Note

Antibiotic-resistance of Fecal Coliforms at the Bottom of the Tama River, Tokyo

MASAHIKO OKAI¹, HANAKO AOKI², MASAMI ISHIDA¹, AND NAOTO URANO³

¹Department of Ocean Sciences, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan
²Department of Ocean Sciences, School of Marine Science, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan
³Department of Marine Resources and Energy, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan

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We investigated the midstream bottom of the Tama River, which flows through Tokyo, to evaluate the occurrence and degree of antibiotic-resistant fecal coliforms including multidrug-resistant fecal coliforms. The genera Klebsiella and Escherichia were the major isolates among the fecal coliforms. For the genus Klebsiella, the highest antibiotic resistance was observed for ampicillin (100%), followed by kanamycin, tetracycline, cefotaxime, and cefoxitin. The highest resistance to E. coli was found for kanamycin (44.4%), followed by ampicillin, tetracycline, chloramphenicol, amoxicillin-clavulanate, cefotaxime, ceftazidime, and aztreonam. Multidrug resistance (MDR) was observed in three E. coli isolates. A double disc synergy test confirmed the production of extended-spectrum β-lactamases by the six-antibiotic-resistant isolate E. coli hfa7, and the strain had CTX-M-1 group gene. Assessments of antibiotic-resistant fecal coliforms at the bottom of the Tama River are important toward the goals of preventing the spread of antibiotic-resistant fecal coliforms in humans, animals, and the environment.

Key words: Antibiotic resistance / ESBL / Fecal coliform / Tama River.

Antibiotics are widely used to treat infections in humans, animals, food, and plants. The uncontrolled and broad use of antibiotics against pathogens has resulted in the occurrence of antibiotic-resistant bacteria. Reports of extended-spectrum β-lactamase (ESBL)-producing bacteria continue to accumulate (Padmini et al., 2017). ESBLs are enzymes that hydrolyze beta lactam antibiotics such as penicillin, cephalosporin, and monobactam. ESBLs are classified into several groups according to their amino acid sequence homology; the major groups of ESBLs are temoniera (TEM), sulphydryl variable (SHV), and cefotaxime (CTX-M) (Ghafoorian et al., 2015). In Japan, the National Action Plan on Antimicrobial Resistance (AMR) 2016-2020 describes the implementation of one health approach against the spread of antibiotic-resistant bacteria that are isolated from humans, animals, food, and the environment (The Government of Japan 2016).

Urban rivers receive wastewater effluents from hospitals, livestock farms, aquaculture farms, and human dwellings. Rivers are thus often contaminated with antibiotic-resistant bacteria. A wide range of the bacterial diversity (including antibiotic-resistant bacteria and genes) in urban rivers has been investigated (Narciso-da-Rocha and Manaia, 2016; Proia et al., 2018). It was suggested that bacterial communities shift due to changes in water quality, affecting the abundance and diversity of antibiotic-resistant genes (Zhou et al., 2017).

Fecal coliforms are generally used as an indicator of bacteriological water quality for drinking. An increase in the number of antibiotic-resistant fecal coliforms in a river changes the ratio of gut flora, and this change leads to the majority of resistant strains in the gut, resulting in a serious public health issue. It is thus essential to
determine the precise relationship between fecal coliforms and antibiotic resistance.

The Tama River is approx. 138 km (85.7 miles) long and flows through metropolitan Tokyo on the borderline between Tokyo and Kanagawa prefectures. Water from the Tama River has been used for drinking in Tokyo since the 17th century. In this study, we investigated the occurrence and variation of fecal coliforms with antibiotic resistance from the bottom of the midstream of the Tama River, and we characterized the ESBL gene types of the E. coli isolate that showed resistance to both cephalosporin and monobactam.

Bottom mud water samples were collected in July 2017 from five midstream sites (35-41 km from the estuary) of the Tama River (Fig. 1). Samples were transported to the laboratory in sterile bottles in contact with ice. Fecal coliforms are defined as coliform bacteria that can grow at 44.5°C and produce acid and gas from lactose within 24 hr. The isolation of fecal coliforms was done by a modification of the method of Ham et al. (2012). A volume of 100 µl of each sample was spread onto MacConkey agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) instead of desoxycholate agar plates, and red-colored colonies were picked up after the incubation at 44.5°C for 24 hr. The identification of each strain was performed by 16S rRNA gene amplification and sequencing, followed by comparison of the sequence with homologous sequences deposited in databases. The total DNA for each fecal coliform isolate was extracted by the alkaline lysis method. The 16S rRNA genes were then amplified by polymerase chain reaction (PCR) using the forward primer 27F (5′-AGA GTTTGATCCTGCTCAG-3′) and the reverse primer 1492R (5′-GGTACCTTGTTCAGACTT-3′). The sequencing was carried out by Eurofins Genomics (Tokyo, Japan). The 16S rDNA sequences of the isolates were subjected to a BLAST analysis of the U.S. National Center for Biotechnology Information (NCBI) databases.

