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Release of Antibiotic-Resistance Genes from Hospitals and a Wastewater Treatment Plant in the Kathmandu Valley, Nepal

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Abstract: Hospitals and wastewater treatment plants (WWTPs) are high-risk point sources of antibiotic-resistance genes (ARGs) and antibiotic-resistant bacteria. This study investigates the occurrence of clinically relevant ARGs (*sul1*, *tet*(B), $bla_{\text{CTX-M}}$, $bla_{\text{NDM-1}}$, *qnrS*) and a class one integron (*intI1*) gene in urban rivers, hospitals, and municipal wastewater in the Kathmandu Valley, Nepal. Twenty-five water samples were collected from three rivers, six hospitals, and a wastewater treatment plant to determine the concentrations of ARGs and *intI1* using quantitative polymerase chain reactions. From the results, all tested ARGs were detected in the river water; also, concentrations of ARGs in WWTP and hospital effluents varied from 6.2 to 12.5 $\log_{10}$ copies/L, highlighting the role of a WWTP and hospitals in the dissemination of ARGs. Except for $bla_{\text{NDM-1}}$, significant positive correlations were found between *intI1* and other individual ARGs (r = 0.71–0.96, $p < 0.05$), indicating the probable implications of *intI1* in the transfer of ARGs. Furthermore, this study supports the statement that the $bla_{\text{NDM-1}}$ gene is most likely to be spread in the environment through untreated hospital wastewater. Due to the interaction of surface water and groundwater, future research should focus on ARGs and factors associated with the increase/decrease in their concentration levels in drinking water sources of the Kathmandu Valley.

Keywords: antibiotic-resistance gene; integron; hospital wastewater; sewage; wastewater treatment

Citation: Thakali, O.; Malla, B.; Tandukar, S.; Sthapit, N.; Raya, S.; Furukawa, T.; Sei, K.; Sherchand, J.B.; Haramoto, E. Release of Antibiotic-Resistance Genes from Hospitals and a Wastewater Treatment Plant in the Kathmandu Valley, Nepal. *Water* **2021**, *13*, 2733. https://doi.org/10.3390/w13192733

Academic Editor: Laura Bulgariu

Received: 13 August 2021
Accepted: 27 September 2021
Published: 2 October 2021

1. Introduction

Antibiotic resistance is one of the biggest threats to global health today and is primarily driven by the overuse of antibiotics. Between 2000 and 2015, antibiotic consumption increased by 65% and is further expected to increase by 200% in 2030 unless significant policy changes are made. The increase in antibiotic consumption was majorly driven by increased usage in low and middle-income countries [1]. Unused antibiotics are often disposed of in drains, and up to 90% of the antibiotics consumed are excreted in the original form or as metabolites [2,3]. The biodegradability test of antibiotics conducted in wastewater has also shown that antibiotics were not degraded in wastewater, resulting in the selection pressure of bacteria [4]. Extensive use of antibiotics for treatment results in the emission of large quantities of antibiotic residues and antibiotic-resistant bacteria (ARB) from hospitals [5]. Similarly, the accumulation of wastewater rich in metals, antibiotic residues, bacteria, and nutrients at wastewater treatment plants (WWTPs) provides conditions to facilitate the proliferation of antibiotic-resistance genes (ARGs) [6]. As such, inadequately treated hospital and municipal wastewater can lead to wider dissemination of ARB and ARGs, thereby posing potential threats to public health.

In developing countries, such as Nepal, the burden of antibiotic resistance is growing due to poor infection control, irrational use of antibiotics, and lack of sufficient surveillance systems for tracking antibiotic use and resistant patterns of bacteria [7,8]. Issues of water, sanitation, hygiene, and wastewater management have not been addressed appropriately. For example, only one functioning WWTP exists, and the majority of the hospitals lack an onsite wastewater treatment facility [9]. Our previous study showed that downstream and midstream sites of the Bagmati River, which flows through the Kathmandu Valley, has a higher abundance of ARGs compared to an upstream site [10]. Downstream and midstream sites are settlement areas where hospitals and WWTPs are present. There are around 115 hospitals in the Kathmandu Valley [11], and the WWTP lacks a chlorine disinfection phase, a critical step [12] in the reduction of ARGs. The WWTP in Kathmandu consists of bar screen, primary sedimentation, oxidation ditch, and secondary sedimentation (Figure S1). Another study conducted in Spain to study the impact of effluents from a hospital and WWTP has also shown that effluents, despite treatment, contributed to the spread of ARGs in receiving river sources [13]. Overall, these statements point out that existing wastewater treatment technologies do not completely remove ARGs and effluents from hospitals and WWTP are a potential source of ARB and ARGs in the Kathmandu Valley. Since the burden of antibiotic resistance and the circulating ARGs vary from one geographical region to another, regional surveillance is necessary to plan management strategies. However, information about the abundance of ARGs in effluents of such point sources and the changes in concentrations of ARGs post-treatment in WWTPs and in onsite treatment plants of hospitals remains unknown.

