Antimicrobial Susceptibility of *Escherichia coli* Isolates from Wild Mice in a Forest of a Natural Park in Hokkaido, Japan

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ABSTRACT. To reveal the antimicrobial susceptibilities of *Escherichia coli* isolates from wild mice, 81 *E. coli* isolates were obtained from 109 voles (*Clethrionomys* spp.), 52 large Japanese field mice (*Apodemus speciosus*) and 19 small Japanese field mice (*A. argenteus*) captured in a forest of a natural park in Hokkaido, Japan. Seventy-eight of the 81 *E. coli* isolates were susceptible to all 10 antimicrobial agents tested. One *E. coli* isolate was resistant to ampicillin, dihydrostreptomycin, kanamycin, chloramphenicol and oxytetracycline. Two isolates were resistant to oxytetracycline. A low prevalence of antimicrobial resistance was maintained among wild mice that inhabited the forest.
KEY WORDS: antimicrobial resistance, *Escherichia coli*, Japan, wild mice.

Antimicrobial-resistant bacteria are recognized as a public health problem. The usage of antimicrobial agents is considered the most important factor in the selection and dissemination of antimicrobial-resistant bacteria. Although antimicrobial agents are not generally administered to wild animals, the prevalence of antimicrobial-resistant *Escherichia coli* isolates was determined in some wild animals [4, 5, 7, 10, 12, 13]. The source of antimicrobial-resistant bacteria among wild animals is still debatable.

To reveal the antimicrobial susceptibilities of *E. coli* isolates from wild mice, we captured wild mice and isolated *E. coli* on MacConkey agar plates with and without 4 μg/ml of the antimicrobial agents enrofloxacin or cefpodoxime.

Wild mice were trapped using Sherman live traps around Tomanbetsu in a forest of Nopporo Shinrin Kouen Prefectural Natural Park (2,051 hectares) seven times between June and October 2006. This Natural Park extends across three cities in Hokkaido (Northern Japan, Sapporo, Ebetsu and Kitahiroshima) (Fig. 1). Permission to capture wild mice was granted by the prefectural government of Hokkaido. The wild mice were anesthetized, and their species were identified as follows: Voles (*Clethrionomys* spp.) were identified by their dark brown back and shorter tail than body length. The large Japanese field mouse (*Apodemus speciosus*) was identified by its brown or orange-brown back, big eyes and ears. The small Japanese field mouse (*A. argenteus*) was identified by its chestnut brown back, small body and short hindfoot [1]. Fifty-three female and 56 male voles, 22 female and 30 male large Japanese field mice and six female and 13 male small Japanese field mice were captured (Table 1). The average weight of the 109 voles was

25.7 g (range 14–41 g); this is lighter than the reported weight of the Yezo red-backed vole (*C. rufocanus bedfordiae*; 27–50 g) [1]. The average head and body (HB) length of voles in this investigation was 90.4 mm (range 50–120 mm), and the lengths were shorter than the reported HB lengths of the Yezo red-backed vole (107–126 mm) [1]. We could not discriminate between the Yezo red-backed vole and the Mikado vole (*C. rutilus mikado*; weight 11.9–27.7 g; HB length 74.2–102.8 mm). Therefore, all the voles were included in one group (*Clethrionomys* spp.). The captured wild mice included the following three species: voles that inhabit grassland or open forests, large Japanese field mice that inhabit forests or areas abundant in dense grasses along riverbanks, and small Japanese field mice that prefer to inhabit climax forests [1]. No house mice, black rats or brown rats that reside in areas associated with humans were captured in this investigation.

The contents of the rectum were inoculated onto MacConkey agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and MacConkey agar plates containing 4 μg/ml enrofloxacin or cefpodoxime. These inoculated isolation media were incubated at 37°C overnight. One or 2 colonies of suspected *E. coli* per sample were picked, subcultured on nutrient agar (Nissui Pharmaceutical Co., Ltd.) and identified by Quick ID GN "Nissui" (Nissui Pharmaceutical Co., Ltd.). One isolate identified as *E. coli* per sample was selected for further tests. All isolates were suspended in 10% skim milk and kept at –80°C.

