Scientific Research Publishing

Advances in Microbiology, 2020, 10, 318-330
https://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Behavior of Antibiotic-Resistant Fecal Coliforms in the Stream of a Sewage Treatment Plant in Tokyo

Naoto Urano[1*], Masahiko Okai[1], Yusuke Tashiro[1], Azusa Takeuchi[1], Rintaro Endo[1], Masami Ishida[1], Masachika Takashio[2]

[1]Laboratory of Marine Biochemistry, Department of Ocean Sciences, Tokyo University of Marine Science and Technology, Tokyo, Japan
[2]Zensho Laboratories of Food Technology, Zensho Holdings Co., Ltd., Tokyo, Japan
Email: *urano@kaiyodai.ac.jp

How to cite this paper: Urano, N., Okai, M., Tashiro, Y., Takeuchi, A., Endo, R., Ishida, M. and Takashio, M. (2020) Behavior of Antibiotic-Resistant Fecal Coliforms in the Stream of a Sewage Treatment Plant in Tokyo. *Advances in Microbiology*, **10**, 318-330.
https://doi.org/10.4236/aim.2020.107023

Received: June 5, 2020
Accepted: July 18, 2020
Published: July 21, 2020

Abstract

We are confronting a new threat in the prevalence of antibiotic-resistant bacteria followed by epidemic spread in aquatic environments in metropolitan areas because damage from river floods is increasing remarkably in Japan due to global extreme weather. The sewer penetration rate is about 100% in Tokyo and reclaimed water from sewage treatment plants accounts for over 50% of all water in both the down- and mid-stream areas of local rivers. The water quality of these rivers, which contain microflora, seems to be seriously affected by reclaimed water. In this study, we collected water samples on July 17, 2018 and examined the behavior of antibiotic-resistant fecal coliforms in the stream of a sewage treatment plant in Tokyo. Extended-spectrum β-lactamase (ESBL)-producing fecal coliforms with encoding genes were found; the CTX-M-1, CTX-M-9, TEM, and SHV groups were found to have survived in the final effluent to the river after sterilization with sodium hypochlorite.

Keywords

Antibiotic-Resistant Bacteria (ARB), Extended-Spectrum β-Lactamase (ESBL), Fecal Coliforms, Sewage Treatment Plant, Reclaimed Water

1. Introduction

The history of the human fight against pathogens goes back to prehistoric times and the development of antibiotics, starting with penicillin in the early 20th century, has made a substantial contribution to our ability to overcome serious in-

fections. However, long-time use and mass use of any one antibiotic can cause an outbreak of antibiotic-resistant bacteria (ARB). The production and use (or overuse) of another antibiotic may then trigger the appearance of new ARB, and so on, creating a vicious circle between humans and pathogens [1]. In the early stages, ARB often arose in places in advanced countries where large amounts of antibiotics were used, such as medical facilities, and livestock or fish farms. In next stage, ARB spread through urban or natural environments, affecting human lives in both advanced and developing countries [2] [3]. Now, β-lactam antibiotics with antibacterial activity that inhibits synthesis of bacterial cell walls have the highest consumption among antibiotics worldwide and thus, medical practice is now seeing β-lactamase-producing bacteria created by natural mutations that are beginning to pose a serious threat. Above all, we have seen the appearance of Enterobacteriaceae with extended-spectrum β-lactamase (ESBL), which are considered to be among the most dangerous ARB in the world [4] [5] [6].

We have been studying the ARB in the stream of the Tama River flowing between Tokyo and Kanagawa Prefecture in Japan for over 10 years and above all, investigating their spread in the midstream bottom of the Tama River to evaluate the occurrence and degree of antibiotic-resistant fecal coliforms (ARFCs). The *Klebsiella* and *Escherichia* genera are the major isolates among ARFCs in the Tama River. Fecal coliforms are used as an indicator of the bacteriological quality of drinking water, and an increase in the ARFC concentration in the river changes gut flora ratios in animals, including humans, in whom this phenomenon might lead to a serious public health issue [7]. Few studies have examined ESBL-producing bacteria from natural environments in Japan; however, in a previous study, we conducted both double disc synergy testing (DDST) and gene amplification followed by sequencing, confirming the production of ESBL by a six-antibiotic-resistant isolate *E. coli* strain in the Tama River that showed the CTX-M-1 group gene [8].

The sewer penetration rate is currently near 100% in Tokyo and Kanagawa and neither industrial nor domestic wastewater flows directly into the Tama River. Reclaimed water from sewage treatment plants near the river accounts for over 50% of all river water in both the down- and mid-stream areas of the Tama River. Thus, the quality of the water of the Tama River, which contains microflora, seems to be seriously affected by this reclaimed water. Several recent studies have assessed the effects of sewage treatment plants on the environmental spread of ARB and their genes, examining coliform and antibiotic-resistant (AR) pathogens in hospital wastewater [9], reporting on increases in ARB and their genes in the environments due to wastewater treatment [10], comparing environmental ARB and genes from urban wastewater treatment plants in 7 European countries [11], analyzing decreases in and remaining ARB in post-treated effluents in India [12], and studying the presence of AR genes in a sewage treatment plant in the USA [13]. Nevertheless, few such studies have been carried out in Japan. The purpose of the present study was to report the behavior of ARFCs

in the stream of a sewage treatment plant in Tokyo.

