Prevalence of ESBL-producing *Escherichia coli* and carbapenem-resistant Enterobacteriaceae in treated wastewater: a comparison with nosocomial infection surveillance

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ABSTRACT

The increasing prevalence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and carbapenem-resistant Enterobacteriaceae (CRE) is a worldwide health threat. Monitoring of these resistant bacteria in the environment can provide regional prevalence reflecting both healthy and infected populations, although the quantitative monitoring of those resistant bacteria, especially CRE, is difficult due to their low proportion in the total Enterobacteriaceae population and the possible interference by autochthonous species with intrinsic resistance. In this study, these resistant bacteria in treated wastewater were quantified at 12 different treatment plants. The proportions of cefotaxime-resistant and ESBL-producing *E. coli* in the total *E. coli* population in the chlorinated effluents in Tokyo were 5.7 and 5.3%, respectively. The estimated proportion of CRE was 0.007% with the constituting species of *Klebsiella* spp. and *Enterobacter* spp., although the conditions during the first incubation may have affected the estimation even after the correction by the proportion of resistant population in the isolates. The observed resistant proportions in this study were lower than those in the surveillance on nosocomial infection not only for inpatients but also for outpatients, and higher than those in the veterinary monitoring.

Key words | antibiotic resistance, CRE, ESBL, *Escherichia coli*, wastewater

HIGHLIGHTS

- The prevalence of antibiotic-resistant bacteria in treated municipal wastewater was carefully quantified in Tokyo and in its suburbs.
- ESBL-producers were 5.3% of the total *E. coli* population in the effluents on average.
- The estimated proportion of carbapenem-resistant Enterobacteriaceae (CRE) was 0.007% in the effluents.
- These proportions of ESBL-producers and CRE were lower than those in the surveillance on nosocomial infection not only for inpatients but also for outpatients.

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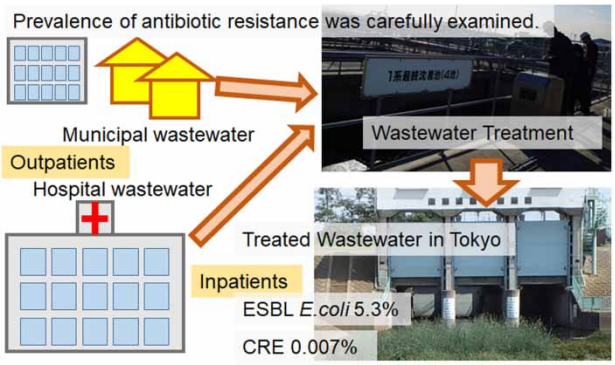
GRAPHICAL ABSTRACT



INTRODUCTION

The increasing prevalence of antibiotic-resistant bacteria (ARB) is a worldwide problem. The growing importance of the 'One health approach' interconnecting human, animal, and environmental health has been announced by WHO and other health organizations with an emphasis on covering quantitative environmental monitoring (Wuijts *et al.* 2017).

Wastewater treatment plants (WTPs) can be hotspots for ARB in the environment (Rizzo *et al.* 2013). An increase in the relative abundance of several resistant genes was observed during the wastewater treatment process (Makowska *et al.* 2020). Antibiotic-resistant *Escherichia coli* from patients with urinary tract infection and those from sewage sludge harbored similar genetic and phenotypic markers, which suggests the interconnection between sewer system and outpatients (Zarfel *et al.* 2013). The close linkage between clinical isolates and environmental isolates suggests the importance of wastewater treatment (Lepuschitz *et al.* 2019). People can possibly be exposed to ARB via water resources (Diab *et al.* 2018) and via the agricultural use of waste sludge (Reinthaler *et al.* 2010).

Since the 1950s, infections caused by Enterobacteriaceae have been treated with β-lactam antibiotics, which bind to enzymes to inhibit bacterial cell wall synthesis. Following the introduction of broad-spectrum third-generation cephalosporins, extended-spectrum β-lactamases (ESBLs) emerged in the family Enterobacteriaceae, particularly in *E. coli* and *Klebsiella* spp. (Korzeniewska & Harnisz 2013; Lepuschitz *et al.* 2019). Pathogens carrying ESBLs represent the main challenges to antibiotic therapy because of a high incidence of multi-resistance to fluoroquinolones (Maheshwari *et al.* 2016; Voigt *et al.* 2020). The prevalence of several *E. coli* clones is considered to be linked to this multi-resistance (Said *et al.* 2016; Chong *et al.* 2018). The resistance is also spreading to carbapenems, a group of broad-spectrum β-lactam antibiotics of last resort against multi-drug resistant infections (Mills & Lee 2019). A study in the US showed that 65 isolates out of 322 *E. coli* resistant to clinically important antibiotics were imipenem-resistant (Hoelle *et al.* 2019).