To address this scenario, ARGs, total bacteria, fecal indicator bacteria, and a mobile genetic element (MGE) were quantified from river water bodies, hospitals, and WWTP effluents. The ARGs/MGEs of focus in this study and the classes of antibiotics it encodes resistance for are given in Table 1. The reason for selecting these specific marker genes includes: (1) The efflux gene, *tet*(B), which confers resistance to both tetracycline and minocycline and exhibits the widest host range among tetracycline resistance genes [14]. (2) *qnrS* gene is a type of plasmid-mediated quinolone resistance (PMQR) gene. The aquatic environment has been proposed as the original source of PMQR genes, while *qnrS* is the most common type in the environment [15]. (3) CTX-M-β-lactamase ($bla_{\text{CTX-M}}$) is the most common variant of extended-spectrum-β-lactamase (ESBL) among ESBL-producing *Escherichia coli* in clinical settings of Nepal [16,17]. (4) New Delhi metallo-β-lactamase-1 ($bla_{\text{NDM-1}}$) originated in India [18], a neighboring country of Nepal, and has been reported as the most common gene among carbapenem-resistant *E. coli* at a university hospital in Kathmandu, Nepal [19]. (5) *sul1* encodes for resistance to sulfonamides and has also been proposed as a marker for ARGs in the environment. (6) Integrons are the best-known MGEs responsible for the spread of antibiotic resistance [20]. Class one integrase genes (*intI1*) are found in greater abundance than other classes of integrons in drinking water, thereby highlighting a greater role of *intI1* in ARG transfer in the environment [21].

Table 1. Marker genes and the class of drugs to which they encode resistance.

Gene	Class of Antibiotics/Importance
16SrRNA	Proxy for total bacterial load
tet(B)	Tetracycline [b]
qnrS	Fluoroquinolones [a]
$bla_{\text{CTX-M}}$	Cephalosporins [a,b], Penicillin, Monobactams [b]
$bla_{\text{NDM-1}}$	Carbapenems [b], Cephalosporins [a,b], Penicillin
sul1	Sulfonamides
intI1	Proxy for the spread of antibiotic-resistance genes [22]

[a] Includes watch group antibiotics; [b] includes reserve group antibiotics ("last resort options") [23].

2. Materials and Methods

2.1. Collection of Water Samples

In this study, 25 grab water samples were collected from three rivers (n = 3), six hospitals (n = 10), and a WWTP (n = 12) at different periods from August 2016 to August 2019. River water samples were collected from the surface water layer in midstream sites of the Manohara, Bagmati, and Bishnumati rivers. Similarly, both private and government hospitals with bed capacities ranging from 25 to 700 that provided consent for sampling were chosen for the study. Two hospitals were sampled twice, one of which had a treatment plant during the second round of sampling. Including that hospital, two hospitals in total had a functioning onsite treatment system during the sampling period. Therefore, wastewater samples before and after treatment were collected from these sites, making 10 hospital wastewater samples. Six samples, each of influent and treated effluents, were collected at the WWTP. All samples were collected in autoclaved 1-liter bottles, transported to the laboratory on ice packs, and processed within 4 h of collection.

2.2. Quantification of Total Coliforms (TC) and Escherichia coli in Water Samples

TC and *E. coli* in the water samples were quantified using the Colilert reagent (IDEXX Laboratories, Westbrook, CA, USA) according to the manufacturer's protocols. One pack of Colilert powder was added to 100 mL of the water sample in an autoclaved glass bottle and shaken gently until it dissolved. Dilutions of up to 10^{-6} were prepared and used in this study. Then, the mixture was poured into a Quanti-Tray/2000 (IDEXX Laboratories) and sealed in a Quanti-Tray sealer (IDEXX Laboratories). The sealed tray was placed in an incubator (LTI-400E; EYELA, Tokyo, Japan) at 37 °C for 24 h. Yellow-colored large and small wells were counted as TC, whereas fluorescent-blue large and small wells under UV light were counted for *E. coli*. The most probable number (MPN) was generated using an MPN generating software 1.4.4 (IDEXX Laboratories), and the MPN per 100 mL of wastewater was calculated considering the dilution ratio.

