Prevalence of Plasmid-mediated Colistin Resistance Gene mcr-1 in Domestic Wastewater

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Abstract. Colistin is one of the last-resort antibiotics. However, the transmissible plasmid-mediated colistin resistance gene encoded by mcr-1 was first detected in China in 2015. The dissemination of mcr-1 is of great concern. This study investigates the prevalence of mcr-1 in urban sewage in Vietnam and Japan because domestic sewage reflects the health status of urban citizens. Water samples were collected from To Lich River and Nhue River in Hanoi, Vietnam, in September 2019. These rivers are severely polluted by untreated domestic wastewater; thus, these samples can be regarded as urban sewage. We also collected wastewater samples from three different municipal wastewater treatment plants (A, B, and C) in Japan in October 2019. DNA was extracted from the samples, and mcr-1 abundance was analyzed via quantitative PCR. Quantitative PCR demonstrated that mcr-1 was detected in all samples, indicating that healthy citizens carry some bacteria harboring mcr-1. The abundance of mcr-1 in the influent of wastewater treatment plant A (1.9 × 10^6 gene copies/L) was lower than that in the other samples in Japan and Vietnam (8.1 × 10^6–1.3 × 10^7 gene copies/L). The log reduction values of mcr-1 in wastewater treatment ranged 1.5–4.2 log_{10}, resulting in abundance of 7.0 × 10^2–7.3 × 10^4 copies/L in the final effluents. The comparable levels of mcr-1 in urban sewage in Vietnam and Japan indicate the global spread of transmissible colistin resistance. Wastewater is considered as an important monitoring target for mcr-1.

1. Introduction
The discovery and invention of antibiotics led to a new era of medical development in the treatment of bacterial infections. Antibiotics are used for medical purposes as well as growth promotion in the livestock industry. The overuse and abuse of antibiotics have resulted in the emergence of antibiotic-resistant bacteria carrying antibiotic resistance genes. The spread of untreatable drug-resistant diseases poses a serious threat to achieving sustainable development goals even though this is not directly mentioned among the 17 goals. Unless actions are taken, by 2050, the number of worldwide deaths related to antibiotic resistance is projected to reach 10 million per year, which is equivalent to approximately one death every 3 s [1].

Colistin is one of the last-resort lines of antibacterial drugs for treating multidrug-resistant bacteria, and it is listed as a critically important antimicrobial agent [2]. Colistin, which is a polycationic peptide, attaches to the outer membrane of gram-negative bacteria cells and then binds to lipopolysaccharide to...
displace calcium and magnesium cations, resulting in the disintegration of membrane structures [3]. In 2015, in China, a transmissible colistin resistance gene (mcr-1) was discovered, and the possible transfer of colistin resistance to other bacterial species including pathogens was suggested [4]. In addition to mcr-1, several variants of mcr have been reported [5]. Moreover, the global import and export of foods and the tourism industry can contribute to the global spread of mcr family including mcr-1 [5–7].

In Vietnam, it was reported that healthy people in rural communities carried colistin-resistant Escherichia coli carrying mcr-1 [8], suggesting that this gene is prevalent in human life. To evaluate the prevalence of mcr-1, domestic wastewater is useful because it reflects the health status of urban citizens. The presence of mcr-1, particularly E. coli isolates carrying mcr-1, in wastewater has been studied in several countries including China, Spain, and Japan [7–10]. In Europe, a comprehensive survey of antibiotic resistance genes in wastewater effluents from 16 wastewater treatment plants in urban areas in 10 countries detected mcr-1 in effluents in the range of $10^4$–$10^5$ copies/L [11]. However, the presence of mcr-1 in urban wastewater in Vietnam has not been examined. Currently, 90% of domestic wastewater is discharged without treatment in Vietnam [12], which is suspected as a major factor of mcr-1 dissemination in aquatic environments. In this study, we determined the level of mcr-1 contamination in domestic wastewater in Vietnam. Moreover, we evaluated the fate of mcr-1 in wastewater treatment plants in Japan.

