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Original Article

Microbiological characteristics of levofloxacin-resistant *Escherichia coli* in the Tone River, Japan

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ABSTRACT — The prevalence of antibiotic residues and antibiotic-resistant bacteria in natural environments due to human and livestock waste emissions, pesticide runoff, and pharmaceutical effluents poses a serious threat to public health. Therefore, in this study, we aimed to examine the characteristics, including antibiotic susceptibility, biofilm formation ability, and virulence genes, of levofloxacin (LVFX)-resistant *Escherichia coli* isolated from the Tone River. Liquid chromatography-tandem mass spectrometry analysis revealed that the upstream, midstream, and downstream river water samples contained 2.2, 19.5, and 15.7 ng/L LVFX, respectively. Despite the very low concentration of LVFX, LVFX-resistant *E. coli* were isolated from the midstream and downstream regions. Based on the antibiotic sensitivity patterns, the following representative strains were selected: TLR101 and TLR104 (from midstream) and TLR105 and TLR108 (from downstream). These four isolates were LVFX and multidrug-resistant, with TLR101 showing high resistance to ampicillin and the others showing high resistance to chloramphenicol and tetracycline. Biofilm formation assays revealed that TLR104 and TLR108 exhibit very strong biofilm-forming abilities. One-step multiplex polymerase chain reaction (PCR) assays were used to detect various virulence genes, including *eaeA*, *bfpA*, *invE*, *ipaH*, *stx1*, *stx2*, *elt*, *sth*, *aggR*, and *astA*. A 100-bp *astA* gene product was detected in TLR104, and DNA sequence analysis confirmed this amplified PCR product, identifying it as a diarrheagenic *E. coli* strain. Overall, this study revealed the presence of multidrug-resistant diarrheagenic *E. coli* in the Tone River, underscoring the need for effective public health measures to prevent the release of untreated sewage and industrial waste into the river.

Key words: Tone River, Levofloxacin-resistant *Escherichia coli*, Pharmaceutical, Pathogenic *Escherichia coli*, Biofilm-forming activity, *astA* gene

INTRODUCTION

Recently, various drug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant Enterococcus, multidrug-resistant *Pseudomonas aeruginosa*, and carbapenem-resistant Enterobacteriaceae, have emerged, posing a serious threat to public health owing to the difficulty in their treatment. Therefore, a comprehensive approach based on the One Health concept is essential for controlling drug-resistant bacterial infections (CDC, 2019).

Antibiotics absorbed by humans are excreted and released into rivers through sewage treatment plants (STPs). Additionally, large amounts of antibiotics used

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to prevent infections and promote animal growth in the livestock and fishery industries are discharged into rivers. The river water is used for drinking and agricultural purposes. Therefore, the contamination of river water with antibiotics and resistant bacteria poses a major public health concern.

Fluoroquinolones (FQs), such as levofloxacin (LVFX) and ciprofloxacin, are an important class of antibiotics widely used in both human and veterinary medicine (Pharm *et al.*, 2019). However, the prevalence of FQ resistance has also increased with the increasing use of FQs (World Health Organization, 2019). Approximately 70% of these antibiotics are excreted from the environment after being administered to humans and animals. FQs are environmental contaminants that pose a high risk of antibiotic resistance.

Many environmental microorganisms do not exist as planktonic cells but remain attached to surfaces and form biofilms (Costerton *et al*., 1999; Davey and O'toole, 2000). Bacterial biofilms are dense bacterial communities enclosed in polymeric matrices (Costerton *et al*., 1999). Biofilms adhere to both inert and living surfaces, and the bacterial communities within them exhibit phenotypes distinct from those of free-floating cells, including increased tolerance to antibiotics (Van Acker *et al*., 2014). Notably, bacteria within biofilms are up to 1,000 times more resistant to antimicrobial agents than those in planktonic cultures (Furuno *et al*., 2008). Furthermore, these bacterial communities can be pathogenic owing to their virulence and antibiotic resistance. Biofilms promote infection through complex interactions between virulence factors and antibiotics (Schroeder *et al*., 2017).