2.3. Extraction of Bacterial DNA

Initially, 10 mL of river water and wastewater were filtered through a disposable filter unit (diameter, 47 mm; pore size, 0.22 µm; Nalgene, Tokyo, Japan). Next, DNA was extracted using a CicaGeneus DNA Extraction Reagent (Kanto Chemical, Tokyo, Japan) from the Manohara River water sample in August 2016 and the WWTP samples in September 2017, as well as April, June, and August 2018. The filter membrane was transferred into a 50-mL plastic tube, and 5 mL of Tris-EDTA buffer (pH 7.4) was added. Following repeated mixing by vortexing and shaking at 50 °C with a speed of 300 rpm, 160 µL of the resuspended sample was used to extract 350 µL DNA as described previously [24] and stored at −25 °C.

Hospital wastewater and the two other river water samples were collected and processed in February and August 2019. For DNA extraction from hospital wastewater and river water samples, the filter membrane was vortexed after adding a 10-mL elution buffer containing 0.2 g/L $Na_4P_2O_7$ $10H_2O$, 0.3 g/L $C_{10}H_{13}N_2O_8Na_3$ $3H_2O$, and 0.1 mL/L Tween brand polysorbate 80. Then, the supernatant was transferred into a new 50-mL tube. The same procedure was repeated with a 5-mL elution buffer that resulted in a final volume of 15 mL. Then, the obtained sample was centrifuged at 2000 × *g* for 10 min at 4 °C, after which the supernatant was discarded. Subsequently, two hundred microliters of phosphate-buffered saline was added to the resulting pellet and vortexed to obtain bacterial concentrate. This step was repeated until 1 mL of bacterial DNA concentrate was obtained. One hundred microliters of bacterial DNA was then extracted from 250 µL of bacterial concentrate using the DNeasy PowerSoil kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.4. Quantitative PCR (qPCR) Assays

In all the qPCR runs, the samples were run in duplicate on a Thermal Cycler Dice Real-Time System III (Takara Bio, Kusatsu, Japan). The specific primers, probes, and amplicon sizes are listed in Table 2. Except for *qnrS* and *tet*(B), which were amplified using the SYBR Green approach, all marker genes were amplified using a probe-based assay. For SYBR Green approach amplification, a 2.5 µL DNA sample was added to a mixture containing 12.5 µL TB Green Premix Ex Taq II (Takara Bio), 0.1 µL each of forward and reverse primers (100 pmol/µL), and 9.8 µL of PCR-grade water. Thermal conditions included an initial denaturation at 95 °C for 30 s, followed by 45 cycles at 95 °C for 5 s, and at 60 °C for 30 s. Then, the melting curve analysis was performed immediately at 95 °C for 15 s, at 60 °C for 30 s, and at 95 °C for 15 s. For all TaqMan probe-based amplifications, the PCR mixture contained 2.5 µL DNA, 12.5 µL Probe qPCR mix with UNG (Takara Bio), 0.1 µL forward and reverse primers (100 pmol/µL), 0.05 µL probe (100 pmol/µL), and 9.75 µL PCR-grade water. Furthermore, the thermal conditions included incubation at 25 °C for 10 min, followed by 95 °C for 30 s, 45 cycles of 95 °C for 5 s, and 60 °C for 30 s, except for the 16S rRNA amplification that was run for 30 cycles. A standard curve was generated from the threshold cycle (C_t) values of 10-fold serial dilutions of a known concentration of artificially synthesized plasmid DNA, and the negative control comprised PCR-grade water. Cutoff values for the threshold cycle were 25 for 16S rRNA and 40 for the remaining marker genes. The PCR run efficiency was 107 ± 7.8%, while the correlation coefficient (*r*) of standard curves ranged from 0.989 to 0.999.

Table 2. Nucleotide sequences of primers and probes used in the study.

