 gene were detected in 35 subjects (91%) of ampicillin-resistant isolates and 6 subjects (100%) of kanamycin-resistant isolates, respectively. The *tet* (A) gene was detected in 62 subjects (46%) of CTC-resistant isolates, and the *tet* (B) gene was detected in 25 subjects (8%) of those in IAM. The present results suggest that some IAMs were the carrier of antimicrobial-resistant bacteria and their genes, and the frequencies of these resistances were different between two IAM species.

KEY WORDS: antimicrobial-resistance of invasive alien mammals, antimicrobial-resistant *Escherichia coli*, Japanese weasel, small Asian mongoose, small isolated island.

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Recent studies have shown that antimicrobial-resistant bacteria are present in wild animals that have not been exposed to antibiotics [2, 6, 7, 20], and wild animals may be as a reservoir of antimicrobial resistance.

Although there are many studies of antimicrobial resistance in a wide variety of animals, there is no study on invasive alien mammals (IAMs). If wild animals are actually reservoirs of antimicrobial resistance, the expansion of IAMs into new areas and their robust ability to survive and reproduce would allow them to act not only as a reservoir but also a carrier of antimicrobial-resistant microorganisms, which might affect other wild animals, livestock and humans.

In the present study, we investigated the frequencies of antimicrobial-resistant *E. coli* in two species of IAMs inhabiting the island of Okinawa prefecture, Japan. One species was the small Asian mongoose (SAM) (*Herpestes javanicus*) which was introduced to the main island of Okinawa in 1910 for the extermination of field mice and snakes, and the other was the Japanese weasel (JW) (*Mustela itatsi*), which became established on a small isolated island, Zamami located approximately 40 km west of the main island in 1958. The SAM does not exist on Zamami, and the JW is

not present on the main island of Okinawa [14, 19]. Currently, the SAM population is estimated approximately 30,000 on the main island (personal communication). Both species have been responsible for the loss of biodiversity in the natural environment in which they have become established [14, 19]. Therefore the projects intended the capture of both IAMs had been carried out in order to protect the natural environment. The population of human, cattle, pig and chicken in the main island (approximately 1200 km²) is approximately 1,230,000, 80,000, 230,000 and 2,000,000, respectively; whereas the population of human and cattle in Zamami Island (approximately 6.5 km²) is approximately 600 and 20, respectively. In view of the situation that two IAMs have inhabited under the different environment, we focused on them inhabiting the two separate locations, and compared between their prevalence of antimicrobial-resistant *E. coli*.

The *E. coli* isolates were randomly recovered from fecal samples of 26 SAMs and 18 JWs that were captured during the project intended to protect the natural environment. We attempted to pick up 4 to 8 colonies showing typical *E. coli* morphology, after seeding and incubating the fecal samples on MacConkey agar plates. Isolates were identified as *E. coli* by testing with indole, methyl red, Voges-Proskauer and citrate, or with a commercial kit (BD BBL ENTEROTUBE® II, Fukushima, Japan), after Gram staining. If isolates recovered from the same individual had the same

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phenotype for antimicrobial resistance, they were regarded as a single isolate in order to avoid duplication. As a result, a total of 128 isolates, comprising 67 from the SAMs and 61 from the JWs, were obtained.

Antimicrobial susceptibilities were determined by the agar dilution method, as recommended by the Clinical and Laboratory Standards Institute (CLSI) [4], for seven antimicrobials: ampicillin (AMP), chloramphenicol (CHL), chlortetracycline (CTC), kanamycin (KAN), streptomycin (STR), nalidixic acid (NAL) and sulfadimethoxine (SDMX). Breakpoints suggested by the CLSI were used for determination of resistance except for STR which has no recommended breakpoints in the CLSI guidelines. Therefore, the breakpoint of STR was assumed to be the same as that recommended by the CLSI for KAN, which has biochemical similarity to STR, as described by Orden *et al.* [15].

The presence of antimicrobial resistance genes in AMP-, KAN-, STR- and CTC- resistant isolates was studied by polymerase chain reaction (PCR) with representative primers. The genetic marker was *bla*_{TEM} for AMP-resistant isolates, *tet* (A-E) and *tet* (M) for CTC-resistant ones, *aphA1* for KAN-resistant ones and *aadA1* for STR-resistant ones. The sequences of the primers and the conditions used for these reactions were as reported previously [6, 13]. The results were statistically compared between the two IAMs by chi-square test. P-value of less than 0.05 was considered significant.