Escherichia coli is a major bacterium in the intestinal flora of humans and animals. It easily contaminates the surrounding environment when discharged through feces and is often used as an indicator of antibiotic resistance worldwide (Korzeniewska *et al*., 2013; Kostyla *et al*., 2015). Most strains of *E. coli* are harmless and rarely cause disease. However, pathogenic strains, particularly diarrheagenic *E. coli* (DEC), can cause food poisoning in humans. To date, six categories of pathogenic *E. coli* have been reported: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998; Kaper *et al*., 2004). Additionally, other *E. coli* strains, such as enteroaggregative *E. coli* heat-stable enterotoxin 1 gene-possessing *E. coli* (EASTEC), adherent-invasive *E. coli*, and cell-detaching *E. coli*, can cause diarrhea (Wakushima *et al*., 2010). Each pathotype possesses specific virulence genes that are associated with the disease symptoms. For example, *bfpA* encodes a bundle-forming pilus in EPEC; *ipaH* encodes the invasive plasmid antigen H in EIEC; and *stx1* and *stx2* encode Shiga toxins I and II, respectively, in EHEC. DEC can be rapidly identified by multiplex polymerase chain reaction (PCR) of virulence genes (Zhou *et al*., 2002; Hegde *et al*., 2012; Yandag *et al*., 2023).

Several studies have reported an association among bacterial phylotypes, virulence, and antibiotic resistance in many countries, including Denmark, Switzerland, Portugal, Mexico, and China (Bukh *et al*., 2009; Chen *et al*., 2011; Pereira *et al*., 2013; Ramírez Castillo *et al*., 2013; Zurfluh *et al*., 2014). However, little is known about river water contamination with drug-resistant bacteria in Japan. Therefore, we determined the amount of pharmaceutical residues in the Tone River flowing through the metropolitan area and revealed the presence of antibiotics, such as clarithromycin (CAM) (Murahashi *et al.*, 2024). Here, we report the microbiological characteristics of LVFX-resistant *E. coli* isolated from the Tone River.

MATERIALS AND METHODS

Chemicals and media

Tetracycline (TC) and chloramphenicol (CP) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). CAM and LVFX were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Ampicillin (ABPC) was purchased from Merck (Darmstadt, Germany). All other reagents were purchased from FUJIFILM Wako Pure Chemical Corporation unless otherwise indicated. Soybean casein digest (SCD) agar and AccuDia Blue Light Broth were purchased from Shimadzu Diagnostics Corporation (Tokyo, Japan). MacConkey Agar Medium was purchased from FUJIFILM Wako Pure Chemical Corporation. Mueller–Hinton broth was purchased from BD Difco (Tokyo, Japan).

River water sampling

Fig. 1 shows the sampling sites along the Tone River. On April 10, 2023, sampling was performed at three sites: upstream (Taisho Bridge, 36°29'48"N, 139°70'51"E; Site A), midstream (Tonegawa Bridge, 36°14'33"N, 139°42'18"E; Site B), and downstream (Joso-ohashi Bridge, 35°52'57"N, 140°20'53"E; Site C). Water samples (500 mL, three bottles) were collected from the center of each bridge using a HEYROHT water sampler (SIBATA Scientific Technology Ltd., Saitama, Japan).

