Occurrence of antibiotic resistance genes as emerging contaminants in watersheds of Tama River and Lake Kasumigaura in Japan

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Abstract. Antibiotic resistance is one of the important health issues. Since antibiotic resistance genes (ARGs) can transfer to pathogenic bacteria in the circulation of human-animal-ecosystems, they are regarded as emerging contaminants. However, little is known about the prevalence of ARGs in water environment. In our study, we investigated the occurrence of ARGs in two watersheds in Japan. Water samples were collected in Tama River in Tokyo in October 2018. Another sampling was conducted in Lake Kasumigaura in Ibaraki Prefecture in November 2018. Four ARGs (blaTEM encoding extended spectrum β-lactamase, sulI encoding the resistance to sulphonamide, tetA encoding the resistance to tetracycline, ere(A) encoding the resistance to macrolide-lincosamide-streptogramin) and one mobile genetic element (intI1 encoding integron) were determined by quantitative PCR. In Tama River, blaTEM and ere(A) were below quantification limit at the most upstream point before receiving wastewater effluent. However, they jumped up in the order of 10⁶–10⁷ copies/L just after receiving the effluent from wastewater treatment plants. The concentrations of the other ARGs and intI1 also increased due to the impact of wastewater effluent. In Lake Kasumigaura, the sampling point which was close to the discharge point of wastewater effluent and the mouth of Sakura River showed the highest copy numbers of intI1, sulI, and ere(A). All target genes were detected at the farthest point in the lake, suggesting that ARGs were persistent in water environment. In order to maintain sustainable water environment, monitoring of ARGs released from wastewater treatment plant is necessary.

1. Introduction
A large amount of antibiotics has been used for human as well as livestock in our society. The abuse of antibiotics results in the occurrence of antibiotic resistant bacteria (ARB) to which antibiotics are not effective anymore. Thus, antibiotic resistance is regarded as one of the global biggest health issues [1].

Wastewater treatment plant (WWTP) is one of the important sources disseminating ARB or antibiotic resistance genes (ARGs) to water environment. While WWTP can remove them, it can be a reservoir for horizontal gene transfer (HGT) [2, 3]. WWTP effluent can increase occurrence of ARB and concentrations of ARGs in receiving water bodies [4–6]. However, little information is available on anthropogenic antibiotic resistance in water environments.

The objective of this study is to evaluate the occurrence and fate of different ARGs in two watersheds (Tama River and Lake Kasumigaura) in Japan. Tama River collects wastewater from the western suburb of Tokyo. The previous study demonstrated that ratio of E. coli resistant to fluoroquinolone and
cephalosporins increased from the middle to downstream of Tama River [5]. In this study, occurrence of ARGs from upstream to downstream of Tama River were evaluated. Lake Kasumigaura is the second largest lake in Japan. It receives many impacts of watershed activities including WWTP effluent. The persistency of ARGs during longer water retention time of Lake Kasumigaura was discussed.

2. Materials and methods

2.1. Sampling
Sampling in the watershed of Tama River in Tokyo was conducted on 24 October 2018. The coverage of sewer system in the watershed reaches almost 99%. Along the river, 9 WWTPs directly discharge effluent to main flow of Tama River. As a result, river flow in the middle and downstream is mainly composed of wastewater effluent. Sampling sites were selected to evaluate the impacts of wastewater effluent as shown in Figure 1. As a reference sample, additional sample was collected from Kanda River in Bunkyo Ward in Tokyo on 19 October 2018. Almost all flow in Kanda River is derived from effluent from a WWTP.

Another sampling was performed in the watershed of Lake Kasumigaura on 7 November 2018. Surface water samples were collected at five points in the lake as shown in Figure 2. In addition, influent and effluent of a WWTP were collected on the same occasion. Moreover, river water sample was collected from Sakura River which was the major inflow to Lake Kasumigaura.

2.2. Water quality
Temperature and conductivity were measured with a HI98129 (Hanna) on site. *E. coli* and total coliform were determined by incubating samples on Chromocult Coliform Agar (Merck Millipore) at 37°C for 18–24 hours. Total cell counts were analyzed with a BD Accuri C6 (BD Biosciences) by staining samples with SYBR Green at 37°C for 10 min. After filtrating samples through a 0.2-µm mixed cellulose ester filter, ammonium nitrogen was measured by HACH Kit with a DR2800 Spectrophotometer (HACH).

