〈Original Article〉

Multidrug-resistant Enterobacteriaceae strains in the Tone River, Japan

Akihisa Hata, Kaori Sekine and Noboru Fujitani

Summary **Objectives:** The increase in the incidence of multidrug-resistant bacterial infection is a global health problem. Environmental resources such as water and soil may harbor drug-resistant bacteria. In this study, we sought to determine the drug resistance of Enterobacteriaceae strains detected in the Tone River, Japan.

Study design: Systematic microbiological testing.

Methods: Water was sampled at 5 points along the Tone River at 4 time points in 2011 and 2012. The drug resistance of the Enterobacteriaceae strains was tested by using the agar plate dilution technique.

Results: A total of 312 Enterobacteriaceae strains were detected in the river samples. *Enterobacter* spp. was the most commonly found Enterobacteriaceae strain, accounting for approximately 40% (n = 122) of detected strains. The prevalence rates of drug resistance to the following drugs among the 312 detected strains were as follows: cephalothin, 53.8%; ampicillin, 52.8%; cefotaxime, 11.9%; chloramphenicol, 10.6%; imipenem, 5.8%; ceftazidime, 5.1%; tetracycline, 4.2%; gentamicin, 1.9%; amikacin, 1.6%; and ciprofloxacin, 1.0%. Drug-resistant strains were more commonly found at sampling points located near densely populated areas.

Conclusions: Our findings suggest that river water is an important source of a variety of drug-resistant bacteria, particularly in more populated areas.

Key words: Multidrug resistant bacteria, Enterobacteriaceae, River water

1. Introduction

The World Health Organization considers the increase in the incidence of multidrug-resistant bacterial infection a global public health problem[1]. Gram-negative bacteria produce endotoxins, which cause sepsis. Enterobacteriaceae strains, which are gram-negative bacilli, are universally found in the bowels of animals, in soil, and in water. Therefore, resistance to multiple drugs is considered a serious problem. In 2013, the United States Centers for Disease Control issued a warning regarding an increase in the incidence of carbapenem-resistant Enterobacteriaceae infection[2].

Drug-resistant Enterobacteriaceae strains have been detected not only in infected humans and animals

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during antimicrobial treatment but also in healthy humans and animals[3, 4]. Environmental resources such as soil and water may act as repositories for these multidrug-resistant bacteria[5-10]. Furthermore, Perry et al. indicated that resistance genes are able to move from the host into pathogens[11].

Previous reports confirmed the presence of multidrug-resistant Enterobacteriaceae strains in river water flowing through an urban area[9, 12]. These resistant bacteria also spread to the environment. However, information on the spread of resistant bacteria from urban areas to natural environments in Japan is insufficient.

Accordingly, we undertook an investigation of drug-resistant Enterobacteriaceae in the Tone River, which has a basin in the urban area near metropolitan Tokyo. In a previous study, we analyzed bacteria from water in the environment using DNA microarray analysis and whole genome amplification[13]. Bacteria detected in the Tone River are not indigenous to the environment; they have been isolated from domestic animals and humans. Thus, the bacterial flora of in Tone River may have been affected by human activities. The 322-km long Tone River flows through the Kantõ region of Japan, and approximately 12 million people live in the Tone River basin. The river water is used for agriculture and drinking, and the drainage water returns to the river. Therefore, contamination of the river by drug-resistant bacteria may pollute the basin environment, resulting in a public health problem.

In this study, we investigated the extent of drug resistance among Enterobacteriaceae strains found in the Tone River, to provide baseline information for future planning to reduce drug-resistant strains in the environment and manage potential infections.

2. Material and Methods

1. Water sampling

We sampled water using a high-route water sampler (Miyamoto Riken Ind., Japan) from a bridge at 5 different points along the Tone River (Figure 1): point A, Minakami-town, Gunma Prefecture; point B, Honjo-city, Saitama Prefecture; point C, Bando-city, Ibaraki Prefecture; point D, Kouzaki-town, Chiba Prefecture; and point E, Choshi-city, Chiba Prefecture. Water was sampled on 4 separate occasions: May 2011, August 2011, July 2012, and August 2012. Water sampling was conducted in a public place at all