As shown in Tables 1 and 2, 19 of 67 isolates from SAMs and 36 of 61 isolates from JWs were resistant to one or more antimicrobials tested. In particular, resistance to AMP, CTC, NAL, and SDMX were frequently found in JW. In Japan, *E. coli* isolates resistant to AMP, tetracycline, STR, NAL, and sulphonamide have been frequently isolated from cattle and pigs [1]. Our results suggest that resistance prevalence in JWs is somewhat similar to those in livestock in Japan except for STR [11]. Costa *et al.* [6] also reported a high prevalence of *E. coli* resistant to these antimicrobials in wild animals inhabiting Portugal. Compared between JWs and SAMs, the prevalence of AMP, CTC and NAL was significantly higher in JWs than in SAMs ($P < 0.05$). In addition, the prevalence of resistance pattern was more diverse in JWs. Osterblad *et al.* [16] have suggested that the geographic difference in resistance prevalence among wild animals may be due to differences in overall antibiotic load, and the number of feeding livestock in the countries concerned. However, the main industry on Zamami Island is tourism, and antimicrobial resistant isolates were hardly detected in cattle there (unpublished data). Sato *et al.* [17] further reported that the prevalence of antimicrobial resistant bacteria and R plasmids were associated with food habit of each animal species. Several researchers [10, 20] reported the different prevalence of resistance among wild birds. Therefore, difference in resistance prevalence between JWs and SAMs may be due to species-specific biology rather than antimicrobial selective pressure. Recently, it was reported that in a remote human community

Table 1. Frequencies of antimicrobial resistance in *E. coli* recovered from small Asian mongoose (SAM) and Japanese weasel (JW)

Antimicrobial ^{a)} (breakpoint)	Numbers of isolates (%)	
	SAM (n=67)	JW (n=61)
AMP (32 µg/ml)	1 (1.5)	19 ^{b)} (31.1)
CHL (32 µg/ml)	0 (0.0)	0 (0.0)
CTC (16 µg/ml)	8 (11.9)	24 ^{b)} (39.3)
KAN (64 µg/ml)	1 (1.5)	4 (4.9)
STR (32 µg/ml)	4 (6.0)	5 (8.2)
NAL (32 µg/ml)	0 (0.0)	21 ^{b)} (34.4)
SDMX (512 µg/ml)	17 (25.4)	17 (27.9)

a) AMP: ampicillin, CHL: chloramphenicol, CTC: chlortetracycline, KAN: kanamycin, STR: streptomycin, NAL: nalidixic acid, SDMX: sulfadimethoxine. b) Significantly higher than that of SAMs ($P < 0.05$).

Table 2. Phenotypes of antimicrobial-resistant *E. coli* isolates recovered from small Asian mongoose (SAM) and Japanese weasel (JW)

Phenotype of resistance ^{a)}	SAM	JW
	(67 isolates)	(61 isolates)
AMP	–	2 (3.3)
CTC	2 (3.0)	4 (6.6)
NAL	–	4 (6.6)
SDMX	10 (14.9)	1 (1.6)
AMP-CTC	–	1 (1.6)
AMP-NAL	–	3 (4.9)
CTC-NAL	–	4 (6.6)
CTC-SDMX	3 (4.5)	–
STR-SDMX	1 (1.5)	–
NAL-SDMX	–	2 (3.3)
AMP-CTC-NAL	–	1 (1.6)
AMP-CTC-SDMX	–	2 (3.3)
CTC-NAL-SDMX	–	1 (1.6)
CTC-KAN-SDMX	1 (1.5)	–
CTC-STR-SDMX	1 (1.5)	–
AMP-CTC-KAN-SDMX	–	1 (1.6)
AMP-CTC-STR-SDMX	1 (1.5)	2 (3.3)
AMP-CTC-NAL-SDMX	–	5 (8.2)
CTC-STR-NAL-SDMX	–	1 (1.6)
AMP-CTC-KAN-STR-SDMX	–	2 (3.3)
Susceptible	48 (71.6)	25 (41.0)

Numbers in parenthesis indicate the percentage. a) AMP: ampicillin, CTC: chlortetracycline, SDMX: sulfadimethoxine, STR: streptomycin, KAN: kanamycin, NAL: nalidixic acid.

antimicrobial resistance was highly prevalent unrelated to consumption of antimicrobials [2, 8]. Kinjo *et al.* [12] indicated that wild serow seemed to readily change to harbor resistant *E. coli* almost as soon as they were reared in human areas. Therefore, we are concerned about the spread of antimicrobial resistant bacteria on Zamami Island. Further study would be needed to clarify the origins of antimicrobial-resistant *E. coli* in IAMs.