2.3. ARGs analysis

2.3.1. DNA extraction. Water samples were filtered by 0.2-µm polycarbonate membranes. The membranes dissolved in Phenol-Chloroform-Isooamyl alcohol solution (Nippon Genes) were subjected to bead-beating treatment for DNA extraction by using FastDNA Kits for Soil (MP Biomedicals) following manufacturer's instructions.

2.3.2. Quantitative PCR. Quantitative PCR (qPCR) assays were performed with a LightCycler480 (Roche). LightCycler 480 SYBR Green I Master solution (Roche) was used for all assays. Table 1 shows the list of primers used in this study. The targets were 16S rRNA genes, 4 ARGs (blaTEM encoding...
extended spectrum $\beta$-lactamase, sulI encoding the resistance to sulphonamide, tetA encoding the resistance to tetracycline, ere(A) encoding the resistance to macrolide-lincosamide-streptogramin) and class one integron (intI1). Duplicate qPCR reactions were conducted for each sample. Additionally, positive and negative control experiments were also included. Thermal conditions consisted of initial denaturation at 95°C for 5 min, following by 45 amplification cycles of denaturation at 95°C for 15 sec, annealing at the specified temperature for each gene (55°C for 20 sec for 16S rRNA gene and intI1; 56°C for 20 sec for sul1; 60°C for 20 sec for tetA, 55°C for 30 sec for blaTEM and ereA), and extension at 72°C for 30 sec. To confirm the specific amplification, melting curve analysis was performed by rising temperature from 65°C to 95°C after qPCR. Standard curves were constructed by cloning the PCR products.

| Target genes | Primer   | Primer sequences                  | Reference |
|--------------|----------|-----------------------------------|-----------|
| 16S rRNA     | 515F     | 5'-CCTACGGGAGGCAGCAG-3'           | [7]       |
|              | 806R     | 5'-ATTACCGCGGCTGCTGGCA-3'         |           |
| tetA         | tetA-F   | 5'-GCTACATCCCTCTTGCCTTC-3'        | [8]       |
|              | tetA-R   | 5'-CATAGATCGCCGTGAAGAGG-3'        |           |
| sul1         | sul1-F   | 5'-CCCGGAACACATCGCTGCA-3'         | [9]       |
|              | sul1-R   | 5'-AAGTTCGCCGCAAGGCT-3'           |           |
| blaTEM       | blaTEM-F | 5'-TTTCCGTGTCGCCCTATTC-3'         | [10]      |
|              | blaTEM-R | 5'-CCTGACTCCGCTCGTGTAG-3'         |           |
| ere(A)       | ereA-F   | 5'-AACACCTGAACCCAAGGAGC-3'        | [11]      |
|              | ereA-R   | 5'-CTTCACATCCGGATTGCTGCA-3'       |           |
| intI1        | intI1-F  | 5'-CTTCCCACAGATGATC-3'            |           |
|              | intI1-R  | 5'-TCCACGCATCGTACGGC-3'           | [12]      |

3. Result and Discussion

3.1. Tama river samples

3.1.1. Water quality. Water quality in Tama River is summarized in Table 2. Due to entry of WWTP effluent, water temperature, electrical conductivity, ammonium, E. coli, total coliform, and total cell counts increased from TM1 to TM2. From TM2 to TM5, these parameters were almost stable except for ammonium. At the most downstream point (TM6), ammonium and total coliform increased significantly. Simultaneous increase of electric conductivity at TM6 suggests intrusion of contaminated coastal water from Tokyo Bay. In case of Kanda River where almost all flow is derived from wastewater effluent, water quality of Kanda River was similar to that at TM6. High electric conductivity in Kanda River also suggests intrusion of coastal water at the time of sampling.
Table 2. Water quality at six sampling sites in Tama River

| Sampling point | Water temp. (°C) | Electrical conductivity (µS/cm) | NH₄⁺-N (mg/L) | E. coli (CFU/mL) | Total coliform (CFU/mL) | Total cell counts (cells/mL) |
|----------------|------------------|--------------------------------|----------------|------------------|-----------------------|-----------------------------|
| TM1            | 19.1             | 139                            | 0.001          | 0                | 31                    | 1.3×10⁶                     |
| TM2            | 19.6             | 275                            | 0.009          | 7                | 157                   | 2.3×10⁶                     |
| TM3            | 20.4             | 314                            | 0.034          | 24               | 151                   | 4.3×10⁶                     |
| TM4            | 20.9             | 301                            | 0.044          | 10               | 128                   | 4.0×10⁶                     |
| TM5            | 20.1             | 294                            | 0.024          | 8                | 147                   | 3.6×10⁶                     |
| TM6            | 17.1             | 3999                           | 1.490          | 22               | 273                   | 5.7×10⁶                     |
| KD             | 20.5             | 5000                           | 0.794          | 52               | 247                   | 2.4×10⁶                     |