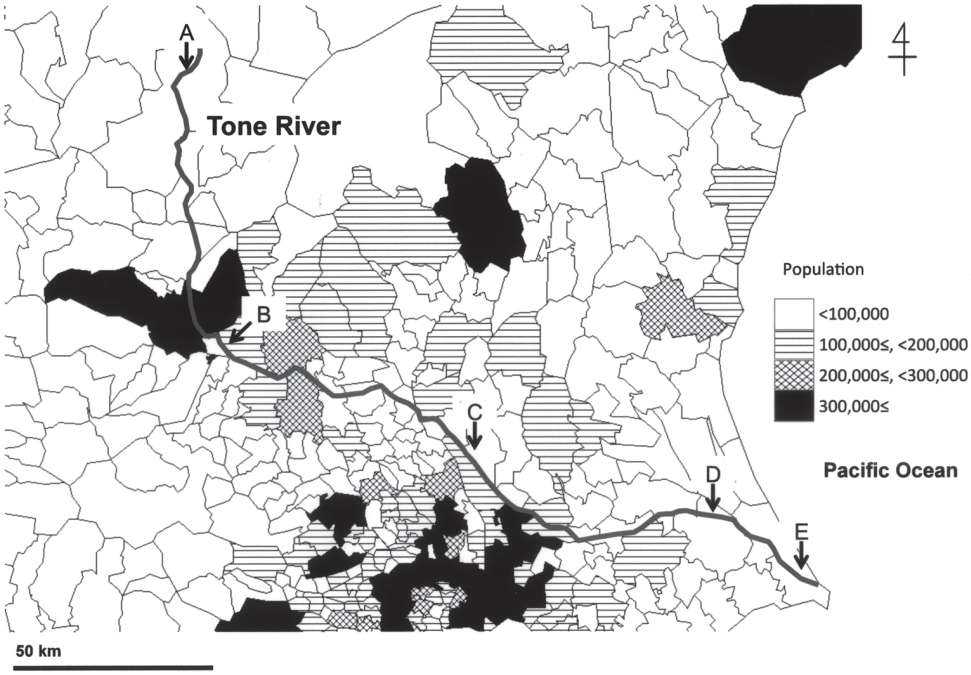

Fig. 1 Five water sampling points (A, B, C, D, and E) in the Tone River, Japan.

points and on all occasions, and specific permission was not required. Water samples were stored at 4℃ in sterilized bottles and were transported to our laboratory within 12 hours. The populations of the local cities around the basin were determined using data from the Japan Statistical Yearbook 2012[14].

2. Detection of Enterobacteriaceae

Deoxycholate agar (Kanto Chemical, Japan) was used to isolate Enterobacteriaceae strains. River water (100 μL) was poured on an agar plate, and the plate was incubated under aerobic conditions at 37℃ for 24 hours. Observed colonies that were able to decompose lactose were chosen for analysis. These colonies were further purified on a standard agar plate (Nissui Pharmaceutical, Japan). Gram staining and oxidase tests (Nissui Pharmaceutical, Japan) were performed on the isolated strains, and the 16S rDNA sequences of the identified gram-negative and oxidase-negative strains were analyzed. Partial 16S rDNA sequences (about 0.5 kbp) were determined by Takara Bio, Inc. (Tokyo, Japan). The 16S rDNA sequences were analyzed on the basis of their homogeneity using the Basic Local Alignment Search Tool (BLAST) program. The genus of isolated strains was identified based on a ≥ 97.5% homology in the 16S rDNA sequence with the Enterobacteriaceae strain. This study did not involve endangered or protected species.

3. Drug sensitivity test

We used the agar plate dilution method to test the drug sensitivity of Enterobacteriaceae strains, according to the performance standards issued by the Clinical and Laboratory Standards Institute[15]. American Type Culture Collections (ATCC) 25922 (*Escherichia coli*) and 35218 (*E. coli*) were used as control strains. Antimicrobial sensitivity tests were performed using the following antibiotics (the resistance-determining concentration is given in parentheses): ampicillin (ABPC, ≥ 32 μg/mL), cephalothin (CET, ≥ 32 μg/mL), cefotaxime (CTX, ≥ 4 μg/mL), ceftazidime (CAZ, ≥ 16 μg/mL), imipenem (IPM, ≥ 4 μg/mL), tetracycline (TC, ≥ 16 μg/mL), gentamicin (GM, ≥ 16 μg/mL), amikacin (AMK, ≥ 64 μg/mL), chloramphenicol (CP, ≥ 32 μg/mL), and ciprofloxacin (CPFX, ≥ 4 μg/mL).