Using MacConkey agar plates, *E. coli* was isolated from 40 of 109 voles (36.7%), 34 of 52 large Japanese field mice (65.4%) and seven of 19 small Japanese field mice (36.8%, Table 1). No *E. coli* isolate was obtained on MacConkey agar plates containing enrofloxacin or cefpodoxime. The isolation frequency was much lower than that in previous reports that assessed livestock [8]. Kozak *et al.* also

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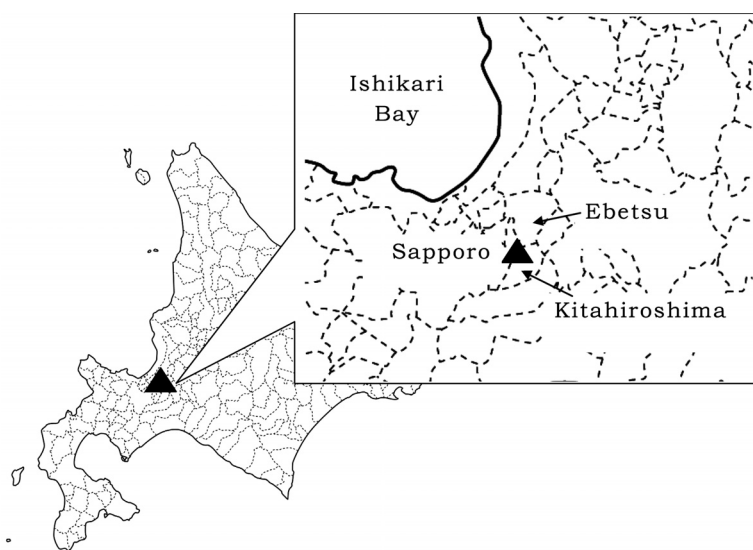   e-mail: tamuray@rakuno.ac.jp

Fig. 1. The geographic location of Nopporo Shinrin Kouen Prefectural Natural Park in Hokkaido, Japan. The black triangle shows the location of the natural park where we captured wild mice for this investigation. This park extends across three cities, Sapporo, Ebetsu and Kitahiroshima.

Table 1. Isolation and antimicrobial resistance of *Escherichia coli* from wild mice

Origins	Isolation rate (%)	Antibiotic resistant rate (%)	Antibiotic resistance pattern* (No. of isolates)
Vole (*Clethrionomys* spp.)	40/109 (36.7%)	2/40 (5%)	AMPC-DSM-KM-CP-OTC** (1) OTC (1)
Large Japanese field mouse	34/52 (65.4%)	0/34 (0%)	
Small Japanese field mouse	7/19 (36.8%)	1/ 7 (14.3%)	OTC (1)
Total	81/180 (45.0%)	3/81 (3.7%)	

* Antibiotic resistance patterns of only antimicrobial-resistant isolates.
** AMPC, ampicillin; DSM, dihydrostreptomycin; KM , kanamycin; CP, chloramphenicol; OTC, oxytetracycline.

reported a low detection frequency of *E. coli* isolates (48.8%) from wild mice, voles and shrews even though enrichment for *E. coli* was performed [10]. The distribution of intestinal bacterial flora including *E. coli* might differ between wild mice and livestock.

The minimal inhibitory concentrations (MICs) of the following 10 antimicrobial agents was determined using the agar dilution method with Mueller-Hinton agar (Becton, Dickinson and Co., Sparks, MD, U.S.A.) according to the Clinical Laboratory Standards Institute (CLSI) guidelines: ampicillin, cefazolin, dihydrostreptomycin, kanamycin, gentamicin, apramycin, chloramphenicol, oxytetracycline, nalidixic acid and enrofloxacin. The following breakpoints were adapted according the CLSI guidelines [3] or a previous study [9]: 64 μg/m*l* for kanamycin and apramycin; 32 μg/m*l* for ampicillin, cefazolin, dihydrostreptomycin, chloramphenicol and nalidixic acid; 16 μg/m*l* for gentamicin and oxytetracycline; and 2 μg/m*l* for enrofloxacin. The antimicrobial resistance genes were detected by PCR or multiplex PCR using Go Taq Green Master Mix (Promega, Madison, WI, U.S.A.). DNA from the isolates was