3.1.2. ARGs abundance. Figure 3 summaries the absolute abundances of target ARGs and intI1 as well as relative abundances of target genes to 16S rRNA genes at different sampling points in Tama River. The concentrations of four ARGs and intI1 were in the order of 10⁴–10⁵ copies/L or below quantification limit at TM1. However, all of them increased by 20–89 times at TM2 due to the impact of discharge from 3 WWTPs, indicating that WWTP is the important source of ARGs and intI1 into water environment. Additional discharge from 4 WWTPs increased the concentrations of tetA, sul1, ere(A) and intI1 from TM2 to TM3. It means that percentage of wastewater effluent in river flow increased during this segment. Simultaneously, ratios of these genes to 16S rRNA genes also increased from TM1 to TM3. The concentrations of blaTEM did not increase from TM2 to TM3 and the ratio of blaTEM to 16S rRNA genes decreased from TM2 to TM3. This trend of blaTEM suggests that effluents from WWTPs between TM1 and TM2 could be more enriched with ARB with blaTEM.

From TM3 to TM5 with discharge from 2 WWTPs, no significant increases were observed for all target genes probably because abundances of ARGs and intI1 in river water reached almost the similar level as those in wastewater effluent. The ratios of ARGs and intI1 to 16S rRNA genes decreased from TM3 to TM5 due to more increase of 16S rRNA genes than the other target genes. Abundances of all ARGs and intI1 increased at TM6 without entry of wastewater effluent. This change was probably caused by the intrusion of polluted coastal water in Tokyo Bay which was suggested by increase of electrical conductivity. In fact, a large WWTP discharges effluent to near the mouth of Tama River. The levels of ARGs and intI1 at TM6 were comparable to those in Kanda River where intrusion of coastal water was also suspected.

Among four ARGs, sul1 showed the highest levels (10⁷–10⁸ copies/L) after TM2, which were equivalent to sul1 concentrations in Ter River receiving wastewater effluent in Spain [5]. The ratios of sul1 to 16S rRNA genes from TM2 to TM6 were the highest (2.7×10²–1.3×10³), which was 10–100 times higher than the ratio in river water receiving wastewater effluent in Beijing in China [13]. Sulfonamide-resistant bacteria could be dominant in Tama River watershed.
Figure 3. Absolute and relative abundances of ARGs (a-d) and \textit{intI1} (e) in Tama River and Kanda River. Arrows indicate the entry point of wastewater effluent. Open bars and open circles indicate below the quantification limits.

3.2. Lake Kasumigaura samples

3.2.1 Water quality. Table 3 shows water quality of samples collected from Lake Kasumigaura, Sakura River, and influent and effluent of a WWTP. Wastewater treatment efficiently removed ammonium, \textit{E. coli} and total coliform, whose concentrations in the effluent were equivalent to those in lake water. Total cell counts in the effluent was nearly two times higher than those in lake water. The levels of \textit{E. coli} and total coliform in Sakura River were higher than those in lake water. Among the lake water sampling points, KS2 and KS5 showed lower ammonium concentrations. \textit{E. coli} and total coliform increased at KS5.
Table 3. Water quality parameters in Lake Kasumigaura