3. Results

In total, 312 Enterobacteriaceae strains were detected, with concentrations ranging from 0 to 153 colony-forming units (CFU)/mL at different sampling points and on different sampling dates. Data on water temperature, pH, and number of detected Enterobacteriaceae strains are presented in Table 1. The number of strains and rate of detection of the following genera among the 312 identified strains were as follows: *Enterobacter* spp., 122 (39.1%); *Klebsiella* spp., 43 (13.8%); *Pantoea* spp., 39 (12.5%); *Citrobacter* spp., 23 (7.4%); *Serratia* spp., 19 (6.1%); *Escherichia* spp., 17 (5.4%); *Raoultella* spp., 8 (2.6%); *Buttiauxella* spp., 4 (1.6%); and other genera, 36 (11.5%).

Drug resistance was not detected in 108 strains (34.6%). The number of strains and the rate of detection of resistance to the following drugs among

Table 1 Number of Enterobacteriaceae strains, water temperature, and water pH at each sampling point in the Tone River, Japan

Smpling point	Water pH (Range)	Water temperature (℃) (Range)	Number of detected Enterobacteriaceae strains (CFU/mL)			
			May 2011	Aug 2011	Jul 2012	Aug 2012
A	6.4-9.5	7.8-15.0	5	45	50	15
B	6.9-7.6	12.8-23.0	15	153	50	75
C	7.3-7.6	18.8-28.0	18	148	23	118
D	7.5-7.8	17.5-29.2	3	17	17	8
E	7.5-8.0	18.0-28.4	3	28	7	0

the 312 strains were as follows: CET, 168 (53.8%); ABPC, 165 (52.8%); CTX, 37 (11.9%); CP, 33 (10.6%); IPM, 18 (5.8%); CAZ, 16 (5.1%); TC, 13 (4.2%); GM, 6 (1.9%); AMK, 5 (1.6%); and CPFX, 3 (1.0%). The resistance rates at each sampling time are shown in Figure 2, and the resistance rates at each sampling point are shown in Table 2. Seventy-six strains were resistant to only 1 drug (37.3% of the 204 drug-resistant strains), and multidrug resistance was noted in the remainder of the strains: resistance to 2 drugs, 71 strains (34.8% of the 204 drug-resistant strains); 3 drugs, 25 (12.3%); 4 drugs, 19 (9.3%); 5 drugs, 4 (2.0%); 6 drugs, 3 (1.5%); 7 drugs, 4 (2.0%); and 8 drugs, 2 (1.0%). Details of the strains resistant to ≥ 3 drugs are presented in Table 3. The most common combinations of drugs showing resistance were ABPC-CET (2-agent-resistant strains); ABPC-CET-CP (3-agent-resistant strains); ABPC-CET-CTX-CP (4-agent-resistant strains); ABPC-CET-CTX-CAZ-IPM, ABPC-CET-CTX-IPM-CP, ABPC-CET-CTX-CAZ-CP, and ABPC-CET-CTX-IPM-CPFX (5-agent-resistant strains); ABPC-CET-CTX-CAZ-IPM-AMK

Table 2 Drug resistance of the Enterobacteriaceae strains detected at each sampling point in the Tone River, Japan

Sampling point	n	Number of antibiotic-resistant bacterial strains (%)									
		ABPC	CET	CTX	CAZ	IPM	GM	AMK	CP	TC	CPFX
A	23	15	19	0	0	0	0	0	0	0	0
	%	(65.2)	(82.6)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)
B	117	66	66	14	10	12	2	4	17	8	0
	%	(56.4)	(56.4)	(12.0)	(8.5)	(10.3)	(1.7)	(3.4)	(14.5)	(6.8)	(0.0)
C	122	57	56	16	3	4	3	1	12	4	2
	%	(46.7)	(45.9)	(13.1)	(2.5)	(3.3)	(2.4)	(0.8)	(9.8)	(3.3)	(1.6)
D	27	18	17	7	3	2	1	0	3	1	1
	%	(66.7)	(63.0)	(25.9)	(11.1)	(7.4)	(3.7)	(0.0)	(11.1)	(3.7)	(3.7)
E	23	9	10	0	0	0	0	0	1	0	0
	%	(39.1)	(43.5)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(4.3)	(0.0)	(0.0)

ABPC, ampicillin; CET, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; TC, tetracycline; GM, gentamicin; AMK, amikacin; CP, chloramphenicol; CPFX, ciprofloxacin

