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Original Research

Insufficient decontamination in Sewage treatment plants induce the risk of artificial selection of extended-spectrum β-lactamase producing *Escherichia coli*

A. Gundogdu[1,2*], H. Kilic[2], C. Kurekci[3], E. Alp[4]

[1] Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Erciyes University, Kayseri, Turkey
[2] Division of Metagenomic, Genome and Stem Cell Centre (GenKok), Erciyes University, Kayseri, Turkey
[3] Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Mustafa Kemal University, Hatay, Turkey
[4] Infectious Diseases and Clinical Microbiology University, Faculty of Medicine, Erciyes University , Kayseri, Turkey

Correspondence to: agundogdu@erciyes.edu.tr

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Abstract: Quantitative data about extended-spectrum beta-lactamase producing *E. coli* (ESBLEC) in the wastewaters are scarce, especially in developing countries. These data could be useful to raise awareness about the potential risk of spreading ESBLEC strains in the community. Water samples were collected weekly over a 10-week period, from one urban sewage treatment plant (STP), one rural STP and one hospital complex's wastewater (HWW) in Turkey. Mean *E. coli* and ESBLEC loads were determined for each sampling point. For the 580 ESBLEC isolated, antimicrobial resistance profiles, phylogenetic grouping, presence of common beta-lactamese-typesand integrons were studied using PCR. The mean ESBLEC ratio was accounted for 0.58%, 0,12%, 1.53% of the total *E. coli* in urban, rural untreated wastewater and HWW, respectively. These values were higher for the outlets. The mean number of different antimicrobial classes to which the strains were resistant was highest in urban STP (4.0± 1.6). The antimicrobial resistance ratios of ESBLEC strains isolated from HWW were observed to be in between those of urban and rural STPs. The most common phylogenetic group was C composing (29.7%) and the most susceptible strains belonged to phylogroup B1. Wastewater treatments without sufficient decontamination, resulting in artificial selection of ESBLEC might lead to public health risk as these strains reach communities through environment. To avoid such risks and protect the human health as well as the environment, well-established decontamination measures imposing barriers against this artificial selection should be implemented.

Key words: ESBL-producing *E. coli*; Sewage treatment plants; Hospital wastewater; Phylogenetic grouping; Integrons.

Introduction

The intensive use of antimicrobial agents in clinical settings, agriculture, aquaculture and livestock industries has led to emergence of multidrug resistant (MDR) bacteria in a variety of environments (1-4). The primary media for the dissemination of MDR strains and the resistance genes are defined as human and animal microbiome, hospitals, contaminated/wastewater systems, and soil (4-6). Among these media, water systems have special importance as they are all in contact with others with an unstable nature. Therefore, contaminated water systems can not only be defined as a public health risk factor to due to the presence of resistant pathogenic microorganisms, but also as the vehicle for the transfer of resistance genes in the ecosystem.

As members of gut microbiome, *E. coli* strains are continuously released into the municipal sewage systems via defecation. These strains may gain resistance genes via mobile genetic elements leading to emergence of extended-spectrum-beta-lactamase producing strains (ESBLEC) due to the favorable environment of wastewater (7-8). Up to now,very limited data are provided by strain-level quantitative studies of ESBLEC in wastewater systems (9-10) with even lesser data from developing countries, where the misuse and/or overuse of antibiotics are common (11).

The objectives of this study are, therefore to (i) quantify the ESBLEC load of a hospital complex and an urban area as well as a rural sewage system network in a metropolitan city in Turkey, (ii) to compare the *E. coli* and the ESBLEC loads between hospital / rural / urban wastewater, (iii) to determine the effect of wastewater treatment on antimicrobial resistance profiles of *E. coli* and ESBLEC strains, and (iv) finally to characterize ESBLEC with respect to their phylogenetic groups, ESBL-subtypes and the presence of different classes of integrons.

Materials and Methods

Sites and sampling

This study was carried out in a metropolitan city with about 1 million inhabitants, in Turkey. A tertiary hospital complex's (five hospitals with 1300-bed) wastewater outlets, one urban sewage treatment plant (serving $1x10^6$people) and one rural sewage treatment plant (serving $2x10^4$people) were selected for this study. The hospital complex's wastewater was not subjected to any treatment and is directly connected to urban sewage canals via two main outlets. The urban sewage treatment plant receives effluent from the community sewage, several industrial areas, hospital sites (including selected hospital complex), and livestock farming.The

rural sewage treatment plant receives effluent only from household waste and included the study as a reference of human contribution.The urban sewage is subjected to biological treatment with four typical processes (pre-treatment, primary treatment, secondary treatment and biological nutrient removal) and discharges the treated effluent into the nearby River. The rural sewage treatment plant includes pre-treatment and primary-treatment stages and dischargesthe treated effluent into the near waterway.