In order to elucidate the role of the environment in the spread of resistant bacteria, quantitative monitoring of the resistance is needed. Although quantitative polymerase chain reaction techniques significantly contribute to the quantitative monitoring of antibiotic resistance, culture-based methods also are widely used for the characterization of isolates to obtain information on the linkage to pathogens (Hiller *et al.* 2019; Pazda *et al.* 2019). As to the phenotype detection of resistance, the Clinical and Laboratory Standards Institute (CLSI) lowered the minimum inhibitory concentration (MIC) breakpoints for resistant Enterobacteriaceae to the third-generation cephalosporins in 2010. The MIC breakpoint for cefotaxime (CTX), which has been used for the selection of ESBL-producing Enterobacteriaceae, was lowered from 64 to 4 μg/mL (Wang *et al.* 2011; Kristo *et al.* 2013). On the same occasion, CLSI also lowered

the MIC breakpoints for carbapenems (e.g. meropenem 16 to 4 μg/mL). The revisions of the test guidelines classified more clinical Enterobacteriaceae isolates as resistant (Hombach *et al.* 2013). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) distributes MIC breakpoint tables slightly different to those by CLSI (e.g. 2 μg/mL for CTX and 8 μg/mL for meropenem) (EUCAST 2019). The comparison with current clinical prevalence becomes possible by applying these new breakpoints consistently in the environmental monitoring.

A wide variety of resistant mechanisms which are usually found in clinical pathogens have been found even in microorganisms taken from pristine environments (Wright 2019). Autochthonous species with intrinsic resistance to carbapenems, such as *Stenotrophomonas maltophilia*, often form more colonies than the target carbapenem-resistant bacteria on carbapenem-containing agar plates (Hrenovic *et al.* 2017; Paulshus *et al.* 2019). The presence of carbapenem-resistant *Aeromonas* spp. is also reported (Wu *et al.* 2012). Due to the presence of these autochthonous species, the studies on quantitative monitoring for carbapenem-resistant Enterobacteriaceae (CRE) are rarely reported and their reliability has to be carefully checked (Hrenovic *et al.* 2017).

This study aimed to quantitatively evaluate the prevalence of ESBL-producing *E. coli* and CRE in treated wastewater. A set of mild selection pressures were imposed at the first incubation step to obtain presumptive resistant population before the second correction step considering the real resistant proportion in the presumptive resistant population. The quantitative results enabled the discussion on the linkage of the environment with the health of both humans and animals through the comparison with national surveillance on nosocomial infections and veterinary monitoring.

MATERIALS AND METHODS

Sample collection

Treated wastewater samples after chlorination were collected from 12 WTPs located in Tokyo and its suburbs in 2018–2019 (Table 1). All treatment plants except for E were operated by municipalities treating domestic wastewater, including a part of industrial and hospital wastewater. The samples were taken to avoid rain events because the combined sewage system was partly employed by some of the target treatment plants (B1, B4, B5, and B6). The plants D1 and D2 were operated with the oxidation ditch process, whereas other plants were operated with the activated sludge process. The plant E is a community plant treating kitchen and toilet wastewater from the Hachioji campus of Tokyo University of Technology. The water samples were collected in sterilized polypropylene bottles in the morning and immediately brought back to the laboratory for analysis in the afternoon of the same date of the sampling.

Table 1 | The list of WTPs for the sampling

WTP	Capacity (m^3/d)	Characteristics
A1	280,000	Large-scale WTPs located in central Tokyo. Hospital wastewater is included. The activated sludge process (partly A_2O operation) is applied.
A2	290,000	
A5	160,000	
B1	30,000	Large-scale WTPs located in the suburbs of Tokyo. Hospital wastewater is included. The activated sludge process is applied.
B3	120,000	
B4	410,000	
B5	150,000	
B6	210,000	
B7	310,000	
D1	9,000	The oxidation ditch process is applied. The plants are located in rural areas.
D2	6,000	
E	300	A community plant in a university campus without medical facilities.