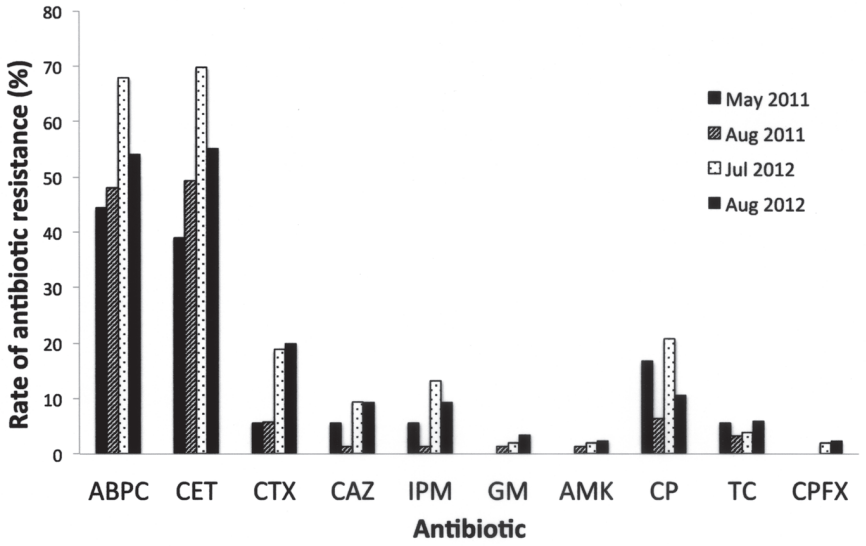

Fig. 2 The rate of drug resistance at each sampling time.
ABPC, ampicillin; CET, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; TC, tetracycline; GM, gentamicin; AMK, amikacin; CP, chloramphenicol; CPFX, ciprofloxacin

Table 3 The multidrug resistant (≥ 3 drugs) strains detected in the Tone River

Sampling		Enterobacteriaceae strains	Antibiotic resistance									
Point	Date		ABPC	CET	CTX	CAZ	IPM	GM	AMK	CP	TC	CPFX
B	Aug 2012	*Enterobacter* spp.	■	■	■	■	■		■	■	■	
D	Jul 2012	*Enterobacter* spp.	■	■	■	■		■		■	■	■
B	May 2011	*Serratia* spp.	■	■	■	■	■			■	■	
B	Jul 2012	*Enterobacter* spp.	■	■	■	■	■		■	■		
B	Jul 2012	*Enterobacter* spp.	■	■	■	■	■			■	■	
C	Aug 2012	*Shigella* spp.	■	■	■	■				■	■	■
B	Aug 2011	*Enterobacter* spp.	■	■	■	■	■		■			
B	Aug 2011	*Escherichia* spp.	■	■	■	■	■		■			
C	Aug 2012	*Enterobacter* spp.	■	■	■		■			■	■	
B	Jul 2012	*Enterobacter* spp.	■	■	■		■			■		
B	Aug 2012	*Escherichia* spp.	■	■	■	■				■		
C	Aug 2012	*Shigella* spp.	■	■	■		■					■
D	Aug 2012	*Enterobacter* spp.	■	■	■	■	■					
B	Aug 2011	*Cronobacter* spp.	■	■	■					■		
B	Aug 2011	*Escherichia* spp.	■	■	■					■		
B	Jul 2012	*Enterobacter* spp.	■	■		■	■					
B	Jul 2012	*Enterobacter* spp.	■	■	■		■					
B	Jul 2012	*Enterobacter* spp.	■	■		■	■					
B	Aug 2012	*Enterobacter* spp.	■	■	■					■		
C	Aug 2011	*Klebsiella* spp.	■	■	■					■		
C	Aug 2011	*Klebsiella* spp.	■	■	■					■		
C	Aug 2011	*Enterobacter* spp.	■	■	■					■		
C	Aug 2011	*Escherichia* spp.	■	■	■					■		
C	Jul 2012	*Pantoea* spp.	■	■	■		■					
C	Jul 2012	*Leclercia* spp.	■	■	■					■		
C	Aug 2012	*Escherichia* spp.	■	■	■		■					
C	Aug 2012	*Klebsiella* spp.	■	■	■	■						
C	Aug 2012	*Enterobacter* spp.	■	■						■	■	
C	Aug 2012	*Escherichia* spp.	■	■				■		■	■	
D	Jul 2012	*Enterobacter* spp.	■	■	■					■		
D	Jul 2012	*Enterobacter* spp.	■	■	■					■		
D	Aug 2012	*Enterobacter* spp.	■	■	■	■						
D	Aug 2012	*Pantoea* spp.	■	■	■		■					
B	Aug 2011	*Enterobacter* spp.	■	■				■				
B	Aug 2011	*Raoultella* spp.	■	■							■	
B	Aug 2011	*Pantoea* spp.	■	■				■				
B	Aug 2011	*Enterobacter* spp.	■	■							■	
B	Aug 2011	*Serratia* spp.	■	■							■	
B	Aug 2011	*Escherichia* spp.	■	■							■	
B	Aug 2011	*Klebsiella* spp.	■	■	■							
B	Jul 2012	*Enterobacter* spp.	■	■						■		
B	Jul 2012	*Ewingella* spp.	■	■						■		
B	Jul 2012	*Citrobacter* spp.	■	■						■		
B	Jul 2012	*Pantoea* spp.	■	■						■		
B	Aug 2012	*Enterobacter* spp.	■	■						■		
B	Aug 2012	*Enterobacter* spp.	■	■		■						
B	Aug 2012	*Enterobacter* spp.	■	■	■							
C	May 2011	*Klebsiella* spp.	■	■						■		
C	Aug 2011	*Enterobacter* spp.	■	■						■		
C	Aug 2012	*Raoultella* spp.	■	■				■				
C	Aug 2012	*Klebsiella* spp.	■	■	■							
C	Aug 2012	*Enterobacter* spp.	■	■	■							
C	Aug 2012	*Enterobacter* spp.	■	■	■							
C	Aug 2012	*Enterobacter* spp.	■	■	■							
C	Aug 2012	*Enterobacter* spp.	■	■						■		
D	Jul 2012	*Klebsiella* spp.	■	■	■							