2. Materials and Methods

2.1. Isolation of Antibiotic-Resistant Fecal Coliform-Like Bacteria

On July 17, 2018, we collected water samples from 4 compartments at a sewage treatment plant in Tokyo. The 4 compartments were 1) in-flow sewage in the sedimentation basin, 2) activated sludge in the aeration tank, 3) treated water, and 4) the final effluent to the river after sterilization with sodium hypochlorite. The samples were rapidly transported to the laboratory of Tokyo University of Marine Science and Technology in sterile bottles on ice and were stored in a refrigerator at 4˚C - 6˚C. The isolation of fecal coliforms was carried out by a modification of the method described by Ham *et al.* [14]. Briefly, an aliquot from each sample was spread onto MacConkey agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and cultivated at 44.5˚C for 24 h. Growing red-colored colonies were selected because the red colonies were thought to be coliform-like bacteria and because the growth of cells at 44.5˚C was also thought to be characteristic of fecal bacteria. Single-colony isolation was performed on the colonies, and the isolates were again cultivated at 44.5˚C for 24 h and then stored at −80˚C.

2.2. Antibiotic Susceptibility Test on the Isolates

An antibiotic susceptibility test was performed on the isolates using the disc diffusion method with the following 14 antibiotics, 8 of which have a β-lactam structure: a penicillin compound (ampicillin, AMPC), 5 cephem compounds (cefotaxime, CTX; cefoxitin, CFX; ceftazidime, CAZ; ceftriaxone, CTRX; and cefpodoxime, CPDX), a monocyclic-lactam compound (aztreonam, ATM), 2 carbapenem compounds (imipenem, IPM; and meropenem, MEPM), 2 aminoglycoside compounds (kanamycin, KM; and gentamicin, GM), a tetracycline compound (tetracycline, TC), a quinolone compound (ciprofloxacin, CPFX), and a chloramphenicol compound (chloramphenicol, CP). Each isolate was suspended in 0.9% (w/v) physiological saline and the 0.5 McFarland standard was used to adjust its turbidity. The suspensions were spread on the surface of Mueller-Hinton agar plates (Nissui Pharmaceutical), antibiotic disks (Japan Becton-Dickinson, Tokyo, Japan) were placed on the plates, and they were incubated at 37˚C for 24 h. Growth inhibitory zones around the disks were interpreted using the Clinical and Laboratory Standards Institute (CLSI) criteria [15]. The antibiotic susceptibilities of the isolates were evaluated for their degrees of sensitivity (S), intermedium (I), and resistance (R).

2.3. Identification of the Isolates

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 a database. The total DNA in 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'-AGAGTTTGATCCTGGCTCAG-3'), and the reverse primer 1492R (5'-GGC TACCTTGTTACGACTT-3'). The sequencing was carried out by Eurofins Genomics (Tokyo, Japan). The 16S rRNA gene sequence of the isolates was subjected to a basic local alignment search tool (BLAST) analysis using the US National Center for Biotechnology Information (NCBI) databases.

2.4. Detection Test for ESBL-Producing Bacteria

First, the ESBL production test was performed for the multi-ARB strains by conducting double disc synergy test (DDST), using a Sensi Disc of AMPC/CVA along with CTX, CAZ, and ATM (Japan Becton-Dickinson). Second, in the ESBL-producing bacteria-like strains, ESBL-encoding genes were amplified by PCR using specific primers for the CTX-M-1 group, CTX-M-2 group, CTX-M-9 group, TEM group, SHV group, and ampC [16] [17] [18] under the conditions shown in Table 1. The sequencing was carried out by Eurofins Genomics. The sequences were subjected to a BLAST analysis of the NCBI databases.

3. Results

3.1. Isolation of Antibiotic-Resistant Fecal Coliform-Like Bacteria

Figure 1 shows the colony forming units (CFUs) of fecal coliform-like bacteria

Table 1. ESBL gene primers and PCR conditions.

Primer name	Primer sequences 5'-3'	Denaturation (˚C/sec)	Annealing (˚C/sec)	Elongation (˚C/sec)	Cycle	PCR products
CTX-M-1 group	F-GCTGTTGTTAGGAAGTGTGC	94/60	55/60	72/90	30	516
	R-CCATTGCCCGAGGTGAAG					
CTX-M-2 group (*)	F-ACGCTACCCCTGCTATTT	94/60	55/60	72/90	30	779 or 780
	R-CCTTTCCGCCTTCTGCTC					
CTX-M-9 group (**)	F-GCAGATAATACGCAGGTG	94/60	55/60	72/90	30	393
	R-CGGCGTGGTGGTGTCTCT					
TEM group (***)	F-CCGTGTCGCCCTTATTCC	94/60	55/60	72/90	30	824
	R-AGGCACCTATCTCAGCGA					
SHV group (****)	F-ATTTGTCGCTTCTTTACTCGC	94/60	55/60	72/90	30	1051
	R-TTTATGGCGTTACCTTTGACC					
ampC	F-GGGGCGGTTTCTCATGCAGCCAACG	94/60	55/60	72/90	30	1313
	R-GAAGCGCTCATGGCACCATCATAGCC					
(*) only in A-12		95/30	63/30	72/60		
(**) only in D-18		94/60	58/60	72/120	35	
(***) only in A-12		95/30	63/30	72/60		
(***) only in D-18		95/30	61/30	72/60		
(****) only in A-12		95/30	63/30	72/60		