Assay	Function	Sequence (5′–3′)	Size (bp)	Reference
16S rRNA	Forward primer	CGGTGAATACGTTCYCGG	142	[25]
	Reverse primer	GGWTACCTTGTTACGACTT		
	TaqMan probe	FAM-CTTGTACAC/ZEN/ACCGCCCGTC-IBFQ		
tet(B)	Forward primer	CGAAGTAGGGGTTGAGACGC	192	[26]
	Reverse primer	AGACCAAGACCCGCTAATGAA		
qnrS	Forward primer	GATATCGAAGGCTGCCACTT	115	[27]
	Reverse primer	CACGGAACTCTATACCGTAGCA		
$bla_{\text{CTX-M}}$	Forward primer	ACCAACGATATCGCGGTGAT	101	[28]
	Reverse primer	ACATCGCGACGGCTTTCT		
	TaqMan probe	FAM-TCGTGCGCCGCTG-BHQ1		
$bla_{\text{NDM-1}}$	Forward primer	ATTAGCCGCTGCATTGAT	154	[29]
	Reverse primer	CATGTCGAGATAGGAAGTG		
	TaqMan probe	FAM-CTGCCAGACATTCGGTGC-TAMRA		
sul1	Forward primer	CCGTTGGCCTTCCTGTAAAG	67	[30]
	Reverse primer	TTGCCGATCGCGTGAAGT		
	TaqMan probe	FAM-CAGCGAGCCTTGCGGCGG-BHQ1		
intI1	Forward primer	GCCTTGATGTTACCCGAGAG	196	[31]
	Reverse primer	GATCGGTCGAATGCGTGT		
	TaqMan probe	FAM-ATTCCTGGCCGTGGTTCTGGGTTTT- BHQ1		

Y denotes C or T; FAM, 6-carboxyfluorescein; ZEN, ZEN internal quencher; IBFQ, Iowa Black fluorescent quencher; BHQ1, black hole quencher 1; TAMRA, 5-carboxytetramethylrhodamine.

2.5. Statistical Analysis

The concentrations of all marker genes were $\log_{10}$ transformed. Paired *t*-tests and Pearson's rank correlation test were carried out using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA). The threshold for significance was $p < 0.05$. The log reduction value (LRV) was calculated as the $\log_{10}$ difference in ARG concentration in the influent and effluent samples. Heatmap was prepared with R statistical software, version 3.6.1, by using RStudio (R Core Team, Vienna, Austria).

3. Results and Discussion

3.1. ARGs in River Water

All of the marker genes tested in this study were detected in all river water samples. As illustrated in Figure 1, the concentrations of 16S rRNA, *intI1*, and ARGs ranged from 10.6–11, 9.9–10.9, and 7.3–10.9 $\log_{10}$ copies/L, respectively. High concentrations of 16S rRNA, *intI1*, and other ARGs have been reported in urban river sites of the Kathmandu Valley throughout the year, showing rivers in the valley are polluted with ARGs and ARB [10]. Therefore, only three river water samples were assessed to further verify those findings and our results show no improvement in ARG pollution control in the past three years. All the river water samples were collected from dense settlements in different periods and processed with different kits, making comparisons between the three rivers difficult. However, the relative abundance (a ratio of the concentration of ARGs or *intI1* to that of 16S rRNA), as shown in Figure 2, was similar in all urban rivers. *sul1* showed the highest relative abundance ($p < 0.05$), whereas the lowest was observed for $bla_{\text{NDM-1}}$. *sul1* encode resistance to sulfonamides, which are among the oldest antibiotics introduced and are recognized as poor or non-biodegradable compounds in the environment [32]. To date, no studies on antibiotic residues in river water and wastewater have been carried out in Nepal. However, the probable presence of sulfonamides in the environment to continuously exert selection pressure can be one of the reasons why *sul1* occurred at a significantly higher concentration than other ARGs ($p < 0.05$).