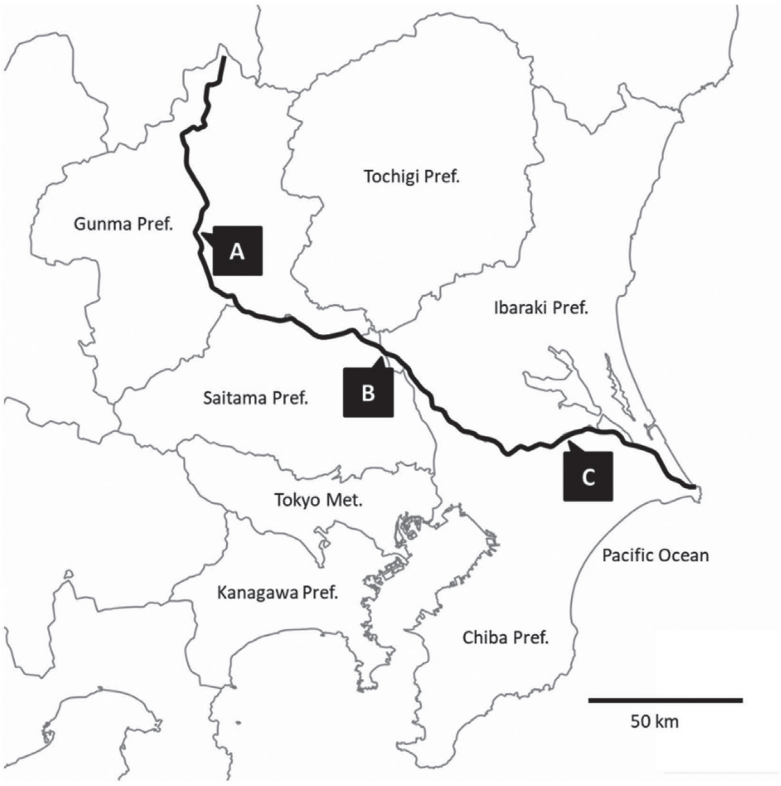

Fig. 1. Sampling locations along the Tone River.

Detection of pharmaceuticals

Pharmaceuticals were extracted from river water using a Waters Oasis HLB Plus cartridge (Milford, MA, USA). The filtered water samples (100 mL) were passed through a cartridge, and the analytes were eluted using methanol (3 mL). The methanol in the extract solution was evaporated to dryness, and the residue was dissolved in 1 mL of ethanol. This solution was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS; SCIEX QTRAP 6500; Framingham, MA, USA).

Bacterial counting and isolation of LVFX-resistant *E. coli*

To count the viable bacteria, 100 µL of river water sample diluted with sterile water was smeared on the SCD plate, cultured at 28°C for two weeks, and the number of colonies formed was counted. The number of *E. coli* was determined using the agar plate mixture method: sample (2 mL) was plated on the MacConkey agar medium, cultured at 36°C for two days, and the number of colonies was counted.

Enrichment cultures were prepared before the isolation of LVFX-resistant *E. coli*. River water (100 mL) collected at three sampling sites was added to the double-concentrated SCD broth and cultured at 33°C for three days. This culture medium was further diluted 10^1–10^7 times. LVFX-resistant *E. coli* strains were isolated on MacConkey agar medium supplemented with an LVFX concentration four times higher than the minimum inhibitory concentration (MIC) of susceptible *E. coli* NBRC3972.

Drug susceptibility test

MICs were determined on Müller–Hinton medium using the two-fold agar dilution method recommended by the Clinical and Laboratory Standards Institute (Wayne, 2023).

Biofilm formation assay

The biofilm formation assay was performed as previously described (O'Toole, 2011), with some modifications. Overnight cultures were prepared to an optical density (OD) of 1.0 using sterile water. The bacterial solution and SCD broth supplemented with 0.25% glucose-added SCD broth were added to each well of a 96-well flat-

bottomed polystyrene microtiter plate at a ratio of 1:40 (4 µL:156 µL). The plates were incubated at 36°C for 48 hr. After incubation, the supernatant from the liquid cultures was gently removed. The wells were gently washed with phosphate-buffered saline. To fix the biofilm on the plate, 25 µL of methanol was added to each well and incubated for 10 min. After removing methanol, the plate was dried at 30°C for 30 min, and 150 µL of 0.1% crystal violet was added. After 10 min, the plate was washed thrice with sterile water, turned upside down, and dried overnight. Finally, 150 µL of 30% acetic acid was added to each well to dissolve the biofilm. The solutions were transferred to a fresh 96-well plate, and the absorbance was measured at 560 nm using an ARVO MX 1420 Multilabel Counter (Perkin Elmer, Yokohama, Japan). The biofilm-forming abilities of the isolates were scored as follows (Aguila-Arcos *et al*., 2017): $OD_{560} < 0.120$, no biofilm formation; $0.120 < OD_{560} < 0.240$, weak biofilm formation; $OD_{560} > 0.240$, strong biofilm formation; $OD_{560} > 1.5$, very strong biofilm formation. Biofilm-forming assay results are represented as the mean ± standard error of the mean (SEM) of at least two independent biological experiments performed in quintuplicate for each strain.