Ten weekly samples were collected from 5 sampling sites between February and May 2015. These include (i) two main outlets of the untreated hospital complex's wastewater (HWW), (ii) the incoming effluent of the urban sewage treatment plant (uSTP-I), (iii) the outlet of the treated effluent water (uSTP-O), (iv) the incoming effluent of the rural sewage treatment plant (rSTP-I), and finally (v) the outlet of the rural sewage treatment plant (rSTP-O). Samples were collected and processed in accordance with the Australian and New Zealand Standards for Water Microbiology and Water Quality Sampling (12-14). HWW samplings were collected from both the main outlets, one serving to the west side of the complex and the other to the east side of it. Each week, two water samples were mixed in equal volumes and the total mixture was analysed.

E. coli and ESBLEC load determination

Total *E. coli* and ESBLEC loads were determined for each sample using a membrane filtration system (including serial dilution method) followed by culture on mFC Agar (Oxoid) without and with 32µg/ml of ceftazidime. Three random colony-forming units per plate were chosen and confirmed as *E. coli* by the presence of *uspA* gene that is the highly specific *E. coli* universal stress protein gene (15). The clearance rate at the STPs was determined as follows: [(mean bacterial load in untreated water – mean bacterial load in treated water)/mean bacterial load in untreated water] × 100.

Isolation and identification of ESBL producing E. coli

HWW and the STPs' incoming samples were processed using serial dilutions and cultured on mFC Agar containing 32µg/ml of ceftazidime. STPs' outgoing effluents were processed by membrane filtration and filtered samples were cultured on two mFC Agar containing 32µg/ml of ceftazidime and up to 28 suspected ESBLEC colonies (where possible) were picked up from the agar plates for further analysis. DNA was extracted using Qiagen Genomic DNA Extraction Kit as per manufacturer instructions. All isolates were confirmed as *E. coli* by *usp*A gene (15).

Phenotypic detection of ESBL production and antimicrobial susceptibility testing

ESBL production of the isolates was initially screened according to the Clinical Laboratory Standards Institute (CLSI) (16). Antimicrobial susceptibility testing was performed by disk diffusion method on Mueller Hinton agar with antibiotic disks according to the CLSI (16). The antibiotic discs used in this study were amoxicillin/clavulanicacid(AMC30µg, Oxoid), cefoxitin (FOX30µg, Oxoid), imipenem (IMI10µg, Oxoid), ciprofloxacin (CIP5µg, Oxoid), ertapenem (ETP10µg, Oxoid) gentamicin (CN10µg, Oxoid), tetracycline (TE30µg, Oxoid), nalidixic acid (NA30µg, Oxoid), norfloxacin (NOR10µg, Oxoid), kanamycin (K30µg, Oxoid), chloramphenicol (C30µg, Oxoid), and tygecycline (TGC15µg, Oxoid).A colistin minimum inhibitory concentration was searched by E-test in accordance with the CLSI (16).

Molecular study

All ESBLEC strains were further tested for the presence of four common ESBL-types encoding TEM-, SHV-, OXA-, and CTX-M-type beta-lactamases (17-19) and class 1, 2, 3 integron-associated integrase (20) using PCR. Representative amplicons were determined based on grouping sample parameters (e.g. sampling point and date, resistance profile, phylogenetic characteristics, existence of integrons) and they were sequenced to be compared to the NCBI GenBank records.

Phylogenetic grouping (A, B1, B2, C, D, E, F and clade 1) was done using multiplex PCR with primers coding for *arp*A,*chu*A and *yja*A genes and the DNA fragment TSPE4.C2 according to Clermont et al. (21).

Statistical analysis

Qualitative characteristics of geographical sampling points based on *E. coli* and ESBLEC counts were reported using their means and standard deviations. Correlation tables between the integrase genes and phylogenetic groups, integron existence and corresponding antibiotic resistance were tabulated and Chi-square and Fisher's exact tests were employed. The tests were performed for each gene and the corresponding p-values were calculated.Matlab (R12) scientific computing platform was used for the statistical calculations.