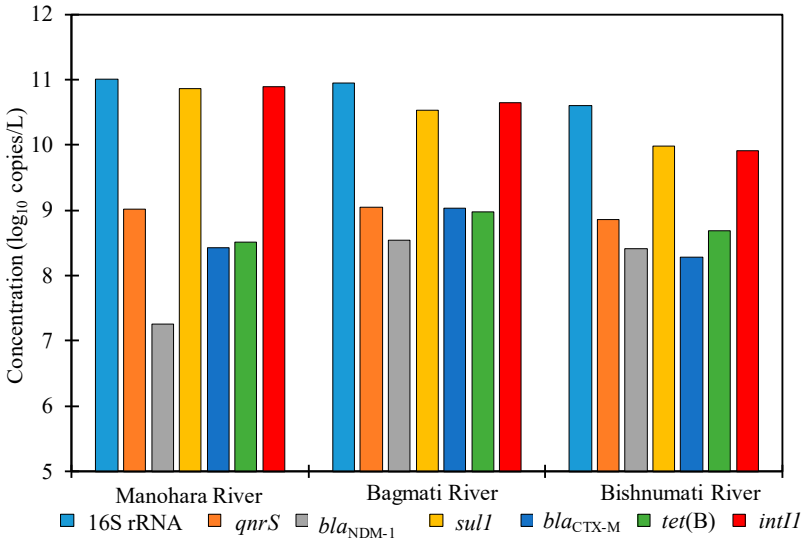

Figure 1. Absolute abundance of ARGs in river water.

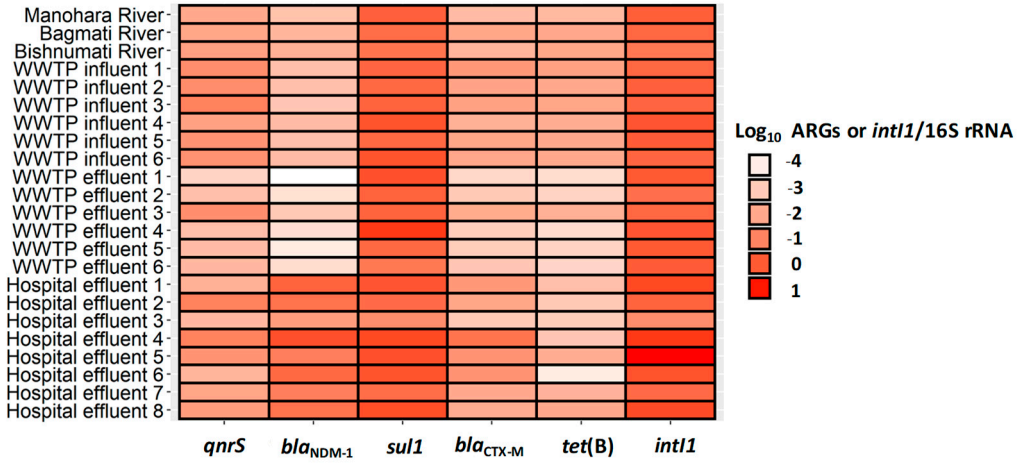

Figure 2. Heat map showing the relative abundance of ARGs and *intI1*.

Urban rivers in the Kathmandu Valley are polluted and not used for drinking. As a cheap source of nutrients, polluted river water is extensively used for irrigation [33]. Since bacterial DNA was extracted by filtering through a 0.22-µm pore size membrane, the detected ARGs were likely to be harbored by potentially pathogenic bacteria, and characterization of the bacterial diversity in the river water of the Kathmandu Valley has shown the presence of 111 potentially pathogenic bacteria [34]. Irrigation water is a pathway for contamination of fresh produce, and the consumption of such produce can lead to outbreaks of antibiotic-resistant bacterial pathogens in the Kathmandu Valley. In China, a study applying high-throughput qPCR to screen for ARGs/MGEs in surface water and groundwater bodies reported the sharing of 69.4% of the total detected ARGs/MGEs between river water and groundwater bodies. This finding provides evidence of surface water infiltration into groundwater [35]. However, currently, no studies that investigated the pollution profile of ARGs in groundwater in the Kathmandu Valley exist. Moreover, due to the acute shortage of drinking water, groundwater is extensively used for household activities. ARB and ARGs in the drinking water may colonize in the gut microbiome or even cause life-threatening infections, and future research should, therefore, focus on ARGs and the factors associated with the increase/decrease in concentration levels in the groundwater of the Kathmandu Valley.