Preparation of genomic and plasmid DNAs

E. coli genomic and plasmid DNAs were extracted using commercial DNA extraction kits (NucleoSpin Tissue, Takara Bio, Shiga, Japan) according to the manufacturer's instructions and subjected to PCR.

One-step multiplex PCR assay

PCR was performed as previously described (Ohmura-Hoshino *et al*., 2022) with slight modifications. *E. coli* isolates were evaluated for the presence of 10 virulence genes: *astA, bfpA, elt, aggR, ipaH, stx1, stx2, eaeA, sth,* and *invE* (Kaper *et al*., 2004; Boerlin *et al*., 2005). An internal control (IC) was used with DNA amplification primers for the *E. coli 16S rRNA* gene to prevent false negatives. The primer sequences, PCR conditions, and product sizes are listed in Table 1. The predicted product sizes of the virulence genes were as follows: *eaeA* (1001 bp) and *bfpA* (326 bp) in EPEC, *elt* (494 bp) and *sth* (172 bp) in ETEC, *aggR* (407 bp) in EAEC, *invE* (235 bp) and *ipaH* (596 bp) in EIEC, *stx1* (693 bp) and *stx2* (794 bp) in EHEC, and *astA* (100 bp) for EASTEC. All primers used for DNA sequencing and PCR were synthesized by FASMAC Inc. (Tokyo, Japan). PCR was performed using a T100 Thermal Cycler (Bio-Rad, Tokyo,

Table 1. Primers used in the one-step multiplex polymerase chain reaction and the resultant amplicon size.

Pathotypesdiarrheagenic *E. coli*	Target genes		Primers(5′–3′) [a]	Product size (bp)
EPEC	*eaeA*	F	CTTCAGTCGCGATCTCTGAACG	1001
		R	GGTAGTCTTGTGCGCTTTGGCT	
	bfpA	F	CTACCAGTCTGCGTCTGATTCC	326
		R	CGTAGCCTTTCGCTGAAGTACC	
EIEC	*invE*	F	GAAATTCTGGATGGCACTCGTAGAAG	235
		R	CTTTCGCGCGAGACAGATTCTCTT	
	ipaH	F	CTTTCCGATACCGTCTCTGC	595
		R	CACCCTCTGAGAGTACTCATTCTCC	
EHEC	*stx1*	F	AGAGGGATAGATCCAGAGGAAGGG	693
		R	AATTGCCCCCAGAGTGGATG	
	stx2	F	GTATACGGACAGAGATATCGACCCC	794
		R	CGCTGCAGCTGTATTACTTTCCC	
ETEC	*elt*	F	TATACCGTGCTGACTCTAGACCCC	494
		R	CGGTGGGAAACCTGCTAATC	
	sth	F	CTTTCTGTATTGTCTTTTTCACCTTTC	172
		R	GCAGGATTACAACACAATTCACAGC	
EAEC	*aggR*	F	GCCTAAAGGATGCCCTGATG	407
		R	TGCTGCTTTGCTCATTCTTG	
EASTEC	*astA*	F	TATATCCGAAGGCCCGCATCCAG	100
		R	CAGGTCGCGAGTGACGGCTTTG	
IC		F	GTTTGATCATGGCTCAGATTGAACGC	1205
		R	CGTAAGGGCCATGATGACTTGAC	

EPEC, enteropathogenic *Escherichia coli*; EIEC, enteroinvasive *E. coli*; EHEC, enterohaemorrhagic *E. coli*; ETEC, enterotoxigenic *E. coli*; EAEC, enteroaggregative *E. coli*; EASTEC, enteroaggregative *E. coli* heat-stable enterotoxin 1 gene-possessing *E. coli*; IC, internal control (*E. coli 16S rRNA*).