TEM β -lactamase, the gene most frequently (84%) associated with AMP resistance in this study (Table 3), as reported in isolates from foods, animals and humans [3]. Recent reports have indicated that *E. coli* isolates containing extended spectrum β -lactamases (ESBLs) are recovered not

Table 3. Resistance genes among antimicrobial-resistant *E. coli* isolates of small Asian mongoose (SAM) and Japanese weasel (JW) origins

Antimicrobial of resistance ^{a)}	SAM				JW	
	Number of isolates	Genes detected		Number of isolates	Genes detected	
		Gene	Number of isolates		Gene	Number of isolates
AMP	1	<i>bla</i> _{TEM}	1	19	<i>bla</i> _{TEM}	16
CTC	8	<i>tet</i> (A)	5	24	<i>tet</i> (A)	11
		<i>tet</i> (B)	2		<i>tet</i> (B)	2
		<i>tet</i> (A) + <i>tet</i> (B)	0		<i>tet</i> (A) + <i>tet</i> (B)	1
		<i>tet</i> (M)	0		<i>tet</i> (A) + <i>tet</i> (M)	1
KAN	1	<i>aphA1</i>	1	4 ^{b)}	<i>aphA1</i>	4

a) AMP: Ampicillin, CTC: Chlortetracycline, KAN: Kanamycin. b) Two of these four isolates also showed streptomycin resistance.

only from humans but also from wild animals and domestic healthy cats and dogs [5, 6], and that the ESBL exists also in TEM β -lactamases [5]. However, since all our isolates resistant to AMP were susceptible to cefotaxime and ceftizoxime that are the third cephalosporin derivative (data not shown), it was thought that ESBLs did not participate in TEM β -lactamases detected in the JW.

The *tet* (A) gene was detected in 62 and 46% of CTC-resistant isolates from SAMs and JWs, respectively, whereas the *tet* (B) gene was detected in 25 and 8% of those from SAMs and JWs, respectively (Table 3). The *tet* (A) and *tet* (B) genes was frequently detected in tetracycline-resistant *E. coli* recovered from humans [21] livestock [9] and wild animals [6] worldwide. On the other hand, the *tet* (M) gene along with the *tet* (A) gene was detected in one of the CTC-resistant isolates from the JW (Table 3). The mechanism responsible for this resistance is known to depend on the expression of ribosome-protective proteins, unlike the *tet* (A-E) genes, for which mechanism is active efflux [18]. The detection of the *aphA1* gene in all of our KAN-resistant *E. coli* isolates indicates that the main mechanism of KAN resistance involves with the expression of phosphotransferases [18]. The *aadA1* gene was not detected in any of our STR-resistant isolates (data not shown). The loci of these resistance genes lie not only on chromosomal DNA but also in plasmids and transposons [18], suggesting that these genes are transferred to other *Enterobacteriaceae* easily via these vectors. On the other hand, resistance to NAL, a quinolone antibacterial agent, has been explained by mutation in the *gyr* and *par* genes on chromosomal DNA [18]. Therefore, it is of interest that a high proportion of JWs harbored NAL-resistant *E. coli*, whereas SAMs did not. It will be necessary to investigate whether natural substances exert as antimicrobials, including quinolone-like chemicals in the habitat of JWs on Zamami Island.

In conclusion, IAMs were found to harbor *E. coli* with antimicrobial resistance genes, which were detected at a higher rate on the small remote island of Zamami than that on the main island of Okinawa which is heavily urbanized. These results suggested that the significant differentiation in the frequencies of antimicrobial resistant bacteria was

observed even in the wild animals inhabiting under the different environment and some IAMs were the carrier of antimicrobial resistant bacteria and resistant genes.

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