Preparation of agar plates

CHROMagar ECC (CHROMagar, Paris, France), which allows simultaneous detection and differentiation between *E. coli* and other coliforms by the coloration of the colonies (Ho & Tam 1997), was used in this study. The numbers of blue colonies on the selective chromogenic medium were counted as the number of presumptive *E. coli* because more than 93% of blue colonies isolated from the same region in Japan by the same incubation procedure were

identified as *E. coli* by the API-10S test in our previous study (Urase & Sato 2016). The same range of specificity (91%) was reported for *E. coli* detection by the chromogenic medium (Paulshus *et al.* 2019). Agar plates of the CHROMagar ECC medium added with CTX (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at concentrations of 0, 0.25, 4, and 64 μg/mL were prepared as well. The concentrations of 4 and 64 μg/mL corresponded, respectively, to the revised (CLSI-M100-S22) and the previous (CLSI-M100-S17) MIC breakpoints separating resistant isolates. Agar plates with a lower CTX concentration of 0.25 μg/mL were also provided in this study because the addition of CTX at 4 and 64 μg/mL allowed the formation of colonies only of the limited population of ESBL-producers (Tsutsui & Urase 2019).

Enterobacteriaceae isolates from the water samples were obtained using the same medium that was used for the determination of *E. coli* because other Enterobacteriaceae forms mauve-colored colonies in addition to blue colonies of *E. coli*. The agar medium containing meropenem (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) with concentrations of 0.125, 0.5, and 1 μg/mL was prepared for the selective growth of meropenem-resistant Enterobacteriaceae. These concentrations added to the plates were lower than the MIC breakpoints of 4 μg/mL (CLSI 2012) and 8 μg/mL (EUCAST 2019) for resistant isolates, anticipating the selection of CRE with broad characteristics at the first incubation step.

Incubation of target bacteria

An appropriate volume of a water sample was filtered through a 47 mm diameter, 0.45 μm pore size nitrocellulose membrane filter (Millipore, Billerica, USA). Agar plates with several steps of sample volume (0.1–100 mL) were prepared with the expectation of 30–300 colonies on an agar plate. The membrane filter was then placed on the plates of the selective chromogenic media and incubated at 37 °C for 24 h. A portion of the plates were incubated at a higher temperature (42 °C) for the determination of CRE based on a report that distinguished environmental population from human-associated population (Hrenovic *et al.* 2017). Several colonies (typically 10–30 colonies for one incubation condition from one water sample) on the plates were randomly picked up and streaked on other plates under the same selective pressure again to obtain isolates.

Identification of isolates

To characterize a part of the isolates obtained from the plates under different selection pressures, full-length 16S rRNA gene sequencing was conducted for 81 isolates in total (8 isolates resistant to CTX obtained from CTX-0.25 μg/mL plates, 70 resistant and 3 intermediate isolates to meropenem obtained from meropenem-containing plates). Before sequencing, DNA was extracted using a DNA extraction kit (Kanto Chemical Co., ltd, Tokyo, Japan). The extracted 16S rRNA gene was amplified by a bacterial 16S rDNA PCR kit (Takara Bio Inc., Shiga, Japan). The amplified DNA was purified by MonoFas DNA clean up kit (GL Sciences Inc., Tokyo, Japan) and sent to Macrogen Japan Corp. (Kyoto, Japan) for sequencing with sequencing primers of 518F (5′-CCAGCAGCCGCGGTAATACG-3′) and 800R (5′-TACCAGGGTATCTAATCC-3′). The obtained two sequences were connected to give a full-length 16S rRNA sequence. The closest species or genera to the connected sequences were determined by the comparison to type strains with the BLASTN program (https://blast.ncbi.nlm.nih.gov/) provided by National Center for Biotechnology Information (NCBI), National Institute of Health, USA.

Disk-diffusion test for antibiotic susceptibility

The disk-diffusion test based on the Kirby–Bauer method was conducted to identify the susceptibility of isolates on the Mueller–Hinton agar medium (Eiken Chemical Co. Ltd, Tokyo, Japan). The antimicrobial disks containing CTX (30 μg) and meropenem (10 μg) (KB disk, Eiken Chemical Co. Ltd, Tokyo, Japan) were used in this study according to the manufacturer's instruction. The susceptibility was interpreted as susceptible (S), intermediate (I), or resistant (R) based on the comparisons of the inhibition-zone diameters around the diffusion disks with the criteria (CLSI 2012). The resistant isolates to CTX were further divided into R+ (MIC: 4–64 μg/mL) and R++ (MIC >64 μg/mL) based on the inhibition-zone diameter. The ESBL production of CTX-resistant *E. coli* was screened by the

double-disk synergy test (DDST) with clavulanic acid targeting CTX. In the DDST, the isolates showing an increase in the inhibition-zone diameter by 5 mm with the addition of clavulanic acid were identified as ESBL-producing *E. coli* based on the instruction by the manufacturer (Eiken Chemical Co. Ltd, Tokyo, Japan).