2. Materials and methods

2.1. Sampling

Water sampling were conducted at To Lich River (TL) and Nhue River (NH) in Hanoi, Vietnam, in September 2019. These urban drainages receive domestic wastewater directly from urban districts. Water samples collected in sterilized bottles were kept on ice and transported to the laboratory.

In Japan, water sampling was performed at three different municipal wastewater treatment plants (A, B, and C) in the Kanto Region in October 2019. The capacities of plants A, B, and C were approximately 1,000,000, 140,000, and 20,000 m$^3$/day, respectively. The sampling points are shown in Figure 1. From plant A, raw sewage (A1), effluent from primary settlement basin (A2), and final effluent after chlorine disinfection (A3) were collected. From plants B and C, raw sewage (B1, C1), effluent from the primary settlement basins (B2, C2), effluent from aeration tanks (B3, C3), effluent from the secondary settlement basins (B4, C4), and final effluent after chlorine disinfection (B5, C5) were collected. Water samples contained in sterilized bottles were kept on ice and transported to the laboratory.

2.2. Enumeration of total cell counts, total coliform, and E. coli

For samples collected in Japan, total cell counts (TCCs) were measured via flow cytometry (BD Accuri C6, BD Biosciences, USA) after staining samples with SYBR™ Green I Nucleic Acid Gel Stain (Thermo Fisher Scientific, USA) at 37°C for 10 min. For samples collected in both Vietnam and Japan, total coliform and E. coli counts were determined via cultivation. One milliliter of diluted samples was incubated on Chromocult Coliform Agar (Merck Millipore, USA) at 37°C for 24 h.

2.3. Cultivation of colistin-resistant bacteria

Colistin-resistant gram-negative bacteria were cultivated on CHROMagar™ COL-APSE with supplement (CHROMagar, France) at 37°C for 18–24 h. The detected colonies were classified according
Table 1. Classification of colonies cultivated on CHROMagar™ COL-APSE[13].

| Color of colonies                                      | Classification                           |
|--------------------------------------------------------|------------------------------------------|
| Dark pink to reddish                                   | Colistin-resistant (COL-R) *Escherichia coli* |
| Metallic blue                                          | COL-R coliform                          |
| Translucent, +/- natural pigmentation cream to green   | COL-R *Pseudomonas*                      |
| Cream                                                  | COL-R *Acinetobacter*                    |
| Colorless, natural pigmentation                        | COL-R other gram-negative bacteria        |

Figure 1. Sampling points at wastewater treatment plants in Japan.

to the manufacturer’s instructions [13] (Table 1). To estimate the percentages of colistin-resistant bacteria, the same samples were cultivated on CHROMagar™ COL-APSE without supplement.

2.4. Analysis of plasmid-mediated colistin resistance gene mcr-1

2.4.1. DNA extraction. A certain volume (50–100 mL) of each sample was filtered through a 0.22-μm polycarbonate membrane (Merck Millipore). The membranes were put in a Lysing Matrix tube (MP Biomedicals, USA) and then dissolved in Phenol:Chloroform:Isoamylalcohol (25:24:1) solution. DNA extraction was conducted by using a FastDNA Spin Kit for Soil (MP Biomedicals). The concentration of extracted DNA was determined using a microspectrophotometer (NanoDrop 2000, Thermo Fisher Scientific).

2.4.2. Quantitative PCR. The SYBR Green method was selected for quantitative PCR to determine mcr-1 abundance. The primers mcr-1_F (5’-CATCGGGGAGGAATCTCGG-3’) and mcr-1_R (5’-AAATCAACACAGGCTTTAGCAG-3’), which are specific for mcr-1, were used [14]. Quantitative PCR was performed using a LightCycler480 (Roche, Switzerland) and LightCycler® 480 SYBR Green I Master (Roche). The cycle conditions consisted of preincubation for initial denaturation at 95°C for 5 min, followed by 45 cycles of 95°C for 10 s (denaturation), 56°C for 20 s (annealing), and 72°C for 20 s (extension). Melting curve analysis was conducted after PCR by continuously raising the temperature from 65°C to 95°C. Duplicate reactions were conducted for all samples. Standard curves were prepared by serial dilution of the plasmid containing artificially synthesized target gene fragments (5 × 10¹–5 × 10⁷ gene copies/μL).