3.2. ARGs in WWTP

Similar to river water samples, *sul1* was the most abundant ARG, followed by *qnrS*, $bla_{\text{CTX-M}}$, *tet*(B), and $bla_{\text{NDM-1}}$ in both influent and effluent samples (Figure 3). In the influent of the WWTP, the concentrations of ARGs varied from 9.2 to 12.2 $\log_{10}$ copies/L. Sewage and drinking water pipelines are aligned very close to one another in the Kathmandu Valley. Some of the main drinking water pipes are over a century old and prone to leakage. This increases the likelihood of mixing sewage with drinking water and further contamination of drinking water with ARGs, ARB, and other pathogens. Antibiotic resistance in WWTP has been shown to mirror the pattern of antibiotic resistance in clinics [36] and our results provide further evidence of the emergence of highly resistant bacterial pathogens in the Kathmandu Valley. Figure 4 shows the LRV of ARGs and *intI1* at the WWTP. ARGs are removed in biological treatment processes by the mechanism of sorption and biodegradation [37]. The highest and lowest LRVs were observed for *tet*(B) (1.3 ± 0.2) and *sul1* (0.4 ± 0.3), respectively. Removal of ARGs is known to be affected by the treatment system used, and different ARGs displayed different reduction rates [38]. Biological treatment with oxidation ditch is among the most economical means of removing organic matter from wastewater, and the same technology is applied in Nepal. One of the concerns regarding treatment using an oxidation ditch is the long retention time, which can support interactions between bacteria and ARGs and increase exposure to antibiotic residues, thereby promoting antibiotic resistance. This interaction is proposed to be one of the reasons for the increase in the concentration of *intI1* and *sul1* in some effluent sample sets (Figure 3).

Except for *sul1* and *intI1*, the relative abundance of all tested marker genes was significantly reduced in effluents (Figure 2, $p < 0.05$). This result is consistent with previous studies, which showed *sul1* and *intI1* persisted in water environments; thus, *sul1* and *intI1* could be used as an indicator of ARGs [7,9,39]. These marker genes are located on mobile genetic elements that can readily transfer among diverse bacteria and have disseminated throughout the bacterial community in wastewater [40]. Quick regeneration of bacteria and the possibility of horizontal transfer of genes between bacteria explain why *sul1* and *intI1* are persistent during wastewater treatment. Despite the reduction, about 10^{12} gene copies/L of individual ARGs were released into the river. Although the release of bacteria-harboring ARGs included in our study is a public health concern, high concentrations of $bla_{\text{NDM-1}}$ are of special relevance. Bacteria belonging to the family of *Enterobacteriaceae* are associated with nosocomial and community-acquired infections, and such bacteria-harboring $bla_{\text{NDM-1}}$ are resistant to all β-lactam antimicrobials except monobactam [41]. Moreover, bacteria-harboring the $bla_{\text{NDM-1}}$ gene usually harbors other

resistant genes [42]; thus, conferring resistance to almost all antibiotics, which seriously limits therapeutic options.

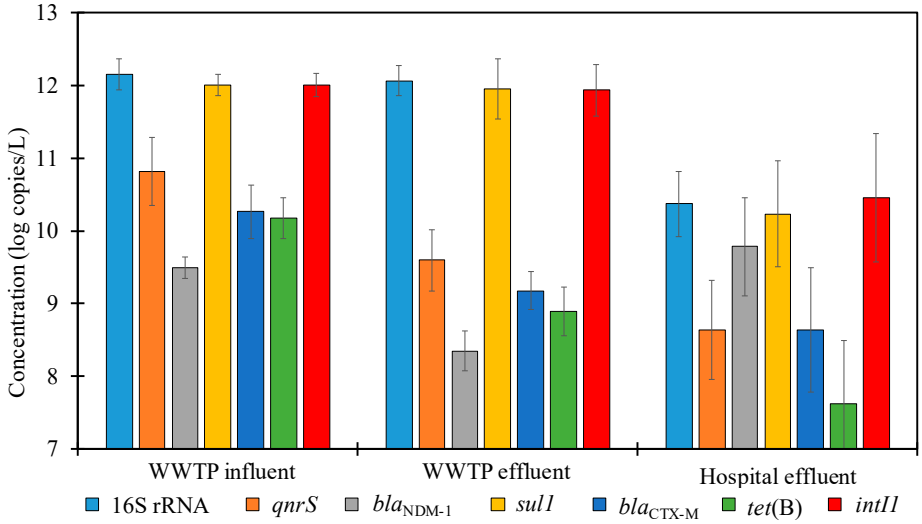

Figure 3. The abundance of 16S rRNA, ARGs, and *intI1* in WWTPs and hospitals.