RESULTS

Colony-counts of *E. coli* and presumptive ESBL-producers

Table 2 shows the colony-counts of presumptive *E. coli* contained in treated wastewater at different locations by using CTX-free and CTX-0.25 μg/mL plates. The median colony-count of total *E. coli* in the treated effluent after chlorination was 18 CFU/mL ($n = 17$) including samples below the detection limit (0.1 CFU/mL). The median colony-count of *E. coli* on the plate containing 0.25 μg/mL CTX was 1.3 CFU/mL including six samples below the same detection limit. The ratios of the colony-counts on CTX-0.25 μg/mL plates (presumptive ESBL-producers) were distributed in a relatively narrow range between 4.4 and 9.3%. The colony-count ratio for treatment plant E, which receives wastewater without hospital effluents, was within the range of other plants. These colony-count ratios of presumptive ESBL-producers in the *E. coli* population of chlorinated effluents in Tokyo and in its suburbs were independent from sampling locations. The average colony-count ratio on CTX-0.25 μg/mL plates was 7.0% of the total *E. coli* colony-counts in treated wastewater.

Colony-counts of Enterobacteriaceae and presumptive CRE

Table 2 also shows that the median colony-count of total Enterobacteriaceae bacteria at 42 °C incubation, contained

Table 2 | Colony-counts of *E. coli* (37 °C) and Enterobacteriaceae (42 °C) on the agar plates

	E. coli			**Enterobacteriaceae 42 °C**		
		Presumptive ESBL-producers			**Presumptive CRE**	
Sample	**Total (CFU/mL)**	**(CFU/mL)**	**(%)**	**Total (CFU/mL)**	**(CFU/mL)**	**(%)**
A1, Dec 2018	19	1.4	7.4	743	0.11	0.015
A2, May 2018	16	0.9	5.6			
A2, June 2018	18	1.3	7.2			
A2, July 2018	0.34	<0.01		43	0.11	0.26
A2, Nov 2018	4.8	0.4	8.3	72.3	0.052	0.072
A5, Sep 2018	138	6.0	4.3			
B1, Jul 2018	72	5.0	6.9	505	0.07	0.014
B1, Nov 2018	59.7	3.9	6.5	305	<0.01	
B3, Sep 2018	<0.1	<0.01		0.15	<0.01	
B3, Nov 2018	0.11	<0.01		8.0	<0.01	
B4, Sep 2018	<0.1	<0.01		<0.1	<0.01	
B5, May 2018	<0.1	<0.01		0.15	<0.01	
B6, Oct 2018	60	5.1	8.5	220	0.68	0.31
B7, Oct 2018	<0.1	<0.01		1.8	<0.01	
D1, Oct 2018	15	1.4	9.3	41.5	<0.01	
D2, Oct 2018	33.7	1.5	4.5	104	<0.01	
E, Apr 2018	26.6	2.3	8.6			

in the treated effluent after chlorination, was 43 CFU/mL ($n = 13$) including samples below the detection limit (0.01 CFU/mL). All of the measured colony-counts were below 3,000 CFU/mL, which is the effluent standard of coliform group bacteria in Japan. The quantitative counts of Enterobacteriaceae on the plate containing 0.125 μg/mL meropenem (presumptive CRE) were obtained for 5 out of 13 samples due to low-resistant proportions to carbapenems in Enterobacteriaceae. The median ratio of colony-counts on the meropenem-containing (0.125 μg/mL) plates to the total counts excluding the samples of below the detection limit was 0.072%.

Characteristics of *E. coli* isolates

Figure 1 shows the results of the disk-diffusion test for the isolates obtained from the agar plates containing different concentrations of CTX. All isolates obtained from the plates containing CTX with higher concentrations than 4 μg/mL were resistant (R+ or R++) to CTX by the disk-diffusion test with a few exceptions which were lost during isolation. The results on the isolates ($n = 264$) from CTX-free plates indicated that the CTX-resistant (R+ or R++) isolates were 5.7% of the total *E. coli* population, followed by 2.3% of intermediate (I). The inhibition-zone diameters of the isolates were dependent on the concentration of CTX used during the isolation process. Most of the isolates from CTX-4 and 64 μg/mL plates were classified into R++ isolates (MIC >64 μg/mL), while the isolates from CTX-free and 0.25 μg/mL plates included a certain number of R+ isolates (MIC: 4–64 μg/mL). The isolates from the plates containing CTX at 0.25 μg/mL consisted of S (10.3%), S (6.6%), R+ (40.0%), and R++ (42.3%). The ratio between R+ and R++ (approximately 1:1) was similar to that in the original population obtained by CTX-free plates (R+: 3.0% and R++: 2.7%). The result on the identification of genera and species of the resistant isolates showed that all eight blue colonies picked up from CTX-0.25 μg/mL plates were identified as *Escherichia/Shigella* spp. based on 16S rRNA genes. This result confirmed the specificity of the chromogenic medium for the selection of *E. coli* even after the addition of CTX to the medium. The ratios of ESBL-producers by DDST among isolates from CTX-free and CTX-0.25 μg/mL plates were 4.9 and 77.0%, respectively, whereas all isolates from CTX-4 and 64 μg/mL plates were ESBL-producers except for lost isolates.