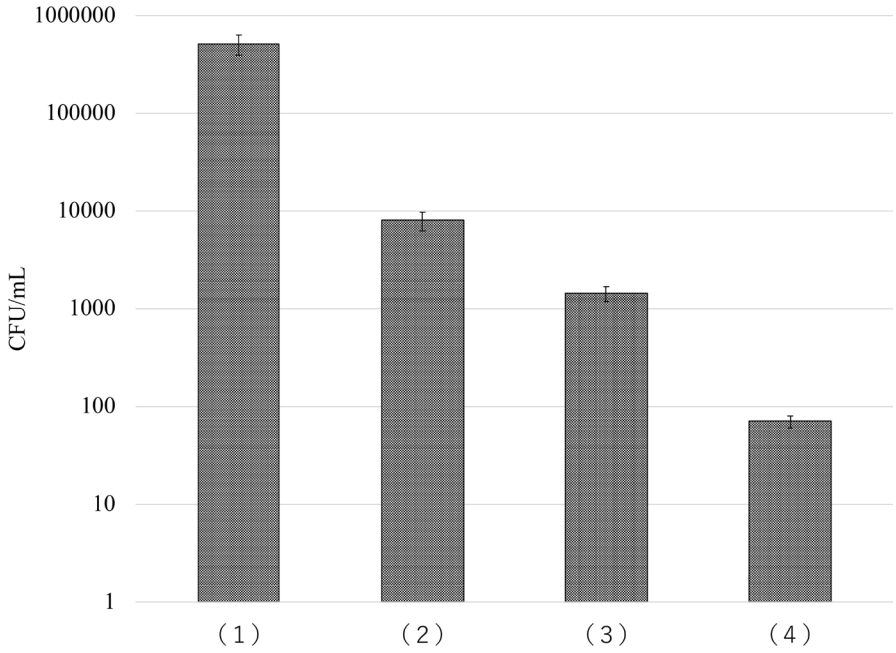

Figure 1. Colony forming units (CFUs) of fecal coliform-like bacteria in the stream water of the sewage treatment plant. (1) In-flow sewage in the sedimentation basin; (2) Activated sludge in the aeration tank; (3) Treated water; (4) Final effluent to the river after sterilization with sodium hypochlorite. The bar graph values are the means of triplicate trials, with their standard deviation (SD) values.

in the stream water of the sewage treatment plant. Each CFU was approximately 5.1×10^5 in the in-flow sewage in the sedimentation basin (1), approximately 8.0×10^3 in the activated sludge in the aeration tank (2), approximately 1.4×10^3 in treated water (3), and approximately 7.0×10 in the final effluent to the river after sterilization with sodium hypochlorite (4). The CFUs gradually decreased from (1) to (4) and fecal coliform cells were found to be living at low concentrations in the final effluent even after sterilization with sodium hypochlorite. Hypochlorite sterilization conditions for fecal coliforms have been thoroughly researched [19] and the final effluent from sewage treatment plants is generally sterilized at a strength sufficient to kill bacteria completely. It remains unknown why some cells in the sterilization tank survive, but recent studies have reported that biofilm formation by various kinds of bacteria leads to a remarkable increase in their stability against environmental stress [20] [21] [22]. It is thought that the existence of fecal coliform biofilm in the tank might prevent sterilization of the bacteria by sodium hypochlorite.

3.2. Identification and Antibiotic Susceptibility of Fecal Coliform-Like Bacteria

Table 2 shows all the isolates in the sewage plant and their strain numbers followed by their antibiotic susceptibility. There were 64 strains of isolates and all were found to be fecal coliforms. In (1), we found 13 strains including 2 genera and 3 species (*Escherichia coli*, *Klebsiella pneumoniae*, and *K. quasipneumoniae*);

Table 2. Identification of coliform-like bacteria and their antibiotic susceptibility in the sewage treatment plant.