extracted with an InstaGene Matrix (Bio-Rad Laboratories Inc., Tokyo, Japan). The genes encoding TEM, SHV and CMY-2 β-lactamase were tested in ampicillin- and/or cefazolin-resistant isolates by multiplex PCR [10]. The *strA, strB* and *aadA* genes were tested in dihydrostreptomycin-resistant isolates [11]. For detection of the *strA* and *strB* genes, the following primer pairs were used: strA-F, TGA CTG GTT GCC TGT CAG AG; strA-R, AAT TGC CGT TAT CAC CAA GC; strB-F, ACG TTT CGC AAC CTG TTC TC; and strB-R; AGG TTT CAA TCC CTT GAC GA. The *aphA1* and *aphA2* genes were tested in kanamycin-resistant isolates by multiplex-PCR [10]. The *tetA* and *tetB* genes were tested in oxytetracycline-resistant isolates [14]. The *cat1* and *cmlA* genes were tested in chloramphenicol-resistant isolates [2].

Seventy-eight of the 81 *E. coli* isolates were susceptible to all antimicrobial agents tested. The MIC ranges for the 78 antimicrobial-susceptible isolates were as follows: ampicillin, 2–4 μg/m*l*; cefazolin, 1–4 μg/m*l*; dihydrostreptomycin, 2–4 μg/m*l*; kanamycin, 2–8 μg/m*l*; gentamicin, 0.5–2 μg/m*l*; apramycin, 4–16 μg/m*l*; chloramphenicol, 4–16 μg/m*l*;

oxytetracycline, 0.5–2 μg/m*l*; nalidixic acid, 1–4 μg/m*l*; and enrofloxacin, ≤ 0.016–0.06 μg/m*l*.

One *E. coli* isolate from a vole (strain 158–2) was resistant to ampicillin (MIC, > 512 μg/m*l*), dihydrostreptomycin (MIC, 512 μg/m*l*), kanamycin (MIC, >512 μg/m*l*), chloramphenicol (MIC, 256 μg/m*l*) and oxytetracycline (MIC, 128 μg/m*l*). Two *E. coli* isolates (strains 54–2 and 81–1), one from a small Japanese field mouse and one from a vole, were resistant to oxytetracycline (MIC, 128 μg/m*l* and 256 μg/m*l*, respectively; Table 1). Strain 158–2 harbored *bla*TEM, *strA*, *strB*, *aphA1*, *cat1* and *tetB*. Strains 54–2 and 81–1 harbored *tetA*.

The percentage of wild mice with antimicrobial-resistant *E. coli* isolates in this investigation (3.7%, 3/81) was as low as that among small wild mammals living in natural areas (10%, 2/20)[10]. It has been reported that the proximity of wild animals to human settlements and the livestock industry increases antimicrobial-resistance among *E. coli* isolates from wild animals [7, 10]. Radhouani *et al.* reported that the high prevalence of antimicrobial resistance among *E. coli* isolated from seagulls in Portugal might result from the birds eating the remains of human food [12]. As few people visit the Tomanbetsu area where the traps were set in this investigation, it is possible that the prevalence of antimicrobial resistance was low in *E. coli* from wild mice living in the forest of the natural park.

Two *E. coli* isolates were resistant to oxytetracycline alone, and another isolate was resistant to five drugs. Oxytetracycline resistance is frequently found amongst *E. coli* isolates from livestock in Japan [9]. The chloramphenicol resistance gene, *cat1,* detected in strain 158–2 isolated from a vole was the same as that in chloramphenicol-resistant *E. coli* isolates from cattle, but not pigs, in Japan [6]. There are almost 100 dairy farms in Sapporo, Ebetsu and Kitahiroshima, where the forest of Nopporo Shinrin Kouen Prefectural Natural Park is located. Indeed, at least one dairy farm was located within 1 km of the area where wild mice were captured. In order to reveal the origin of these antimicrobial-resistant *E. coli* isolates from wild mice, comparison with antimicrobial-resistant isolates from domestic animals and humans by molecular analysis is needed.

In conclusion, antimicrobial resistance was rarely found in *E. coli* isolates from wild mice captured in the forest of a natural park in Hokkaido, and at present, it appears that human activities rarely brought the antimicrobial-resistant *E. coli* isolates to these wild mice.

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