Results

Total E. coli and ESBLEC loads

Whilst the mean *E. coli* loads were similar in urban/ rural untreated wastewater (220000±177828 vs 222600±150421 CFU/mL, respectively) and treated water (1071±1013 vs 1096±1303 respectively), these values were nearly ten-fold lower in HWW (20582±23261 CFU/mL). The mean number of ESBLEC were found to be significantly (Student's t test: $p<0.001$) higher in uSTP-I (1637 CFU/mL) than in rSTP-I (136 CFU/mL). The mean ESBLEC ratio was accounted for 0.58%, 0.12%, 1.53% of the total *E. coli* in urban, rural untreated wastewater and HWW respectively. This ratio was higher in the treated wastewater compared to the untreated wastewaters in both STPs (Table1). In the rSTP, 95% of *E. coli* were removed on average during the treatment stages where that value was 94% for ESBLEC in rSTP. This value in the uSTP was 99% for *E. coli* removal and 98% for ESBLEC removal.

Antibiotic resistance patterns of ESBLEC

Overall, 580 confirmed ESBLEC strains were isolated from HWW (n=107), uSTP-I (n=137), uSTP-O (n=117), rSTP-I (n=115), and rSTP-O (n=104) within ten weeks of sampling. Antimicrobial resistance rates among the ESBLEC strains varied in samples collected from different sites (Table1). The mean numbers of

Table 1. Mean *E. coli* and ESBLEC load in water samples with the antimicrobial resistance rates.

Sampling Sites (No of isolates)	Mean*E. coli*load± Std*	Mean ESBLEC load± Std*	ESBLEC rate**	The rate of resistant strains against selected antimicrobials										
				IMI	AMC	FOX	CIP	ETP	CN	TE	NA	NOR	K	C
uSTP-I (137)	220000 ± 177828	1637±2896	0,5808	14,59[1]	67,15[1]	53,28[1,6]	67,88[1]	11,67[1]	38,68	65,69[1,6]	80,29[1]	67,15[1,6]	44,5[6]	9,48
uSTP-O (117)	1071 ± 1013	8±8,24	0,951	13,67	48,71	40,17	60,68	13,67	58,97[2]	76,06	77,77	53,8	46,15	22,22[2]
rSTP-I (115)	222600±150421	136±177	0,121	5,21	22,6	10,43	47,82	0	61,73[3,8]	50,43	59,13	28,69	33,9	28,69[3]
rSTP-O (104)	1096±1303,2	6±9,42	0,6767	6,73	25	6,73	37,5	6,73[4]	55,76	53,84	46,15	23,28	27,88	23,07
HWW (107)	20582±23261	278±409	1.531	9,34	57[7]	16,82	59,81[7]	7,47[7]	38,31	50,46	76,63	43,9[7]	30,84	33,64[5]

*CFU–colonyforming units in 1mL water sample, **Mean ESBLEC rate in total *E. coli* ,uSTP-I; the incoming effluent of the urban sewage treatment plant, uSTP-O; the outlet of the treated urban effluent water , rSTP-I; the incoming effluent of the rural sewage treatment plant, rSTP-O; the outlet of the treated rural effluent, HWW; untreated hospital complex's wastewater. IMI; imipenem, AMC; amoxicillin/clavulanic acid, FOX;cefoxitin, CIP; ciprofloxacin, ETP;ertapenem, CN; gentamicin, TE; tetracycline, NA;nalidixic acid, NOR;norfloxacin, K; kanamycin, C; chloramphenicol.

Significance level; p=0.05.[1] strains from uSTP-I had significantly higher resistant than strains from the rSTP-I, [2] strains from uSTP-O had significantly higher resistance than the uSTP-I strains, [3] strains from rSTP-I had significantly higher resistance than the uSTP-I strains, [4] strains from rSTP-O had significantly higher resistance than the rSTP-I strains, [5]strains from HWW had significantly higher resistance than the uSTP-I strains, [6]strains from uSTP-I had significantly higher resistance than the HWW strains, [7]strains from HWW had significantly higher resistance than the rSTP-I strains, [8]strains from rSTP-I had significantly higher resistance than the HWW strains.