An antibiotic susceptibility test was performed against 63 isolates of fecal coliforms using the disc diffusion method for the following 13 antibiotics: ampicillin (AMP), amoxicillin-clavulanate (AMX-CVA), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (CFX), aztreonam (ATM), meropenem (MEM), imipenem (IPM), kanamycin (KAN), gentamicin (GEN), tetracycline (TET), chloramphenicol (CHL), and ciprofloxacin (CIP). Each isolate was suspended in 0.9% (w/v) normal saline, and the 0.5 McFarland standard was used to adjust the turbidity. The suspensions were spread on the surface of Mueller-Hinton agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The antibiotic disks (Japan Becton-Dickinson, Tokyo, Japan) were placed on the agar plates, and the agar plates were incubated for 16-24 hr at 37°C. The results of inhibitory zones were interpreted using the Clinical and Laboratory Standards Institute (CLSI) criteria (Clinical and Laboratory Standards Institute, 2016).

We determined the ESBL production of E. coli hfa7 (which was resistant to all of the third generation cephalosporins CTX and CAZ, and the monobactam ATM) by conducting a double disc synergy test (DDST), using a disc of AMX-CVA along with CTX, CAZ, and ATM. ESBL encoding gene type was determined by gene amplification and sequencing, followed by comparison of the sequence with homologous sequences deposited in databases. ESBL encoding genes were amplified by PCR using specific primers for TEM, SHV, CTX-M-1, CTX-M-2, and CTX-M-9 groups (Shibata et al., 2006; Yagi et al., 2000). The sequencing was carried out by Eurofins Genomics. The sequences were subjected to a BLAST analysis of the NCBI databases.

The number of fecal coliforms among the five midstream sample sites in the Tama River ranged from 5.7 × 10^2 to 3.4 × 10^3 CFU/mL. We sequenced 63 randomly selected isolates including Escherichia coli (42.9%), Escherichia sp. (16%), Klebsiella pneumoniae (22.2%), K. variicola (6.3%), K. quasipneu-

![ FIG. 1. Location of midstream sampling sites in the midstream of the Tama River, Tokyo.](image1)

![ FIG. 2. Variation in fecal coliforms isolated from the midstream of the Tama River.](image2)
moniae (3.2%), Klebsiella sp. (15.9%), and Cronobacter sakazakii (7.9%) (Fig. 2).

For the genus Klebsiella, the highest resistance was found for AMP (100%), followed by KAN (13.3%), TET (6.7%), CTX (3.3%), and CFX (3.3%) (Table 1). For E. coli, the highest resistance was found for KAN (44.4%), followed by AMP (14.8%), TET (14.8%), CHL (7.4%), AMX-CVA (3.7%), CTX (3.7%), CAZ (3.7%), and ATM (3.7%). MDR was observed in three isolates of E. coli. One of these isolates was resistant to six antibiotics (AMP, CTX, CAZ, ATM, KAN, and TET); another isolate was resistant to five (AMP, AMX-CVA, KAN, TET, and CHL), and the last to three (KAN, TET, and CHL). Fecal coliforms resistant to MEM, IPM, GEN, and CIP were not detected. Full details of the antibiotic resistance of the isolates are shown in Table 2. The DDSTs confirmed the ESBL production by the six-antibiotic-resistant isolate E. coli hfa7, and the strain had CTX-M-1 group gene.

The Nippon AMR One Health Report (NAOR) describes the antibiotic resistance rate of E. coli (The AMR One Health Surveillance Committee, 2017). In E. coli from humans admitted to medical institutions, the resistance rate to carbapenem antibiotics is low (<0.2%), and those of CTX and CAZ increased each year from 2011 to 2015 (CTX: 14.8% to 24.5%, CAZ: 5.2% to 10.8%). In E. coli from livestock (cattle, pigs, and broiler and layer chickens) on farms, the antibiotic-resistance rate of CTX decreased from 2011 to 2015 and remained at <3% in 2015. Our study detected ESBL-producing E. coli hfa7 which is resistant to CTX and CAZ, and showed the necessity of continuous investigation of antibiotic resistance in the Tama River.