[a]Sequences obtained from the study of Ohmura-Hoshino *et al*., 2022.

Table 2. Various pharmaceutical concentrations in the Tone River.

Sampling site	LVFX	CAM	Carbamazepine	Bezafibrate	Diphenhydramine
A	2.2	2.2	2.6	4.7	1.6
B	19.5	27.4	21.1	29.4	5.6
C	15.7	38.7	59.2	35.7	4.2

Unit, ng/L. LVFX, levofloxacin; CAM, clarithromycin.

Japan) and Quick Taq HS DyeMix kit (TOYOBO, Tokyo, Japan). The PCR mixture (reaction volume: 50 μL) contained the PCR buffer, 1.5 mM $MgCl_2$, 200 μM (each) dNTP, 10 μM primers, 2.5 U rTaq DNA polymerase, and 2 μL DNA extract. PCR was performed for 35 cycles (10 sec at 94°C for denaturation, 30 sec at 60°C for annealing, and 1 min at 72°C for polymerization). The PCR products (10 μL) were resolved electrophoretically using a 2.0% horizontal agarose gel in Tris-acetate-EDTA buffer. The gel was stained with Midori Green Advance Solution (Nippon Genetics, Ltd., Tokyo, Japan) and visualized using a Gel Doc 2000 Documentation System (Bio-Rad). The band sizes of the PCR products were determined using an ExcelBand 100 bp DNA ladder (COSMO BIO, Tokyo, Japan).

Sequence analysis of PCR products

Next, the identities of the representative PCR products of *E. coli* TLR104 were verified via nucleotide sequence analysis using the Big Dye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific K. K., Tokyo, Japan).

Nucleotide sequence accession numbers

The identified nucleotide sequence of the *astA* gene of *E. coli* TLR104 was deposited in the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/) under accession number LC834846.

RESULTS AND DISCUSSION

Determination of pharmaceutical concentrations in the Tone River

Water samples were collected upstream (Site A), midstream (Site B), and downstream (Site C) of the Tone River. LVFX and CAM, which pose high environmental risks, were analyzed by LC-MS/MS after pretreatment with solid-phase extraction. LVFX and CAM were detected at concentrations of 2.2–19.5 and 2.2–38.7 ng/L, respectively (Table 2). The concentrations at the midstream and downstream sites were approximately 10 times higher than those at the upstream site. Because carbamazepine, bezafibrate, and diphenhydramine were also detected at concentrations similar to those of LVFX and CAM, many pharmaceuticals, including antibacterial agents, may exist in river water. We demonstrated the antibacterial activity of the Tone River water extract using a disk diffusion assay (Murahashi *et al*., 2024), while other researchers have reported the presence of resistant bacteria at concentrations much lower than their MICs (Gullberg *et al*., 2011; Chow *et al*., 2021; Pereira *et al*., 2023). These findings suggest that microorganisms may acquire drug resistance in river water. We then investigated the presence of LVFX-resistant bacteria in Tone River water.

Table 3. Numbers of bacteria at the Tone River sampling sites.

Sampling site	Number of bacteria (CFU/mL)	
	Total viable bacteria	*E. coli*
A	1.3×10^4	2.2
B	1.7×10^4	2.3
C	9.0×10^3	0.8

CFU, colony forming unit.

Isolation of antibiotic-resistant *E. coli* strains

Viable bacterial counts from upstream to downstream of the Tone River were approximately 10^4 CFU/mL, with no significant variation observed across sites. *E. coli* counts were below 3 CFU/mL at all sampling points, indicating relatively low *E. coli* contamination (Table 3). Based on these findings, LVFX-resistant *E. coli* strains were isolated from sites B and C, and 10 colonies from each site were randomly selected. The selected LVFX-resistant *E. coli* strains exhibited brick-red colony morphology on MacConkey agar plates and fluorescence in AccuDia blue light broth. The strains were further identified using matrix-assisted laser desorption/ionization time-of-flight MS with a MALDI Biotyper Smart (Bruker Japan, Yokohama Japan). Despite the low concentration of LVFX in the Tone River, LVFX-resistant *E. coli* strains were isolated from both midstream (site B) and downstream (site C) sites.