ABPC, ampicillin; CET, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; TC, tetracycline; GM, gentamicin; AMK, amikacin; CP, chloramphenicol; CPFX, ciprofloxacin

(6-agent-resistant strains); ABPC-CET-CTX-CAZ-IPM-CP-TC (7-agent-resistant strains); and ABPC-CET-CTX-CAZ-GM-CP-TC-CPFX and ABPC-CET-CTX-CAZ-IPM-AMK-CP-TC (8-agent-resistant strains).

Drug resistance differed between the sampling points. The number and rate of detection of drug-resistant strains at each sampling point among the total 204 drug-resistant strains were as follows: A, n = 19, 9.2%; B, n = 80, 38.8%; C, n = 70, 34.0%; D, n = 21, 10.2%; and E, n = 14, 6.8%. The only multidrug resistant strain identified at points A and E was resistant to ABPC-CET. Strains resistant to more than 3 drugs were identified at points B, C, and D. The number and rate of detection of strains resistant to more than 3 drugs among the total 204 drug-resistant strains were as follows: B, n = 28, 13.6%; C, n = 22, 10.7%; and D, n = 7, 3.4%.

4. Discussion

Enterobacteriaceae strains are used as index bacteria for river pollution. The strains previously detected in the Spanish Arga River included 10^2-10^7 CFU/mL *Klebsiella* spp., *E. coli*, *Citrobacter* spp., *Kluyvera* spp., *Enterobacter* spp., *Morganella* spp., *Serratia* spp., *Yersinia* spp., and *Providencia* spp. as fecal coliforms[16]. Meanwhile, approximately 22 × 10^4 CFU/mL *E. coli* were detected in the Chinese Wenyu river[12], and 10^2 CFU/mL *E. coli* were detected in the receiving water of an Austrian sewage treatment plant[17]. In the present investigation, the concentration of Enterobacteriaceae detected in the Tone River was as high as 153 CFU/mL; however, this is considerably lower than previously reported values.

Most of the Enterobacteriaceae strains detected in the river water were drug resistant, and multidrug resistance was more common than resistance to a single drug. Resistance to beta-lactam antibiotics tended to be frequent, particularly to ABPC and CET. Some Enterobacteriaceae strains (*Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp.) have natural resistance to first- and second-generation cephalosporins, including ABPC and CET, owing to the presence of a chromosomal *Amp*C beta-lactamase gene[18]. *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. strains accounted for approximately half of the detected Enterobacteriaceae strains in the current study, and just over 41.5% of these exhibited resistance to at least ABPC and CET.