Compartment	Species	Strain No.	AMPC	CTX	CAZ	CFX	IPM	MEPM	ATM	CTRX	CPDX	KM	GM	TC	CPFX	CP
(1)	*Klebsiella pneumoniae*	A-1	R	S	S	S	S	S	S	S	S	I	S	S	S	S
	Klebsiella pneumoniae	A-2	R	S	S	S	S	S	S	S	S	I	S	S	S	S
	Escherichia coli	A-3	S	S	S	S	S	S	S	S	S	I	S	S	S	S
	Escherichia coli	A-4	R	S	S	S	S	S	S	S	S	R	S	R	R	R
	Klebsiella pneumoniae	A-5	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	A-6	I	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	A-7	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	A-8	R	S	S	S	S	S	S	S	S	S	S	R	S	S
	Escherichia coli	A-9	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	A-11	S	S	S	S	S	S	S	S	S	I	S	S	S	S
	Klebsiella pneumoniae	A-12	S	R	S	R	S	S	R	I	S	S	S	S	S	S
	Klebsiella quasipneumoniae	A-13	I	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	A-14	S	S	S	S	S	S	S	S	S	R	S	R	S	S
(2)	*Klebsiella pneumoniae*	C-1	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	C-2	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella quasipneumoniae	C-4	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	C-5	S	S	S	S	R	S	S	S	S	I	R	S	S	S
	Klebsiella variicola	C-6	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	C-7	I	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	C-9	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	C-10	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	C-11	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	C-12	I	S	S	S	S	S	S	S	S	S	S	S	S	S
	Enterobacter cloacae	C-13	I	S	S	R	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	C-14	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	C-15	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella quasipneumoniae	C-17	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella oxytoca	C-18	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	C-19	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	C-20	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	C-21	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	C-22	R	S	S	S	S	S	S	S	S	S	S	S	S	I
	Citrobacter spp	C-23	R	S	S	S	S	S	S	S	S	S	S	S	S	S
(3)	*Klebsiella pneumoniae*	D-1	I	S	S	S	S	S	S	S	S	I	I	S	S	S
	Citrobacter freundii	D-2	I	S	S	S	S	S	S	S	S	S	S	S	S	S

Continued

	Klebsiella pneumoniae	D-3	I	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	D-4	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	D-5	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	D-6	R	S	S	S	S	S	S	S	S	I	S	R	S	S
	Escherichia coli	D-8	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella quasipneumoniae	D-9	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	D-10	R	S	S	S	S	S	S	S	S	I	R	R	R	S
	Escherichia coli	D-11	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Citrobacter freundii	D-12	R	S	S	I	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	D-13	I	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	D-14	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	D-15	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	D-16	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	D-18	R	R	I	S	S	S	S	R	R	S	S	S	S	S
(4)	*Klebsiella pneumoniae*	B-1	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	B-2	S	S	S	S	S	S	S	S	S	I	S	S	S	S
	Escherichia coli	B-3	S	S	S	S	S	S	S	S	S	I	S	S	S	S
	Klebsiella quasipneumoniae	B-6	R	S	S	S	S	S	S	S	S	I	S	S	S	S
	Klebsiella quasipneumoniae	B-7	R	R	R	S	S	S	R	R	R	I	S	S	S	S
	Klebsiella pneumoniae	B-8	R	R	S	R	S	S	S	R	R	I	S	S	S	S
	Escherichia coli	B-9	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	B-11	R	S	S	I	S	S	R	S	S	I	S	S	S	S
	Klebsiella quasipneumoniae	B-12	R	S	S	S	S	S	S	S	S	S	S	S	S	S
	Klebsiella pneumoniae	B-13	R	I	I	S	I	I	S	S	S	S	S	S	S	S
	Escherichia coli	B-16	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	B-17	S	I	S	S	S	S	S	S	S	S	S	R	S	S
	Escherichia coli	B-18	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	B-19	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Escherichia coli	B-20	S	S	S	S	S	S	S	S	S	S	S	S	S	S

(1) In-flow sewage in the sedimentation basin; (2) Activated sludge in the aeration tank; (3) Treated water; (4) Final effluent to the river after sterilization with sodium hypochlorite; AMPC, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CFX, cefoxitin; IPM, imipenem; MEPM, meropenem; ATM, aztreonam; CTRX, ceftriaxone; CPDX, cefpodoxime; KM, kanamycin; GM, gentamicin; TC, tetracycline; CPFX, ciprofloxacin; CP, chloramphenicol; S, sensitivity; I, intermedium; R, resistance.

in (2), we found 20 strains including 4 genera and 7 species (*Citrobacter* spp., *Enterobacter cloacae*, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *K. quasipneumoniae* and *K. variicola*); in (3), we found 16 strains containing 3 genera and 4 species (*C. freundii*, *E. coli*, *K. pneumoniae*, and *K. quasipneumoniae*); and in (4), we found 15 strains containing 2 genera and 3 species (*E. coli*, *K. pneumoniae*, and

K. quasipneumoniae). There were a total of 25 *E. coli* strains (39% of 64 isolates) and 26 *K. pneumoniae* strains (41% of 64) from the sewage treatment plant. Seven strains showed sensitivity against all antibiotics, 37 showed mono-resistance or mono-intermedium, and 20 showed multi-resistance or multi-intermedium. At the sewage plant in the present study, there were 11 multi-ARB strains (17% of all fecal coliforms).

Figure 2 shows the numbers of ARFCs isolated from the stream water of the sewage treatment plant. The ratios of multi-ARFCs to all antibiotic-resistant and intermedium FCs were 33% in (1), 5% in (2), 25% in (3), and 33% in (4). Multi-ARFCs were also found to survive even after sterilization treatment, and then seemed to increase in the metropolitan aquatic environment.