Table 4. Drug susceptibilities of the *Escherichia coli* strains identified in this study.

E. coli strain	MIC (μg/mL)				
	LVFX	ABPC	CAM	CP	TC
LR101	16	>128	64	8	1
LR104	0.5	8	64	>128	64
LR105	0.5	8	64	>128	64
LR108	0.5	8	64	>128	64
NBRC3972	0.016	2	64	8	0.5

MIC, minimum inhibitory concentration; LVFX, levofloxacin; ABPC, ampicillin; CAM, clarithromycin; CP, chloramphenicol; TC, tetracycline.

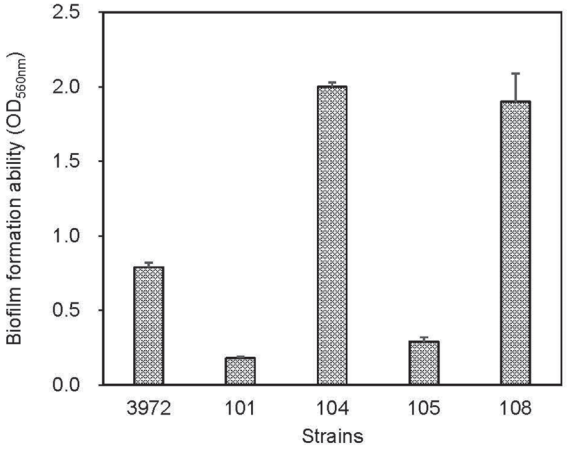

Fig. 2. Biofilm-forming abilities of the bacteria isolated from the Tone River. 3972: *Escherichia coli* NBRC3972; 101: *E. coli* TLR101; 104: *E. coli* TLR104; 105: *E. coli* TLR105; and 108: *E. coli* TLR108. Biofilm formation assay results are represented as the mean ± standard error of the mean (SEM) of at least two independent experiments performed in quintuplicate for each strain.

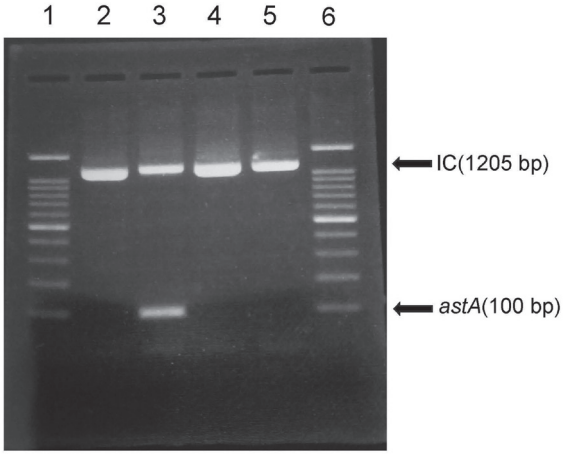

Fig. 3. Multiplex polymerase chain reaction analysis of the bacteria isolated from the Tone River. Lanes 1 and 6: DNA molecular size marker (100 bp ladder); Lane 2: *E. coli* TLR101; Lane 3: *E. coli* TLR104; Lane 4: *E. coli* TLR105; and Lane 5: *E. coli* TLR108. IC, internal control (*E. coli 16S rRNA* gene).

Antimicrobial resistance phenotypes of *E. coli* isolates

Next, the antimicrobial susceptibility of the isolated LVFX-resistant *E. coli* strains was analyzed. Based on the antibiotic sensitivity patterns, the following representative strains from each sampling point were selected: TLR101 and TLR104 (midstream isolates) and TLR105 and TLR108 (downstream isolates). These isolates exhibited high resistance to LVFX (Table 4). In addition to LVFX, TLR101 is highly resistant to ABPC, whereas TLR104, TLR105, and TLR108 are highly resistant to TC and CP. Gram-negative bacteria such as *E. coli* exhibit intrinsic resistance to CAM. The Multiple antibiotic resistance (MAR) index of an individual isolate was calculated by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics it was exposed to (Krumperman, 1983). Four isolates (MAR index > 0.2) were considered to have originated from a highly contaminated source. Multidrug-resistant *E. coli* strains were detected at different sampling points in the midstream and downstream sites of the Tone River, which are close to densely populated areas. These findings were consistent with those reported by Hata *et al*. (Hata *et al.*, 2015).