Sampling site characteristics, including population size, can influence the amount and variety of drug-resistant strains in the sample. Point A is a mountainous region with an upstream dam. The population of the town is approximately 20,000 (Figure 1). Two cities slightly upstream of point B have populations of >300,000. Between points B and C, 2 cities have populations of 200,000-300,000, 4 cities have populations of 100,000-200,000, and over 10 cities have populations <100,000. Between points C and D, 1 city has a population of >300,000, 4 cities have populations of 100,000-200,000, and approximately 10 cities have populations of <100,000. Between points D and E, 5 cities have populations of <100,000. Towns situated around the sampling point A, where resistance to only ABPC and CET was detected, and sampling point E, where resistance to only ABPC, CET, and CP was detected, were more sparsely populated than towns situated around sampling points B, C, and D, where resistance to most of the tests drugs was observed. Similar investigations in rivers in Spain, China, and Switzerland also detected high numbers of resistant bacteria at points downstream of populous locations[9, 12, 16]. These findings are similar to those of the present study.

Antimicrobial tolerance also varies according to the investigation area. The drug-resistant rates of Enterobacteriaceae in the Spanish Arga river were as follows: ABPC, 18.2%; TC, 18.2%; CP, approximately 10%; and aminoglycoside, 0%[16]. The drug-resistant rates of *E. coli* in the China Wenyu river were as follows: TC, 36%; ABPC, 7.9%; CET, 1.9%; and CAZ, 1.1%[12]. The drug-resistant rates of *E. coli* in the Austrian water treatment plant were as follows: TC, approximately 57%; CET, approximately 35%; CP, approximately 35%; ABPC, approximately 18%; CPFX, approximately 2%; CTX, 0%; CAZ, 0%; and aminoglycoside, 0%[17]. These differences may be explained by the specific use of antimicrobials in each area. The Enterobacteriaceae detected in this

study tended to be beta-lactam derivative antimicrobial- and CP-resistant. Beta-lactam derivative antimicrobials comprise at least 40% of the total antimicrobials used in treatment of human bacterial infections in the Japanese population[19]. Conversely, CP is rarely prescribed and is mainly used for rare diseases such as typhoid. In addition, the use of CP for animals has been prohibited since 1998. Previous investigations have reported the presence of CP-resistant strains in the feces of stock animals since 1998, and the CP tolerance is maintained by co-tolerance[20]. Most of the CP-resistant bacteria detected in the present study showed tolerance to another antimicrobial. The strain which showed only CP tolerans was only two strains. The CP-resistance detected in the present study may be a result of co-tolerance with other antibiotic-resistant genes.

In the present study, the strain that was resistant to multiple types of drugs, including third-generation cephalosporin, aminoglycoside, TC, and quinolone reagents, was detected in the river water in an urban area (Table 3). Katrin et al. reported that extended-spectrum beta-lactamase-producing *E. coli* detected in a river in an urban area in Switzerland showed tolerance to not only the beta lactam system but also antimicrobials of other systems such as aminoglycoside, TC, chloramphenicol, and quinolone[9]. In addition, they suggested that antimicrobial use in medical care was one cause of multidrug resistance. Likewise, it has been suggested that human activity around the basin is related to the variety of drug-resistant Enterobacteriaceae detected in the Tone River.

Previous articles have reported that drug-resistant Enterobacteriaceae are detected in human feces and in the feces of animal stock, in agriculture sites, and in sewage-treatment plants[3, 4, 17, 21, 22]. In the present study, commercial areas, residential areas, industrial areas, farmland, and areas with stock co-existed, and sampling points B, C, and D were located in these areas. Therefore, the source of drug-resistant bacteria might not be limited to a single site type. Multidrug-resistant strains were detected at the highest level at Point B, which is near 2 large cities, each with a population of >300,000. Therefore, the primary origin of multidrug-resistant Enterobacteriaceae may be the human enteral environment.

The Enterobacteriaceae strains detected in this study are unlikely to pose an infection risk because most environmental bacteria are not pathogenic to the majority of healthy humans. However, it may lead to infections in persons with low immunity. The Tone River basin is used as farmland, with the river water being used for irrigation, and recent studies suggest that the soil is a drug-resistant genetic hotbed[5, 6]. Multidrug-resistant bacteria may therefore spread through agriculture[22]. The increasing torrential rainfall in recent years has led to more frequent river flooding in Japan, which may also contribute to greater exposure to drug-resistant bacteria.

Our findings suggest that river water is an important source of a variety of drug-resistant bacteria in Japan, particularly in more populated areas.

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Competing interests: None declared.

Ethical approval: Not required.

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