3.3. Detection of ESBL-Producing Bacteria

Antibiotic susceptibility testing showed the following candidate strains of ESBL-producing bacteria: *Klebsiella pneumoniae* A-12 having CTX-, CFX-, and ATM-resistance in (1); *Escherichia coli* D-18 having AMPC-, CTX-, CTRX-, and CPDX-resistance in (3); *K. quasipneumoniae* B-7 having AMPC-, CAZ-, CTX-, CTRX-, CPDX-, and ATM-resistance in (4); *K. pneumoniae* B-8 having AMPC-, CTX-, CTRX-, CPDX-, and CFX-resistance in (4); and *K. pneumoniae* B-11 having AMPC- and ATM-resistance in (4), as shown in Table 3. These strains were examined using the DDST and amplification of ESBL-encoding genes in order to ascertain their ESBL production.

Table 4 shows the DDST results for the candidate strains of ESBL-producing bacteria. *K. pneumoniae* A-12 was pseudo-positive for the CTX-M-2, TEM, and SHV groups. *E. coli* D-18 was positive for the CTX-M-1 and TEM groups, as was

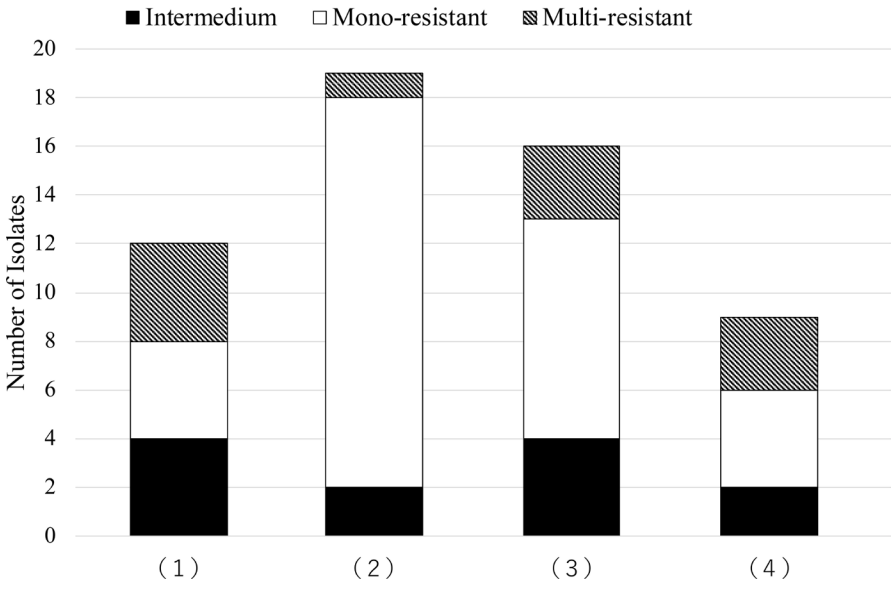

Figure 2. Numbers of ARFCs isolated from the stream water of the sewage treatment plant. (1) In-flow sewage in the sedimentation basin; (2) Activated sludge in the aeration tank; (3) Treated water; (4) Final effluent to the river after sterilization with sodium hypochlorite.

Table 3. Candidate strains for ESBL-producing bacteria by antibiotic susceptibility tests.

Strain No.	Identification			AMPC	CAZ	CTX	CTRX	CPDX	CFX	ATM	MEPM	IPM	KM	GM	TC	CP	CPFX
	Species	e-Value	Homology														
A-12	*Klebsiella pneumoniae*	0.0	99%	S	S	R	I	S	R	R	S	S	S	S	S	S	S
D-18	*Escherichia coli*	0.0	99%	R	I	R	R	R	S	S	S	S	S	S	S	S	S
B-7	*Klebsiella quasipneumoniae*	0.0	99%	R	R	R	R	R	S	R	S	S	I	S	S	S	S
B-8	*Klebsiella pneumoniae*	0.0	99%	R	S	R	R	R	R	S	S	S	I	S	S	S	S
B-11	*Klebsiella pneumoniae*	0.0	99%	R	S	S	S	S	I	R	S	S	I	S	S	S	S

AMPC, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; CTRX, ceftriaxone; CPDX, cefpodoxime; CFX, cefoxitin; ATM, aztreonam; MEPM, meropenem; IPM, imipenem; KM,(kanamycin; GM, gentamicin; TC, tetracycline; CP, chloramphenicol; CPFX, ciprofloxacin; S, sensitivity; I, intermedium; R, resistance.

Table 4. Double disc synergy testing (DDST) of ESBL-producing candidates.

Strain \ Primer	CTX-M-1 group	CTX-M-2 group	CTX-M-9 group	TEM group	SHV group	ampC
Klebsiella pneumoniae A-12	−	±	−	±	±	−
Escherichia coli D-18	+	−	−	+	−	−
Klebsiella quasipneumoniae B-7	+	−	−	+	−	−
Klebsiella pneumoniae B-8	−	−	+	+	−	−
Klebsiella pneumoniae B-11	−	−	−	±	−	−

+, positive; ±, pseudo-positive; −, negative.