The Tama River supplies fresh water to Tokyo, which has a central-area population of over 13 million people. Studies of fecal coliforms and antibiotic resistance in the Tama River have been reported by other groups. Kobori et al. (2012) investigated the antibiotic susceptibility of E. coli from Tama River water and raw sewage samples using 12 antibiotics: AMP, AMX-CVA, KM, TET, CHL, CIP, cefixime (CFI), streptomycin (STR), minocycline (MIN), nalidixic acid (NAL), ofloxacin (OFL), and trimethoprim-sulfamethoxazole (TMP-SMX). They reported that the highest resistance of E. coli isolates in the Tama River water was that of TMP-SMX (24%), followed by AMP (16%) and TET (9%). The antibiotic-resistance rates of E. coli isolates in the raw sewage were as follows: AMP (22%), TMP-SMX (21%), and TET (11%). Six antibiotics (AMP, AMX-CVA, KM, TET, CHL, and CIP) were used in both the study of Kobori et al. and our present investigation, and the antibiotic-resistance pattern of the E. coli isolates from the river bottom tested herein show a rate of antibiotic resistance that is similar to those from the water and raw sewage samples in the Kobori et al. study, except for KM. The resistance rate to KM in our study was higher than that (1%) in the study of Kobori et al., and the resistance rate in Site 3 (the Hodokubo River) was 64% (Table 2). A zoological park is located around the source of the Hodokubo River, and the high resistance rate in Site 3 might be due to KM treatment for animals.

Ham and co-workers collected water samples from upstream to downstream in the Tama River and investigated the concentrations of total coliform, fecal coliform, and antibiotic-resistant E. coli (Ham et al., 2009; Ham et al., 2012). They observed that the E. coli concentra-

### Table 1. The prevalence of antibiotic resistance in total fecal coliforms, the genus Klebsiella and E. coli

| Antibiotic            | No. (%) of total fecal coliforms | No. (%) of Klebsiella spp. | No. (%) of E. coli |
|-----------------------|----------------------------------|----------------------------|-------------------|
| Ampicillin (AMP)      | 36 (57.1)                        | 30 (100)                  | 4 (14.8)          |
| Amoxicillin-clavulanate (AMX-CVA) | 1 (1.6)                        | 0 (0)                     | 1 (3.7)           |
| Cefotaxime (CTX)      | 3 (4.8)                          | 1 (3.3)                   | 1 (3.7)           |
| Ceftazidime (CAZ)     | 1 (1.5)                          | 0 (0)                     | 1 (3.7)           |
| Cefoxitin (CFX)       | 2 (3.2)                          | 1 (3.3)                   | 0 (0)             |
| Aztreonam (ATM)       | 1 (1.5)                          | 0 (0)                     | 1 (3.7)           |
| Meropenem (MEM)       | 0 (0)                            | 0 (0)                     | 0 (0)             |
| Imipenem (IPM)        | 0 (0)                            | 0 (0)                     | 0 (0)             |
| Kanamycin (KAN)       | 17 (27.0)                        | 4 (13.3)                  | 12 (44.4)         |
| Gentamicin (GEN)      | 0 (0)                            | 0 (0)                     | 0 (0)             |
| Tetracycline (TET)    | 7 (11.1)                         | 2 (6.7)                   | 4 (14.8)          |
| Chloramphenicol (CHL) | 3 (4.8)                          | 0 (0)                     | 2 (7.4)           |
| Ciprofloxacin (CIP)   | 0 (0)                            | 0 (0)                     | 0 (0)             |