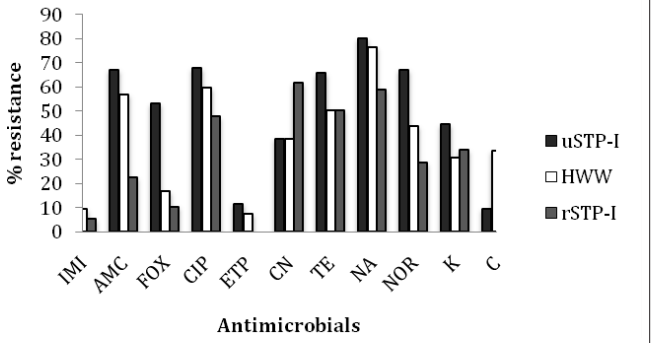

Figure 1. Percentage of multi-drug resistant ESBLEC (n=580) isolated from urban sewage treatment plan inlet (uSTP-I, blackbars) untreated hospital wastewater (HWW, White bars) and rural sewage treatment plant inlet (rSTP-I, greybars). IMI, imipenem; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; CIP, ciprofloxacin; ETP, ertapenem; CN, gentamicin; TE,tetracycline; NA, nalixidicacid; NOR, norfloxacin, K, kanamicin; C, chloramphenicol.

different classes of antimicrobials to which the strains were resistant were determined to be 4.0±1.6 in uSTP-I, 3.8±1.5 in uSTP-O, 2.8±1.6 in rSTP-I, 3.2±1.5 in STP-O, and 3.3±1.8 in HWW. In general, the antimicrobial resistance ratios of ESBLEC strains isolated from HWW were observed to be in between the ratios of urban and rural STP inlet isolates except for kanamycin (Figure 1). All strains included in the study were susceptible to tygecycline and colistin.

Phylogenetic typing

Phylogenetic grouping showed that strains belonging to phylogenetic group C were abundant in UHWW (47.7%), uSTP-I (33.5%) and uSTP-O (30.6%), whereas strains belonging to phylogenetic group A were found to be in significantly ($p<0.001$) higher ratios in rSTP-O (41.3%) samples (Table 2).

Distribution of antimicrobial resistant strains among different phylogenetic groups was analyzed. It was found that the distribution of quinolone resistant strains wassignificantly (Chi-square test, $p<0.01$) different between the phylogenetic groups. CIP resistant strains were more prevalent i.e.78.9% and 63% among B2 and C respectively, this rate was as low as 20% for group A. Prevalence of NA resistant strains for these two phylogroups was 78.9% and 70.4% respectively. This figure for phylogroup A was only 5%. While 68.4% and 48.1% of NOR resistant strains belonged to phylogroupsB2, no NOR resistant strain belonged to phylogroup A. Furthermore, all strains resistant to FOX were found in phylogroup C. The most susceptible strains belonged to phylogroup B1, and the order of their prevalence was as B1>A>E>C>B2.

Prevalence of ESBL and intI genes in ESBLEC strains

According to the PCR results, in the range of 67% and 94 % strains isolated from different sources were found to be carrying CTX-M-type ESBL. 98.6% of the isolates carrying CTX-M-type ESBL also contain TEM-type beta-lactamase. The sequencing analyses showed that only one of them was classified as ESBL (TEM-201). For SHV-type and CTX-M-type following subtypes were detected; SHV-12, SHV-2a, CTX-M-15, CTX-M-3, and CTX-M-1.55% (n=319) of the tested ESBLEC were found to be *Int*1 positive, and 0.4% (n=2) were *Int*2 positive (Table 2).

Table 2. Distribution of beta-lactamase genes, phylogenetic groups and integrons among ESBLEC strains found in STPs and HWW.

	% of positive strains (no of strain)				
ESBL-types	**uSTP-I (n=137)**	**uSTP-O (n=117)**	**rSTP-I (n=115)**	**rSTP-O (n=104)**	**HWW (n=107)**
SHV-type	0	18.3	0	10.3	0
CTX-M-type	92	67	94	74	84
TEM-type	47	55	50	76	68
OXA-type	0	0	0	0	0
Integrase					
intI1	76.6 (105)	54.7 (64)	44.3 (51)	50 (52)	43.9 (47)
intI2	0	1.7 (2)	0	0	0
intI3	0	0	0	0	0
Phylogenetic groups					
A (119)	14.5 (20)	21.4 (25)	21.7 (25)	41.3 (43)	5.6 (6)
B1 (78)	5.1 (7)	5.1 (6)	0	34.6 (36)	27.1 (29)
B2 (107)	24.1 (33)	13.8 (15)	35.6 (41)	4.8 (5)	12.2 (13)
C (172)	33.5 (46)	30.6 (36)	25.2 (29)	9.6 (10)	47.7 (51)
D (0)	0	0	0	0	0
E (81)	20.4 (28)	20.5 (24)	12.1 (14)	9.6 (10)	4.7 (5)
F (0)	0	0	0	0	0
Clade1/2(3)	2.1 (3)	0	0	0	0
Unknown (20)	0	9.4 (11)	5.2 (6)	0	2.8 (3)