| Sampling point | Temp. (°C) | Electrical conductivity (µS/cm) | NH₄⁺-N (mg/L) | E. coli (CFU/mL) | Total coliform (CFU/mL) | Total cell counts (cells/mL) |
|----------------|------------|--------------------------------|----------------|-----------------|------------------------|----------------------------|
| INF            | 22.9       | -                              | 20.9           | 4.2×10⁴         | 2.8×10⁵                | 6.7×10⁷                    |
| EFF            | 22.9       | -                              | 0.022          | 0               | 16                     | 1.2×10⁷                    |
| RS             | 17.8       | -                              | 0.044          | 18              | 274                    | 4.9×10⁶                    |
| KS1            | -          | -                              | 0.056          | 2               | 33                     | 7.0×10⁶                    |
| KS2            | 17.5       | 241.31                         | 0.006          | 1               | 8                      | 8.3×10⁶                    |
| KS3            | -          | -                              | 0.029          | 2               | 15                     | 7.7×10⁶                    |
| KS4            | 17.0       | 279.87                         | 0.055          | 3               | 12                     | 6.3×10⁶                    |
| KS5            | 17.7       | 267.14                         | 0.003          | 11              | 71                     | 6.8×10⁶                    |

3.2.2 ARGs abundance. Figure 4 shows the abundances of four ARGs and intI1 in influent/effluent of the WWTP, Sakura River, and 5 sampling points in Lake Kasumigaura. Log removal values of ARGs and intI1 by the WWTP were 1.9–2.4 log10. The effluent contained higher abundances of four ARGs and intI1 than TM5 in the downstream of Tama River. Sakura River contained less abundances of tetA, sul1, ere(A) and intI1 than the effluent. Interestingly, blaTEM in Sakura River was below quantification limit, suggesting that primary source of blaTEM in Lake Kasumigaura was WWTP. In Lake Kasumigaura, water samples were collected from five points to investigate the persistency of ARGs and intI1. At KS1 where it was located close to the discharge point of the WWTP and the mouth of River Sakura, abundances of sul1, ere(A) and intI1 were higher than those at the other points. The highest abundance of tetA and blaTEM were observed at KS4 and KS2/KS5, respectively. In Lake Tai in China, the level of sul1 in lake water was higher than that in Lake Kasumigaura, while the levels of blaTEM in both lakes were almost comparable [14].

Figure 4. Abundances of ARGs and intI1 in the watershed of Lake Kasumigaura. Open bars indicate below quantification limit.
Figure 5 shows relative abundances of ARGs and intI1 to 16S rRNA genes. Wastewater treatment reduced the relative abundances of all target genes, indicating that bacteria possessing ARGs and intI1 were preferentially removed by the treatment. However, the ratios in effluent were still much higher than those in the lake water. The sulI/16S rRNA genes and intI1/16S rRNA genes in River Sakura were much higher than those of the lake water samples. It is possible that there were some sources in the watershed of River Sakura where sulI and intI1 were enriched. Among the lake water samples, tetA/16S rRNA genes and blaTEM/16S rRNA genes were relatively stable. On the other hand, sulI/16S rRNA genes, ere(A)/16S rRNA genes and intI1/16S rRNA genes decreased from KS1 to centre of the lake and then increased to KS5. The ratios of sulI/16S rRNA genes in urban lakes in China, 21 Swiss lakes, and Lake Geneva were \(10^{-3} - 10^{-2}\) [15], which were higher than those in KS2–KS5 in Lake Kasumigaura.

![Figure 5. Relative abundances of ARGs and intI1 to 16S rRNA genes in the watershed of Lake Kasumigaura.](image)

The levels of ARGs and intI1 in the lake are compared as relative to those at KS1 in Figure 6. From KS1 to KS3, the abundances of tetA, sulI, ere(A) and intI1 decreased continuously, while blaTEM fluctuated. From KS3 to KS5, tetA and blaTEM increased and sulI and intI1 remained lower levels. On the other hand, ere(A) jumped from KS4 to KS5. Further study is necessary to identify the factors causing these changes in the lake. Additional point and non-point sources as well as effects of dilution should be confirmed. It is also possible that fates of ARGs and intI1 could be dependent on the fates of their hosts. Some host can be easily removed by settlement or predation. However, it should be emphasized that all ARGs and intI1 were still detected at the farthest point (KS5) in the lake. In addition, the abundances of tetA, blaTEM, ere(A) were even higher than those in Sakura River flowing into Lake Kasumigaura. The results demonstrated that ARGs were persistent in water environment.
4. Conclusion

Our study aimed to investigate the occurrence of ARGs in Tama River and Lake Kasumigaura in Japan. The results demonstrated that wastewater effluents had a significant impact on ARGs in receiving water environment and ARGs released to water environment were persistent.

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Figure 6. Abundances of ARGs and intI1 relative those at KS1 in Lake Kasumigaura