The prevalence of antibiotic resistance in total fecal coliforms, the genus Klebsiella and E. coli.
| Sampling site | Strain ID | Organism                          | Antibiotics |
|---------------|-----------|-----------------------------------|-------------|
|               |           |                                   | AMP| AMX-CVA| CTX| CAZ| CFX| ATM| MEM| IPM| KAN| GEN| TET| CHL| CIP |
| Site 1        | nfa2      | Cronobacter sakazakii             | R | S | S | S | S | S | S | S | S | S | S | S | S |
|               | nfa3      | Escherichia coli                  | R | R | I | S | S | S | S | S | I | R | S | R | R |
|               | nfa4      | Escherichia coli                  | I | I | S | S | S | S | S | S | S | I | S | S | S |
|               | nfa17     | Klebsiella pneumoniae             | R | I | S | S | S | S | S | S | I | S | S | S | S |
|               | nfa47     | Escherichia coli                  | I | I | I | S | S | S | S | S | I | I | R | S | S |
|               | nfa48     | Escherichia coli                  | I | I | S | S | S | S | S | S | I | S | S | S | S |
|               | nfa52     | Klebsiella pneumoniae             | R | I | S | S | S | S | S | S | I | S | S | S | S |
|               | nfb1      | Klebsiella sp.                    | R | I | S | S | S | S | S | I | I | I | S | S | S |
|               | nfb2      | Klebsiella pneumoniae             | R | S | S | S | S | S | S | I | I | R | S | S | S |
|               | nfb3      | Klebsiella variicola              | R | I | S | S | S | S | S | S | I | S | S | S | S |
|               | nfb5      | Escherichia coli                  | S | I | S | S | S | S | S | S | S | I | S | S | S |
|               | nfb19     | Klebsiella sp.                    | R | I | S | S | S | S | S | S | S | S | S | S | S |
| Site 2        | gfa1      | Escherichia coli                  | S | S | S | S | S | S | S | S | S | S | S | S | S |
|               | gfa2      | Klebsiella variicola              | R | I | I | S | I | S | S | S | I | S | S | S | S |
|               | gfa3      | Klebsiella variicola              | R | I | I | S | I | S | S | S | S | S | S | S | S |
|               | gfa5      | Escherichia coli                  | R | I | I | S | I | S | S | S | S | S | S | S | S |
|               | gfa6      | Escherichia coli                  | R | I | I | S | I | S | S | S | S | S | S | S | S |
|               | gfa11     | Klebsiella sp.                    | R | S | I | S | S | S | S | S | R | S | S | S | S |
|               | gfa12     | Klebsiella pneumoniae             | R | S | S | S | S | S | S | S | I | S | S | S | S |
|               | gfa15     | Cronobacter sakazakii             | I | I | I | S | I | S | S | S | S | S | S | S | S |
|               | gfa16     | Escherichia coli                  | I | I | I | S | S | S | S | S | S | S | S | S | S |
|               | gfa18     | Klebsiella sp.                    | R | S | S | S | S | S | S | S | I | S | S | S | S |
|               | gfa20     | Escherichia coli                  | I | I | I | S | S | S | S | S | I | S | S | S | S |
|               | gfa21     | Klebsiella pneumoniae             | R | S | R | S | S | S | S | S | S | S | S | S | S |
|               | gfa24     | Klebsiella pneumoniae             | R | S | S | S | S | S | S | S | S | S | S | S | S |
|               | gfb2      | Klebsiella sp.                    | R | S | S | I | I | I | S | S | S | S | S | S | S |
|               | gfb4      | Klebsiella variicola              | R | S | I | S | S | S | S | S | S | S | S | S | I |
| Site 3        | hfa1      | Escherichia sp.                   | I | I | I | S | S | S | S | S | S | I | S | S | S |
|               | hfa2      | Escherichia coli                  | I | I | I | I | S | S | S | S | S | S | S | S | S |
|               | hfa3      | Cronobacter sakazakii             | S | S | I | S | R | S | S | S | I | S | S | R | I |
|               | hfa4      | Escherichia coli                  | I | I | I | I | S | S | S | S | I | R | S | R | R |
|               | hfa6      | Klebsiella sp.                    | R | I | I | S | R | S | S | S | I | S | S | I | S |
|               | hfa7      | Escherichia coli                  | R | I | R | R | R | S | R | S | S | R | S | R | S |
|               | hfa8      | Escherichia coli                  | I | I | I | S | S | S | S | R | S | S | S | S | S |
|               | hfa9      | Escherichia coli                  | S | I | I | S | S | S | S | S | I | R | S | S | S |
|               | hfa10     | Escherichia coli                  | S | S | S | S | S | S | S | S | S | S | S | S | S |
|               | hfa19     | Escherichia coli                  | I | I | I | S | S | S | S | S | R | S | S | I | S |
|               | hfa20     | Escherichia coli                  | I | I | I | S | S | S | S | S | R | S | S | S | S |
|               | hfa21     | Escherichia coli                  | S | S | S | S | S | S | S | S | S | S | S | S | S |
|               | hfa22     | Escherichia coli                  | S | S | I | S | S | S | S | S | I | S | S | S | S |
|               | hfa24     | Cronobacter sakazakii             | R | S | I | S | I | S | S | S | I | S | S | I | S |
|               | hfa36     | Klebsiella sp.                    | R | I | I | S | I | S | S | I | I | I | S | S | I |
|               | hfa38     | Klebsiella sp.                    | R | I | I | S | I | S | S | I | I | I | S | I | S |
ations were positively correlated with the prevalence of antibiotic-resistant *E. coli*. The concentration of fecal coliforms in the examined mud water of the river bottom in our study was approx. 18-fold higher than that of the water samples in the previous studies. Thus, the assessment of antibiotic-resistant *E. coli* in both surface water and bottom water is recommended when considering how to prevent the spread of antibiotic-resistant fecal coliforms in humans, animals, and the environment.

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