Characteristics of Enterobacteriaceae isolates

Figure 2 shows the resistance to meropenem examined for Enterobacteriaceae isolates from agar containing different concentrations of meropenem. Almost all of the isolates obtained from meropenem-free (MPM-free) plates were susceptible to meropenem regardless of the incubation temperature in accordance with a low expected proportion of CRE in total Enterobacteriaceae population (Hrenovic

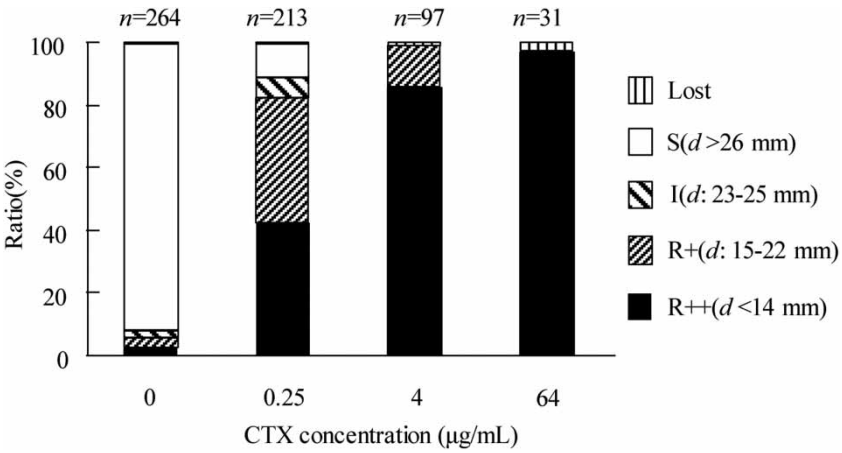


Figure 1 | Resistance to CTX of the isolates from agar plates containing different concentrations of CTX. *n* denotes the number of isolates examined for their resistance to CTX, and *d* denotes the inhibition-zone diameter around the disk containing CTX.

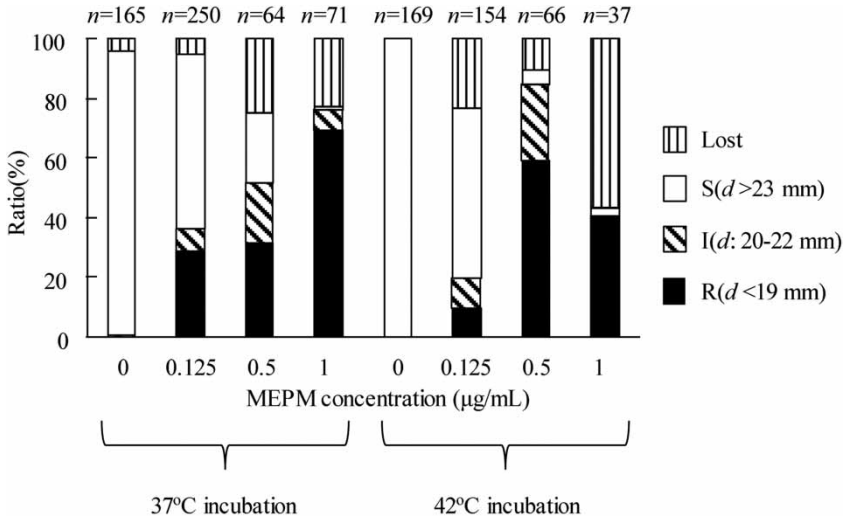


Figure 2 | Resistance to meropenem of the isolates from agar containing different concentrations of meropenem under different temperatures. *n* denotes the number of isolates examined for their resistance to meropenem, and *d* denotes the inhibition-zone diameter around the disk containing meropenem.

et al. 2017). On the other hand, a considerable number of the colonies were lost during the isolation process from meropenem-containing plates. More than 50% of the colonies were lost during isolation at meropenem concentration at 1 μg/mL under 42 °C temperature, although an increase in the meropenem concentration in the agar medium increased the proportion of real resistant isolates in the presumptive resistant population. Only 9.7% of the isolates obtained from 0.125 μg/mL plates were resistant to meropenem.