K. quasipneumoniae B-7. *K. pneumoniae* B-8 was positive for the CTX-M-9 and TEM groups. And *K. pneumoniae* B-11 was pseudo-positive for the TEM group. All five strains were assayed by the amplification of ESBL-encoding genes.

Table 5(a) and Table 5(b) show the results of the amplification of ESBL-encoding genes (AEEG) for ESBL-producing bacteria-like strains. The homology of AEEG sequences in the BLAST analysis is summarized in Table 5(a). In *K. pneumoniae* A-12, the product amplified by the TEM primer showed 93% homology to TEM β-lactamase in *Burkholderia* sp. LLH-Slr-7 and that amplified by the SHV primer showed 83% homology to Class A β-lactamase SHV-152 in *K. pneumoniae*, but that amplified by the CTX-M-2 primer showed only 54% homology to lipoate-protein ligase Lp1A in *K. pneumoniae*. In *E. coli* D-18, the product amplified by the TEM primer showed 97% homology to blaTEM-84_1_AF427130 and that amplified by the CTX-M-1 primer showed 92% homology to β-lactamase CTX-M-1 in *K. pneumoniae*. In *K. quasipneumoniae* B-7, the product amplified by the CTX-M-1 primer showed 98% homology to β-lactamase CTX-M-15 and that amplified by the TEM primer showed 96% homology to class A ESBL TEM-143 in *E. coli*. In *K. pneumoniae* B-8, the product amplified by the CTX-M-9 primer showed 94% homology to ESBL CTX-M-14 in *K. pneumoniae* while that amplified by the TEM primer showed only 61% homology to β-lactamase TEM in *E. coli*. In *K. pneumoniae* B-11, the product amplified by the TEM primer showed 98% homology to TEM β-lactamase in *E. coli*.

Table 5. (a) Homology of ESBL-encoding gene sequences in the BLAST analysis (*). (b) ESBL-encoding genes for ESBL-producing candidates.

(a)

Strain	Primer	Results of search for ESBL-encoding gene sequence homology	e-Value	Homology
A12	TEM	TEM β-lactamase, partial [*Burkholderia* sp. LLH-Slr-7]	6.00E−141	93%
A12	CTX-M-2	Lipoate-protein ligase LplA, partial [*Klebsiella pneumoniae*]	5.00E−32	54%
A12	SHV	Class A β-lactamase SHV-152 [*Klebsiella pneumoniae*]	3.00E−155	83%
D18	CTX-M-1	β-lactamase CTX-M-1, partial [*Klebsiella pneumoniae*]	9.00E−84	92%
D18 (**)	TEM	BlaTEM-84_1_AF427130 [*Klebsiella pneumoniae*]	1.00E−167	97%
B7	CTX-M-1	β-lactamase CTXM-15 [*Escherichia coli*]	2.00E−103	98%
B7 (**)	TEM	Class A extended-spectrum β-lactamase TEM-143 [*Escherichia coli*]	6.00E−167	96%
B8	CTX-M-9	Extended-spectrum β-lactamase CTX-M-14, partial [*Klebsiella pneumoniae*]	6.00E−46	94%
B8	TEM	β-lactamase TEM [*Escherichia coli*]	6.00E−72	61%
B11 (**)	TEM	TEM β-lactamase, partial [*Escherichia coli*]	8.00E−94	98%

(*) A BLAST analysis was used to search for sequence homology (http://blast.ncbi.nlm.nih.gov/Blast.cgi). (**) Carried out with Taq polymerase treated with DNase.

(b)

Strain \ Primer	CTX-M-1 group	CTX-M-2 group	CTX-M-9 group	TEM group	SHV group	ampC
Klebsiella pneumoniae A-12	−	−	−	+	+	−
Escherichia coli D-18	+	−	−	+	−	−
Klebsiella quasipneumoniae B-7	+	−	−	+	−	−
Klebsiella pneumoniae B-8	−	−	+	−	−	−
Klebsiella pneumoniae B-11	−	−	−	+	−	−

+, positive; −, negative.

Therefore, the five strains of the ESBL-producing candidates were found to have ESBL-encoding genes as follows: *K. pneumoniae* A-12 was positive for the TEM and SHV groups; *E. coli* D-18 and *K. quasipneumoniae* B-7 were positive for the CTX-M-1 and TEM groups; *K. pneumoniae* B-8 was positive for the CTX-M-9 group; and *K. pneumoniae* B-11 was positive for the TEM group (Table 5(b)). The positive and pseudo-positive groups identified by DDST were partially different from the groups identified as positive in AEEG. In conclusion, all five strains were found to be ESBL-producing bacteria having one or two kinds of ESBL-encoding genes.

4. Discussion

Extreme environmental destruction occurred during the high economic growth period in the 1960s and 1970s in Japan. Aquatic areas in metropolitan areas (rivers, lakes, marshes, canals, coasts, etc.) were filled with trash, oil, and detergent foam, accompanied by an unbearable stench, and seemed to be essentially

lifeless. In the 1960s, the sewer penetration rate was about 20% - 40% in Tokyo and both industrial and domestic wastewater was leaked directly into aquatic areas. Over 50 years have passed since then and the quality of outdoor water has improved remarkably; the sewer penetration rate is now about 100% in Tokyo. Reclaimed water from sewage treatment plants accounts for over 50% of all water in both the down- and mid-stream areas of local rivers and the quality of their water, which contains microflora, seems to be seriously affected by this reclaimed water [23].