3. Result and Discussion

3.1. Total cell counts, total coliform, and *E. coli* in wastewater

Figure 2 shows the results of total cell counts (TCCs), total coliform counts, and *E. coli* counts. TCCs were measured for samples collected from wastewater treatment plants in Japan. Influent of plant A contained the highest TCCs (4.2 × 10⁷ cells/mL). The TCCs in the influents of plant B and C were lower
than those in the effluents from the subsequent primary settlements. In particular, the TCCs in plant B were extremely low compared with the total coliform and *E. coli* counts. These inconsistent trends were likely attributable to fluctuation of the water quality matrix in influents containing high levels of particulates. The TCCs in the final effluent after chlorine disinfection at plant A was higher (1.4 × 10^7 cells/mL) than those at plants B (6.8 × 10^5 cells/mL) and C (2.2 × 10^6 cells/mL). Although TCCs differed among the plants, the abundances of total coliform and *E. coli* in influent samples from the three plants were approximately 10^8 and 10^6 CFU/mL, respectively. The total coliform counts in all final effluents complied with the standard (3000 CFU/mL). No *E. coli* was detected in the final effluents at all three plants. For samples collected in Vietnam, the total coliform and *E. coli* counts in TL (total coliform, 4.5 × 10^5 CFU/mL; *E. coli*, 1.0 × 10^5 CFU/mL) were higher than those in NH (total coliform, 3.9 × 10^4 CFU/mL; *E. coli*, 5.2 × 10^3 CFU/mL). The high levels of total coliform and *E. coli* in TL and NH indicate that these rivers in Hanoi receive untreated wastewater.

### 3.2. Detection of colistin-resistant bacteria

To evaluate the ratios of culturable colistin-resistant bacteria, A1, B2, and C2 samples were incubated on CHROMagar™ COL-APSE with and without antibiotic supplement. The detected colonies were classified into four bacterial groups based on their colors. Figure 3 shows the abundances of total and colistin-resistant bacterial groups. The abundance of *E. coli* detected on CHROMagar™ COL-APSE without supplement was almost equivalent to the result for A1 on Chromocult Coliform Agar. Conversely, 4–8-fold higher *E. coli* counts were detected on CHROMagar™ COL-APSE without supplement in B2 and C2. The ratios of colistin-resistant *E. coli*, coliforms, and *Pseudomonas* ranged 39%–71% (average, 57%), 10%–40% (average, 21%), and 13%–100% (average, 56%), respectively. Meanwhile, no resistant *Acinetobacter* species were detected in these wastewater samples. Figure 3 shows the relative compositions of resistant bacteria. Colistin-resistant *E. coli* comprised 69%, 81%, and 97% of all resistant bacteria in A1, B2, and C2 samples, respectively. Although these data cannot distinguish intrinsic colistin resistance from plasmid-mediated colistin resistance including that induced by *mcr-1*, it has been reported that most colistin-resistant *E. coli* isolates carry *mcr-1* [15, 16]. It is likely that *E. coli* could be the potential host of *mcr-1* in these wastewater samples.