Figure 3 shows the results on the identification of the genera of the meropenem-resistant isolates. Of 39 resistant isolates obtained from 37 °C-incubation plates, 59% of the tested isolates were *S. maltophilia*, which carry intrinsic resistance to carbapenems, although only mauve-colored colonies

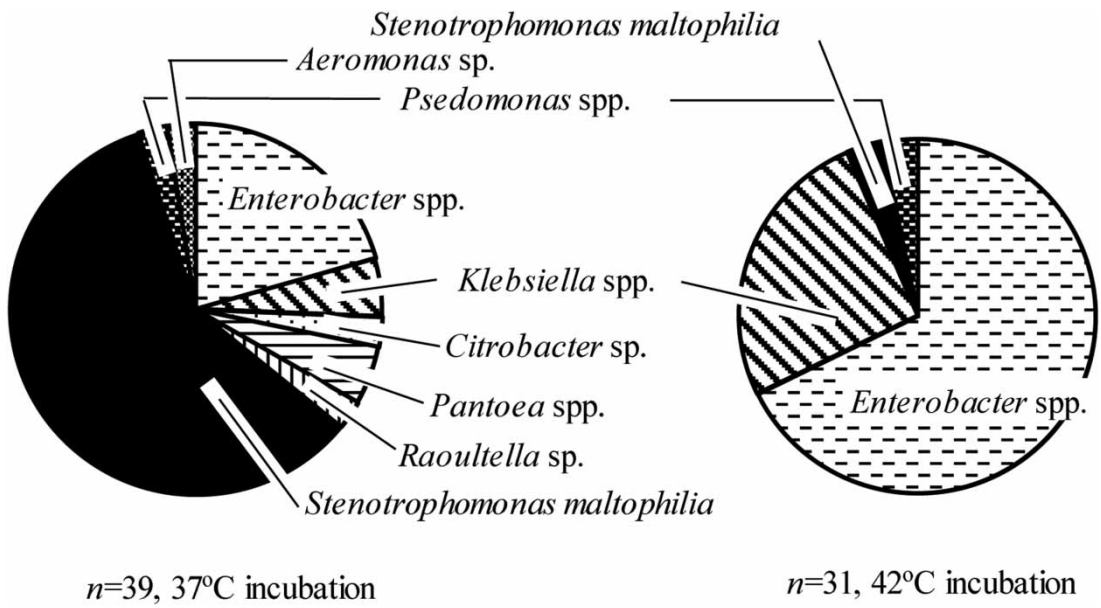


Figure 3 | The results on the identification of the genera of the meropenem-resistant isolates under different incubation temperatures. The resistance to meropenem had been confirmed in advance by the disk-diffusion test for all examined isolates.

(presumptive Enterobacteriaceae) were picked up for isolation. Incubation at 42 °C obviously suppressed the growth of *S. maltophilia*. The other isolates from 42 °C-incubation plates were identified as Enterobacteriaceae (*Enterobacter* spp. and *Klebsiella* spp.) except for one isolate of *Pseudomonas* sp. The detected genera were similar to a previous report in which CHROMagar ESBL and CHROMagar KPC were used (Haller *et al.* 2018). Blue-colored, meropenem-resistant colonies (presumptive carbapenem-resistant *E. coli*) were not observed in this study, although three colonies with intermediate resistance to meropenem were identified as *Escherichia/Shigella* spp. by the examination of 16S rRNA.

DISCUSSION

Detection of ESBL-producing *E. coli* and CRE

Environmental monitoring for treated wastewater on antibiotic resistance can elucidate the average prevalence of ARB in watersheds targeting total population in the area. In addition, environmental monitoring can detect emerging pathogens because the carriers of the pathogens do not always go to hospitals. A general procedure for the determination of resistance in bacteria is incubation first without antimicrobials before determining resistance/susceptibility according to the guidelines (CLSI 2012; EUCAST 2019). However, several difficulties may be associated with the procedure, if it is applied for the quantitative monitoring of environmental samples targeting the resistance of low incidence because it requires the examination of a few hundreds of colonies to obtain reliable resistant ratios.