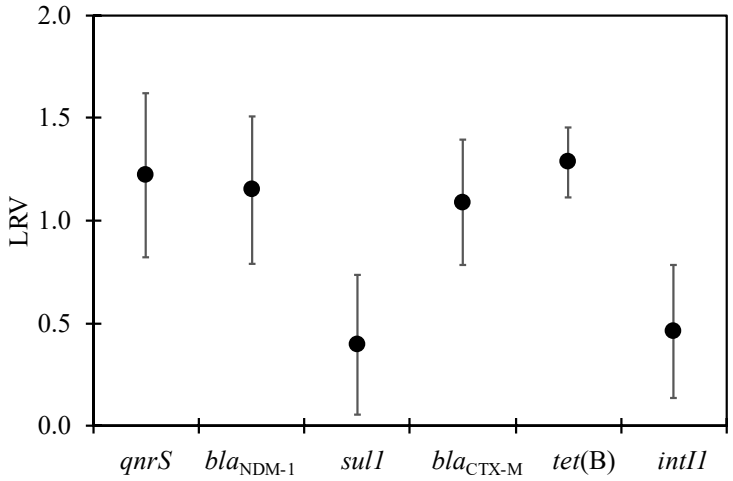

Figure 4. LRVs of ARGs and *intI1* in WWTPs. n = 6, except for *sul1* (n = 4) and *intI1* (n = 3).

The concentration of *E. coli* in effluents of WWTPs ranged from 6.2 to 8.1 $\log_{10}$ MPN/100 mL. In this study, we did not observe the antibiotic resistance of bacterial isolates from WWTPs, but in neighboring countries of India and Bangladesh [43–45], *E. coli*, and bacteria belonging to the group TC have been shown to harbor $bla_{\text{NDM-1}}$, suggesting a likelihood of certain percentage of TC and *E. coli* to be carbapenem-resistant and $bla_{\text{NDM-1}}$ positive in our samples. TC and *E. coli* belong to the family *Enterobacteriaceae*, and carbapenem-resistant *Enterobacteriaceae* have been placed in the critical category of the World Health Organization's list for an urgent need for new antibiotics due to an increase in resistance towards available antibiotics. In addition, $bla_{\text{NDM-1}}$ has been found to transfer at 30 °C more frequently than at other temperatures among the *Enterobacteriaceae* family via conjugation [46]. Since the temperature of 30 °C is common during summer, environmental transfer of $bla_{\text{NDM-1}}$ among pathogens can occur.

3.3. ARGs in the Hospital Wastewater

The occurrence of ARGs in the hospital wastewater is shown in Figure 3. In some sample sets, *sul1*, $bla_{\text{NDM-1}}$, and *intI1* were found to be in higher concentrations than 16Sr RNA marker genes. Similar results have been reported previously [45,47], reflecting high

concentrations of bacteria harboring the ARGs. The concentration of $bla_{\text{NDM-1}}$ in the hospital wastewater of New Delhi, India, has been reported to be as high as 16 $\log_{10}$ copies/L [43], which is six logs higher than our findings in Nepal. Despite the widespread dissemination of $bla_{\text{NDM-1}}$ in the Indian subcontinent [42], the difference in concentrations of $bla_{\text{NDM-1}}$ in the hospital wastewater of these two countries could be explained by the larger size of hospitals surveyed in India, where overcrowding by up to three times the designated capacity was also noted. In comparison to WWTP samples, the relative abundance of only $bla_{\text{NDM-1}}$ was significantly high ($p < 0.05$) in hospital effluent samples, as shown in Figure 2. The use of carbapenem drugs is more common in hospitals, and bacteria-harboring genes encoding resistance to carbapenems in rivers have been shown to be of hospital origin [48]. In Dhaka, Bangladesh, $bla_{\text{NDM-1}}$ was detected in 71% of wastewater samples from hospital adjacent areas in comparison to 12.1% of wastewater samples from community areas [44]. The variation in the ARG profile in hospital wastewater suggests hospital effluents as a diffusion reservoir of $bla_{\text{NDM-1}}$-producing bacteria outside clinical settings and deserves special attention [49]. In comparison to hospital wastewater, ARGs have been extensively studied in WWTP. Hospital wastewater can be often overlooked since it is extensively diluted in municipal sewage and contributes less than one percent of total wastewater in WWTP [50]. A limitation of our study is that we only screened for a limited number of ARGs, and thus, we may have missed other important ARGs. Subsequent studies based on a metagenomics approach that will provide clear information of the overall ARG profile to picture the antibiotic resistance landscape of the study area is suggested.