We are now confronting a new threat in the prevalence of ARB followed by epidemic spread in aquatic environments in metropolitan areas because damage from river floods is increasing remarkably due to global extreme weather. As mentioned in the *Introduction* above, several studies have reported on ARB in sewage treatment plants and their environmental spread worldwide [9] [10] [11] [12] [13]. However, there have been few studies on the relationship between the spread of ARB and sewage treatment plants in Japan. In our previous study, we found that ESBL-producing bacteria inhabit the Tama River in Tokyo [8] and in the present study, we also found that ESBL-producing bacteria live in the final effluent to the river after sterilization with sodium hypochlorite. Thus, ARB contamination in the sewage treatment plant is thought to be directly connected to the prevalence of ARB in aquatic environments, and more thorough sterilization of microorganisms in the effluents is indispensable. However, there is the risk of increasing environmental pollution with increased concentrations of disinfectants such as sodium hypochlorite. The combination of ozone treatment with chemicals also seems to be valid for sterilization [24]. Future studies should aim to develop a complete sterilization method for ARB without river pollution in the final effluent from sewage treatment plants.

5. Conclusion

We collected water samples on July 17, 2018 and examined the behavior of antibiotic-resistant fecal coliforms in the stream of a sewage treatment plant in Tokyo. Fecal coliforms containing mainly *Escherichia coli* and *Klebsiella pneumoniae* with extended-spectrum β-lactamase (ESBL)-encoding genes were found; the CTX-M-1, CTX-M-9, TEM, and SHV groups survived in the final effluent to the river after sterilization with sodium hypochlorite.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Labanovska, M. and Pilla, G. (2017) Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? *Yale Journal of Biology and Medicine*, **90**, 235-245.

[2] Levy, J.Y. and Marshall, B. (2004) Antibacterial Resistance Worldwide: Causes,

Challenges and Responses. *Nature Medicine*, **10**, S122-S129. https://doi.org/10.1038/nm1145

[3] Nguyen, V.T., Carrique-Mass, J.J., Ngo, T.H., Ho, H.M., Ha, T.T., Campbell, J.I., Nguyen, T.N., Hoang, N.N., Pham, V.M., Wagenaar, J.A., Hardon, A., Thai, Q.H. and Schultsz, C. (2015) Prevalence and Risk Factors for Carriage of Antimicrobial-Resistant *Escherichia coli* on Household and Small-Scale Chicken Farms in the Mekong Delta of Vietnam. *Journal of Antimicrobial Chemotherapy*, **70**, 2144-2152.

[4] Pitout, J.D. and Lasupland, K.B. (2008) Extended Spectrum Beta-Lactamase-Producing Enterobacteriaceae: An Emerging Public-Health Concern. *The Lancet Infectious Diseases*, **8**, 159-166. https://doi.org/10.1016/S1473-3099(08)70041-0

[5] Woerther, P.L., Budet, C., Chachary, E. and Andremont, A. (2013) Trends in Human Fecal Carriage of Extended-Spectrum Beta-Lactamases in the Community: Toward the Globalization of CTX-M. *Clinical Microbiology Reviews*, **26**, 744-758. https://doi.org/10.1128/CMR.00023-13

[6] Luvsansharav, U.O., Hirai, I., Nakata, A., Imura, K., Yamauchi, K., Niki, M., Komalamisura, C., Kusolsuk, T. and Yamamoto, Y. (2012) Prevalence of and Risk Factors Associated with Fecal Carriage of CTX-M β-Lactamase-Producing Enterobacteriaceae in Rural Thai Communities. *Journal of Antimicrobial Chemotherapy*, **67**, 769-774. https://doi.org/10.1093/jac/dks118

[7] Maier, L., Pruteanu, M., Kuhn, M., Zeller, G., Telzerow, A., Anderson, E.E., Brochado, A.R., Fernandez, K.C., Dose, H., Mori, H., Patil, K.R., Peer Bork, P. and Typas, A. (2018) Extensive Impact of Non-Antibiotic Drugs on Human Gut Bacteria. *Nature*, **555**, 623-628. https://doi.org/10.1038/nature25979

[8] Okai, M., Aoki, H., Ishida, M. and Urano, N. (2019) Antibiotic-Resistance of Fecal Coliforms at the Bottom of the Tama River in Tokyo. *Biocontrol Science*, **24**, 173-178. https://doi.org/10.4265/bio.24.173

[9] Asfaw, T. (2018) Review of Hospital Wastewater as a Source of Emerging Drug Resistance Pathogens. *Journal of Research in Environmental Science and Toxicology*, **7**, 47-52. https://doi.org/10.14303/jrest.2018.020

[10] Rodriguez-Molina, D., Mang, P., Schmitt, H., Chifiriuc, M.C., Raden, K. and Wengenroth, L. (2019) Do Wastewater Treatment Plants Increase Antibiotic Resistant Bacteria or Genes in the Environment? Protocol for a Systematic Review. *Systematic Reviews*, **8**, 304. https://doi.org/10.1186/s13643-019-1236-9