It is attractive to directly count resistant colonies by adding antibiotics to agar plates for the selective growth of resistant bacteria. Several selective agar media are commercially available and successfully applied also to wastewater examination from hospitals (Haller *et al.* 2018). As shown in the Results section, the addition of antibiotics at a MIC breakpoint to agar plates would be overdose; as shown in Figure 1, most of the isolates from the plates containing CTX at 4 μg/mL had higher MICs than 64 μg/mL (the previous MIC breakpoint). This result shows that the addition of CTX at 4 μg/mL imposes too severe selection pressure to obtain the typical population of ESBL-producers because only 24–66.7% of clinical isolates of ESBL-producers were resistant to CTX with MICs higher than 64 μg/mL (Wang *et al.* 2011; Hombach *et al.* 2013; Kristo *et al.* 2013). These results, discussing the effect of the revision of the guidelines on MIC breakpoints, also showed that 78.7–100% of ESBL-producers were resistant to CTX, which suggests that CTX is a suitable antimicrobial reagent to obtain ESBL-producing population.

In the case of the isolation of CRE, as shown in Figure 3, the use of 42 °C incubation is preferable to avoid the interference by autochthonous species with intrinsic resistance to carbapenems, such as *S. maltophilia*, while the incubation at 42 °C reduced the number of the colonies of these species. The number of colonies with coloration representing Enterobacteriaceae were reduced by 42 °C incubation to 58.7% (MPM-free plates, $n = 6$), 21.4% (MPM 0.125 μg/mL, $n = 4$), 34.6% (MPM 0.5 μg/mL, $n = 2$), and 15.6% (MPM 1 μg/mL, $n = 2$) of the number of colonies with 37 °C incubation, respectively. One of the serious problems associated with the 42 °C incubation is that a considerable part of the isolates were lost during isolation with meropenem dose at 0.5 and 1 μg/mL due to excessively severe selection pressures, as shown in Figure 2. Another problem is that the quantitative monitoring of CRE is difficult even with a small concentration of meropenem added to the plates at 0.125 μg/mL. As shown in Table 1, colonies on meropenem-containing plates were countable only for 5 out of 13 samples due to low proportions of CRE in the total population of Enterobacteriaceae.

As mentioned in the Results section, the average colony-count number on CTX-0.25 μg/mL plates was 7.0% of the total *E. coli* colony-count number in treated wastewater. The average colony-count number of 7.0% can be reduced to 5.3% for ESBL-producers and 5.7% for CTX-resistant population, considering the ratios of ESBL-producers (77%) and CTX-resistant population (82%) in the presumptive ESBL-producers obtained from 0.25 μg/mL plates. The estimated proportion of ESBL-producers in this study was higher than those in the river environment in Japan (Urase & Sato 2016; Yamashita *et al.* 2017). A monitoring in Norway reported that the proportions of ESBL-producing *E. coli* were 11.5% in hospital wastewater, 6.9% in community wastewater, and 3.7% in urban wastewater (Paulshus *et al.* 2019). Another study in Ireland

estimated the proportions of CTX-resistant *E. coli* in the effluents from municipal WTPs were 4.3% with hospital wastewater and 2.5% without hospital wastewater (Harris *et al.* 2014). A similar study in Sweden showed that CTX-resistant *E. coli* were 2.1–4.4% without hospital wastewater and 14% with hospital wastewater (Kwak *et al.* 2015). It is noted that the difference between influent and effluent must be considered for the comparison of our results with some of these studies. Our observations on the proportion of ESBL-producing *E. coli* in this study would be within an expected range for effluents from large-scale WTPs which receive hospital wastewater.

As to CRE, our observation in Table 2 showed that the median ratio of colony-counts on the meropenem-containing (0.125 μg/mL) plates to the total Enterobacteriaceae counts was 0.72%. Considering the proportion (9.7%) of meropenem-resistant isolates in the presumptive CRE obtained from 0.125 μg/mL plates, the average colony-count number of 0.072% can be reduced to 0.0070% of meropenem-resistant population in total Enterobacteriaceae contained in the effluent samples. The quantitative results on the environmental CRE were rarely reported, probably due to the difficulty associated with low prevalence and the interference by the autochthonous species with intrinsic resistance to carbapenems during detection. A study in Croatia reported that carbapenem-resistant population was 0.0006% in the influents and 0.002% in the effluents of WTPs (Hrenovic *et al.* 2017). To discuss the accuracy of the estimated ratio of CRE, it is necessary to consider a large deviation of the colony-count on the plates containing meropenem, as shown in Table 1, unlike the case of the relatively stable colony-count ratio of presumptive ESBL-producers. Another limitation on the estimation of CRE proportion might be associated with the incubation temperature at 42 °C and the concentration of meropenem added to the plates. It may be expected that different selection pressures on the first incubation may affect the estimation of CRE proportion even after the correction by the real CRE ratio in the isolates of presumptive CRE. The appropriateness of the use of meropenem for the selection for CRE must also be considered.