Biofilm-forming ability of multidrug-resistant *E. coli* isolates

Biofilm-forming bacteria are up to 1000 times more antibiotic-resistant than their planktonic counterparts (Mah and O'Toole, 2001; Stewart and Costerton, 2001) and are responsible for chronic or persistent infections (Costerton *et al.*, 1999). Biofilm formation is a crucial aspect of pathogenicity that combines virulence and antibiotic resistance (Davey and O'toole, 2000; Sauer *et al*., 2002). Therefore, we investigated the biofilm-forming ability of the four isolates. As shown in Fig. 2, the mean optical density of *E. coli* NBRC3972 was 0.79 ± 0.03. TLR104 (2.0 ± 0.03) and TLR108 (1.9 ± 0.19) produced twice as much biofilm, whereas TLR101 (0.18 ± 0.01) and TLR105 (0.29 ± 0.03) produced less biofilm than NBRC3972. According to the criteria described by Águila-Arcos *et al.* (Águila-Arcos *et al*., 2017), the isolates and NBRC3972 were classified into three groups based on their biofilm-forming abilities: non-biofilm-forming (TLR101 and TLR105), weak biofilm-forming (NBRC3972), and very strong biofilm-forming (TLR104 and TLR108) isolates. These results revealed that TLR104 and TLR108 exhibited very high biofilm-forming abilities, indicating that they may pose a threat to public health.

Detection of pathogenesis gene from multidrug-resistant *E. coli* isolates

The four isolates were subjected to one-step multiplex PCR, and the following virulence genes were detected: *eaeA* and *bfpA* from EPEC, *invE* and *ipaH* from EIEC, *stx1* and *stx2* from EHEC, *elt* and *sth* from ETEC, *aggR* from EAEC, and *astA* from EASTEC. IC (*E. coli 16S rRNA* gene) was also used for all analyzed isolates. As shown in Fig. 3, the *astA* gene product of 100 bp was detected in TLR104, and DNA sequence analysis confirmed this amplified PCR product. *astA* encodes enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1), one of the main pathogenic factors in diarrheagenic *E. coli* (Nataro and Kaper, 1998). Although the diarrheagenic potential of EAST1 remains unknown, food poisoning outbreaks caused by *E. coli* strains possessing only the *astA* gene have been reported in various Japanese cities, including Osaka, Hiroshima, Himeji, and Yashio in Saitama Prefecture (IASR, 2015, 2019, 2022). Additionally, virulence genes (*eaeA* in EPEC, *invE* in EIEC, *stx1* and *stx2* in EHEC, *elt* and *est* in ETEC, *aggR* in EAEC, and *lysP* in *E. alberii*) were analyzed using a commercial Cica Geneus Pathogenesis Gene Detection PCR Kit (Kanto Kagaku, Tokyo, Japan); however, none were detected in the isolates.

Our data indicated that the residual amount of LVFX at the midstream and downstream sites of the Tone River was approximately 20 ng/L, and the number of *E. coli* was low (approximately 2 CFU/mL). The detected *E. coli* strains, including diarrheagenic *E. coli* carrying *astA*, were multidrug-resistant and had high biofilm-forming abilities.

In conclusion, this study demonstrated the presence of multidrug-resistant pathogenic *E. coli* strains in the Tone River. River ecosystems and human and animal health are significantly affected by pathogenic and multidrug-resistant *E. coli* strains. The excessive use of broad-spectrum antibiotics in human and veterinary medicine for livestock management has contributed to the emergence of antibiotic resistance. Future studies should investigate LVFX resistance mechanisms in *E. coli* isolates to understand better the prevalence of antibiotic-resistant microorganisms in the Tone River ecosystem. Assessment and control of antibiotic resistance are important for the preservation of river ecosystems.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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