[11] Parnanen, K.M.M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T.U., Cacase, D. and Do, T.T. (2019) Antibiotic Resistance in European Wastewater Treatment Plants Mirrors the Pattern of Clinical Antibiotic Resistance Prevalence. *Science Advances*, **5**, eaau9124. https://doi.org/10.1126/sciadv.aau9124

[12] Nimonkar, Y.S., Yadav, B., Talreja, P., Sharma, A., Patil, S., Saware, S.S., Ranade, D.R. and Prakash, O. (2019) Assessment of the Role of Wastewater Treatment Plant in Spread of Antibiotic Resistance and Bacterial Pathogens. *Indian Journal of Microbiology*, **59**, 261-265. https://doi.org/10.1007/s12088-019-00793-2

[13] Naquin, A., Shrestha, A., Sherpa, M., Nathaniel, R. and Boopathy, R. (2015) Presence of Antibiotic Resistance Genes in a Sewage Treatment Plant in Thibodaux, Louisiana, USA. *Bioresource Technology*, **188**, 79-83. https://doi.org/10.1016/j.biortech.2015.01.052

[14] Ham, Y.S., Kobori, H., Kang, J.H., Matsuzaki, T., Lino, M., and Nomura, H. (2012) Distribution of Antibiotic Resistance in Urban Watershed in Japan. *Environmental Pollution*, **162**, 98-103. https://doi.org/10.1016/j.envpol.2011.11.002

[15] Clinical and Laboratory Standards Institute (2016) Performance Standards for An-

timicrobial Susceptibility Testing, 27th ed., CSCL Supplement M100S-S26. Clinical and Laboratory Standards Institute, Wayne.

[16] Rivoarilala, O.L., Garin, B., Andriamahery, F. and Coliard, J.M. (2018) Rapid *in Vitro* Detection of CTX-M Groups 1, 2, 8, 9 Resistance Genes by Lamp Assays. *PLoS ONE*, **13**, e0200421. https://doi.org/10.1371/journal.pone.0200421

[17] Shibata, N., Kurokawa, H., Doi, Y., Yagi, T., Yamane, K., Wachino, J., Suzuki, S., Kimura, K., Ishikawa, S., Kato, H., Ogawa, Y., Shibayama, K., Kai, K., Konda, T. and Arakawa, Y. (2006) PCR Classification of CTX-M Type β-Lactamase Genes Identified in Clinically Isolated Gram-Negative Bacilli in Japan. *Antimicrobial Agents and Chemotherapy*, **50**, 791-795. https://doi.org/10.1128/AAC.50.2.791-795.2006

[18] Yagi, T., Kurokawa, H., Shibata, N., Shibayama, K. and Aarakawa, Y. (2006) A Preliminary Survey of Extended-Spectrum β-Lactamases (ESBLs) in Clinical Isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. *FEMS Microbiology Letters*, **184**, 53-56. https://doi.org/10.1016/S0378-1097(00)00016-1

[19] Bari, M.L., Nazuka, E., Sabina, Y., Todoriki, S. and Isshiki, K. (2003) Chemical and Irradiation Treatments for Killing *Escherichia coli* O157:H7 on Alfalfa, Radish, and Mung Bean Seeds. *Journal of Food Protection*, **66**, 767-774. https://doi.org/10.4315/0362-028X-66.5.767

[20] Tachikawa, M. and Yamanaka, K. (2014) Synergistic Disinfection and Removal of Biofilms by a Sequential Two-Step Treatment with Ozone Followed by Hydrogen Peroxide. *Water Research*, **64**, 94-101. https://doi.org/10.1016/j.watres.2014.06.047

[21] Kubota, H., Senda, S., Nomura, N., Tokuda, H. and Uchiyama, H. (2008) Biofilm Formation by Lactic Acid Bacteria and Resistance to Environmental Stress. *Journal of Bioscience and Bioengineering*, **106**, 381-386. https://doi.org/10.1263/jbb.106.381

[22] Kubota, H., Senda, S., Tokuda, H., Uchiyama, H. and Nomura, N. (2009) Stress Resistance of Biofilm and Planktonic *Lactobacillus plantarum* subsp. *plantarum* JCM1149. *Food Microbiology*, **26**, 592-597. https://doi.org/10.1016/j.fm.2009.04.001

[23] Bureau of Sewerage, Tokyo Metropolitan Government (2018) Sewerage in Tokyo. https://www.gesui.metro.tokyo.lg.jp/english/aboutus/ourprofile/index.html

[24] Ebihara, K., Mitsugi, F., Ikegami, T., Yamashita, Y., Hashimoto, Y., Yamashita, T., Kanazawa, S., Stryczewska, H.D., Pawlat, J., Teii, S. and Sung, T.-L. (2016) Sterilization Characteristics of Ozone-Mist Spray for Chemical Free Agriculture. *International Journal of Plasma Environmental Science and Technology*, **10**, 11-15. http://www.iesj.org/content/files/pdf/IJPEST_Vol10_No1_03_pp011-015.pdf