Comparison with national surveillance

The quantitative results on the prevalence of clinically important resistant bacteria in the environments enabled the discussion on the linkage of the environment with the health of both human and animals through the comparison with national surveillance on nosocomial infections and veterinary monitoring. A recent global surveillance for the nosocomial infection database showed that the proportion of ESBL-producing *E. coli* is in the order of 15% in Europe, 10% in North America, 20–40% in southeast and east Asia, and 60–70% in China (Chong *et al.* 2018). Table 3 shows the resistance of clinical isolates reported in Japan Nosocomial Infections Surveillance (JANIS 2019),

Table 3 | The resistance of clinical isolates reported in Japan Nosocomial Infections Surveillance (JANIS 2019)

Susceptibility to CTX			**R (%)**	**I (%)**	**S (%)**
E. coli	$n = 251{,}068$	Inpatients	27.6	0.6	71.2
	$n = 230{,}427$	Outpatients	17.0	0.4	81.8
Susceptibility to meropenem			**R (%)**	**I (%)**	**S (%)**
E. coli	$n = 365{,}600$	Inpatients	0.14	0.03	99.5
	$n = 313{,}652$	Outpatients	0.02	0.01	99.5
K. pneumoniae	$n = 175{,}408$	Inpatients	0.49	0.09	99.1
	$n = 91{,}747$	Outpatients	0.08	0.02	99.5
E. cloacae	$n = 71{,}119$	Inpatients	1.06	0.26	98.3
	$n = 20{,}385$	Outpatients	0.30	0.12	99.2
K. aerogenes	$n = 35{,}448$	Inpatients	0.82	0.27	98.8
	$n = 12{,}471$	Outpatients	0.09	0.02	99.6

n denotes the number of isolates examined for their resistance.

which indicates that the resistant ratios to CTX among *E. coli* isolates were 27.5% for inpatients and 17.0% for outpatients in 2018. These proportions were considerably higher than the observation in this study (5.7%) for treated wastewater. A probable interpretation of this result is that the wastewater gathers bacteria not only from inpatients and outpatients but also from healthy people.

The most frequently detected species of CRE in the surveillance in Japan were *Klebsiella aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *E. coli* in 2018. Table 3 shows the resistance to meropenem for those four Enterobacteriaceae species in the surveillance. Meropenem-resistant proportions in clinical isolates of these species were in a range of 0.02–1.06%. Considering that wastewater collects bacteria not only from inpatients and outpatients but also from healthy people living in the area, the obtained CRE ratio in this study (0.007%) would be in an expected range. *Enterobacter* spp. were most frequently detected in the meropenem-resistant isolates probably due to relatively high-resistant incidences of this genera in the surveillance.

According to a veterinary monitoring program, CTX-resistant proportions in *E. coli* isolated from Japanese livestock in 2015 ranged from 0.0% (cattle and pigs at slaughterhouses) to 3.1% (healthy broilers on farms) (JVARMS, 2018). A significant decrease in these resistant ratios was observed during 2008–2015 especially for broilers, due to a regulation against the use of third-generation cephalosporins in preventive inoculation (Hiki *et al.* 2015). The limited spread of ESBL-producing *E. coli* in animals was also reported (Diallo *et al.* 2013). The observed ratio of ESBL-producing *E. coli* in treated wastewater observed in this study was higher than that obtained from veterinary monitoring program, which would be a general tendency for antibiotics that are used mainly for humans but usually not for animals.

CONCLUSION

In this study, the proportions of ESBL-producing *E. coli* and carbapenem-resistant Enterobacteriaceae in treated wastewater after chlorination were quantified at 12 different treatment plants located in Tokyo and in its suburbs. For the quantitative monitoring of these resistant bacteria with low proportions in the total population, the plate-count method with relatively mild selection pressures was combined with the correction by the examination of presumptive resistant isolates. The estimated proportions of ESBL-producers and CTX-resistant isolates were 5.3 and 5.7% of the total *E. coli* population in treated wastewater, respectively. The estimated CRE proportion in the total Enterobacteriaceae population was 0.007% with the constituting species of *Klebsiella* spp. and *Enterobacter* spp., although the selection pressures during the first incubation may have affected the proportion. The observed proportion of resistant isolates were lower than those in the surveillance on nosocomial infection not only for inpatients but also for outpatients, and higher than those in the veterinary monitoring. Bacterial population in treated wastewater might be dependent on the dilution of bacteria originated from inpatients and outpatients with bacteria originated from healthy people.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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