Among the two hospitals with an onsite wastewater treatment facility, one used biological treatment, and another used ultrafiltration with an activated carbon filter as secondary treatment after primary sedimentation. Both treatment methods reduced similar levels of ARGs and *intI1* with LRVs ranging from 0.3 to 1.1. However, we were unable to draw concrete conclusions because the sampling was done only once. Regardless, our results indicate hospital wastewater as an important reservoir of ARGs. To combat antibiotic resistance, the prudent use of antibiotics should be adopted, and installing effective wastewater treatment at hospitals is needed to eliminate ARGs at the point source. Treatment strategies that include tertiary treatment steps to effectively remove ARGs are recommended.

3.4. Correlation between Individual ARGs, intI1, and Fecal Indicator Bacteria

As summarized in Table 3 and Figures S2–S4 in the Supplemental Material, Pearson's rank correlation test was performed on all data sets. Except for $bla_{\text{NDM-1}}$, significant positive correlations were found between *intI1* and all individual ARGs (0.71–0.96, $p < 0.05$), which indicate the probable implications of *intI1* in the transfer of ARGs. In contrast, TC concentrations did not correlate with concentrations of ARGs ($p > 0.05$). Fecal pollution is often associated with ARG contamination, exemplifying the impact of sewage, but high-throughput sequencing to determine the bacterial community at the genus level revealed pathogenic or opportunistic pathogens of genera *Acinetobacter*, *Aeromonas*, *Myroides*, *Proteus*, *Pseudomonas*, and *Streptococcus* to be highly prevalent with ARGs in the hospital wastewater, along with *Escherichia* [47,51]. Especially the species *Acinetobacter baumannii* is a frequent cause of infection and is reported to be resistant to many antibiotics used in hospitals [52,53]. The possibility of a higher percentage of bacteria belonging to different genera harboring these ARGs can explain the non-correlation between TC and ARGs.

Table 3. Pearson's correlation coefficient between *intI1*, ARGs, and fecal indicator bacteria.

Indicator	*sul1*	*tet*(B)	*qnrS*	$bla_{\text{CTX-M}}$	$bla_{\text{NDM-1}}$
intI1	0.96 *	0.73 *	0.71 *	0.80 *	−0.04
TC	−0.16	0.16	0.22	0.18	0.33
E. coli	0.58 *	0.74 *	0.61 *	0.60 *	0.06

* $p < 0.05$.

4. Conclusions

Antibiotic resistance is a global public health problem. However, regional surveillance is the first step to prevent the spread of antibiotic resistance as variation in the burden of antibiotic resistance and circulating ARGs can be observed. Our results demonstrated river water to be contaminated with the studied ARGs. Up to 10^{12} copies/L of individual ARGs were discharged in WWTP and hospital effluents, highlighting the role of WWTPs and hospitals as important point sources of ARGs in the Kathmandu Valley. Positive correlations between *intI1* and most ARGs also indicate the probable implications of *intI1* in the transfer of ARGs. To conclude, more centralized WWTPs, tertiary treatment for WWTP and hospitals with an onsite treatment plant, and effective treatment strategies for hospitals without any treatment is recommended to combat the growing threat of antibiotic resistance via wastewater management.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13192733/s1, Figure S1: Schematic diagram of WWTP, Figure S2: Correlation of *E. coli* with ARGs, Figure S3: Correlation of TC with ARGs, Figure S4: Correlation of *intI1* with ARGs.

Author Contributions: Conceptualization, O.T. and E.H.; investigation, O.T., B.M., S.T., N.S., S.R., T.F., K.S. and E.H.; methodology, O.T., B.M. and E.H.; validation, T.F., K.S., J.B.S. and E.H.; formal analysis, O.T.; resources, J.B.S. and E.H.; writing—original draft preparation, O.T.; writing—review and editing, T.F., K.S., J.B.S. and E.H.; supervision, E.H.; funding acquisition, E.H. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Science and Technology Research Partnership for Sustainable Development (SATREPS) program of Japan International Cooperation Agency (JICA) and Japan Science and Technology Agency (JST), entitled "Hydro-microbiological Approach for Water Security in Kathmandu Valley, Nepal" and the Japan Society for the Promotion of Science (JSPS) through Fund for the Promotion of Joint International Research (Fostering Joint International Research (B)) (grant number JP18KK0297).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors thank Rajani Ghaju Shrestha, Takahiro Doyama, Taizo Mochizuki, and Masaaki Ito (University of Yamanashi, Japan) for their support during the water sampling and laboratory analysis. The authors also thank the workers of WWTP and hospitals for their kind coopereation in sample collection.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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