### 3.3. Prevalence of *mcr-1* in wastewater

Figure 4 shows the numbers of *mcr-1* copies in samples collected from Vietnam and Japan. Urban rivers receiving domestic wastewater in Hanoi (TL and NH) and sewage influents in Japan contained *mcr-1* in the order of 10^6–10^7 copies/L. The results indicate the wide spread of *mcr-1* in urban areas in Vietnam and Japan. The levels of *mcr-1* in raw wastewater in Spain (2.4 × 10^6 copies/L in 2011 and 1.6 × 10^6 copies/L in 2016) were equivalent to or higher than the results of this study [10]. The influent in China contained substantially higher *mcr-1* copy numbers (3.3 × 10^6 copies/L). The levels of *mcr-1* may reflect the differences in the consumption of colistin and the prevalence and dissemination of *mcr-1* in urban environments in each country. Final effluents after chlorine disinfection contained between 7.0 × 10^2 (B5) and 7.3 × 10^3 (A3) copies/L *mcr-1*. In other countries, *mcr-1* concentrations in wastewater effluents ranged 1.2 × 10^5–2.9 × 10^6 copies/L in Spain [10], 1.1 × 10^5 copies/L (median) in European countries [11], and 2.5 × 10^8 copies/L in China [9]. The concentrations of *mcr-1* in final effluents from plants A, B, and C were relatively lower than previously reported values. It is expected that the prevalence of wastewater treatment in Vietnam significantly mitigates *mcr-1* pollution in untreated wastewater.
Figure 2. Total cell counts, total coliform counts, and \textit{E. coli} counts in wastewater samples.

Figure 3. Abundances of total and colistin-resistant \textit{Escherichia coli}, coliforms, \textit{Pseudomonas}, and \textit{Acinetobacter} in the influent of plant A (A1), effluent from the primary settlement basin of plant B (B2), and plant C (C2). The numbers indicate the percentages of resistant bacteria.

The reduction of \textit{mcr-1} copy numbers during treatment process at plants A, B, and C was further discussed as shown in Figure 5. Since the concentrations of \textit{mcr-1} in influents were slightly lower than those in effluents from the primary settlement basins at plants A and C, the reduction of \textit{mcr-1} copy numbers was calculated according to the \textit{mcr-1} copy numbers in effluents from the primary settlement basin. The log reduction values (LRVs) of \textit{mcr-1} in the total treatment were 1.5, 4.2, and 3.7 \(\log_{10}\) at plants A, B, and C, respectively. The LRVs in wastewater treatment in Spain ranged 1.3–1.7 \(\log_{10}\) [10], whereas that in China was 1.1 \(\log_{10}\) [9]. Critical factors governing \textit{mcr-1} removal should be revealed in future research. At plants B and C, treatment of the aeration tank contributed to the largest reduction of \textit{mcr-1} counts (plant B, 3.4 \(\log_{10}\); plant C, 2.7 \(\log_{10}\)). The LRVs of secondary settlements and chlorination were lower at both plants. The LRVs of TCCs, total coliform, and \textit{E. coli} are also shown in Figure 5. Compared with the LRV of TCCs, the LRV of \textit{mcr-1} was much larger. This indicates that hosts of \textit{mcr-1} can be more efficiently removed than other bacteria. Total coliform and \textit{E. coli} counts based on cultivation indicated that treatment in the aeration tank as well as chlorination was effective for their removal. The count of \textit{E. coli}, which was regarded as the major host of \textit{mcr-1} in wastewater samples, was below the detection limit in the final effluents after chlorination (Figure 2). Contrarily, certain amounts of \textit{mcr-1} (7.0 \(\times\) 10^2–7.3 \(\times\) 10^4 copies/L) were discharged to aquatic environments even after chlorination. This suggests that \textit{mcr-1} remains in dead cells even if the host bacteria are sterilized by chlorination. Plasmids carrying \textit{mcr-1} in dead bacteria could be released to water environments and potentially disseminate through horizontal gene transfer.
4. Conclusion
The comparable levels of mcr-1 in urban sewage in Vietnam and Japan showed the prevalence of the transmissible colistin resistance gene. Domestic wastewater is thus considered an important monitoring target for mcr-1 in urban cities. Whereas wastewater treatment could reduce mcr-1 copy numbers, final effluents after chlorination still contained mcr-1. In addition to conventional indices of total coliform and E. coli, it is necessary to control mcr-1 released into aquatic environments from wastewater treatment plants.

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