uSTP-I; the incoming effluent of the urban sewage treatment plant, uSTP-O; the outlet of the treated urban effluent water , rSTP-I; the incoming effluent of the rural sewage treatment plant, rSTP-O; the outlet of the treated rural effluent, HWW; untreated hospital complex's wastewater.

Discussion

Several studies have identified the presence of antibiotic-resistant *E. coli* not only in clinical samples but also in environment (22-24). However, very limited data are available on the strain-level quantitative data of ESBLEC in these sources especially in developing countries (25). This study aimed to exhibit a comprehensive data on this topic. To our knowledge, this is the first comprehensive study from a developing country and also the first study to use an STP treated with only human waste (rSTP) as a reference source to substantiate the contribution of the non-community MDR-ESBLEC strains.

Here we showed that whilst the *E. coli* load in STPs' inlet were similar, ESBLEC load as well as the their ratio were significantly higher in uSTP-I than rSTP-I. This difference can be explained by the urban and rural waste diversities. The rSTP included in this study only receives waste from households, whereas uSTP serves one million population and receives waste from community, animal husbandry, industrial areas as well as hospitals. Thus, it is anticipated that high number of resistant bacteria as well as antimicrobial agents from urban structure are constantly released into the urban sewage, dictating resistance characteristics of microorganisms. In the present study, the ESBLEC strains from uSTP-I found to be resistant against greater number of antibiotics tested than the strains from rSTP-I (4.0± 1.6 versus 2.8±1.6). In accordance with this observation, in the current study ESBLEC isolated from uSTP-I found to be carrying greater rate of *intI*1 (76%) than ESBLEC isolated from other sources. However, as the discharge of resistant bacteria is the important output of the wastewaters (26-27), it has been suggested that factors such as the density of resistant bacteria and antibiotics, duration of exposure to the antibiotics, and presence of a favorable environment are serious determinants for resistance gaining process (28-31).Thus, it is rather difficult to conclude whether the higher prevalence of ESBLEC strains or greater resistance rate in uSTP was due to the constant release of large numbers of these strains from diverse sources (e.g. hospital wastewater, animal husbandry) or due to their tendency-higher prevalence of integrons might pose the emergence of novel resistance strains via horizontal transfers- to gain resistance characteristics in the urban sewage or due to the contribution of both. Nevertheless in all cases composition of the urban sewage showed a greater potential for antimicrobial resistance.

The uSTP clearance rate average for ESBLEC in this study (99%) is similar to previous report (9). However, this rate was higher than the rSTP rate (95%). This might be explained by the lack of a secondary treatment stage of the rSTP. Similar to other findings (8, 32), both water treatment processes increased the mean ESBLEC rates from 0.58% to 0.95% in uSTP and from 0.12 to 0.67 in rSTP. In addition, antimicrobial resistance rates of ESBLEC strains against certain agents (i.e. CN and C for urban sewage and ETP for rural sewage) were found to be significantly increasing after treatment. It has been hypothesized that antibiotic resistant strains have ecological advantages over antibiotic sensitive strains during the treatment process of the STP despite the fact that *E. coli* can be removed more efficiently from the sewage than the other species (33). Although water treatment eliminated majority of ESBLEC, the higher ESBLEC rates found in the outlet of the STPs, supports this hypothesis on ecological advantage of resistant isolates. It is therefore conceivable that some of the ESBLEC survive treatment stages of the both STP to be released into the environment. In the present study we showed that the release of such resistant strains from the STP outlets into the environmental waters occurs with a relatively higher abundance in accordance with the previous studies (22, 34). As the wastewater cycles back to the environment through STPs with a certain resistant strain load, this natural selection of these resistant strains acts to amplified and returns the resistant strains back to the environment. Considering the daily use of the treated water (e.g. agricultural use) results in the exposure of human populations to such resistant strains and poses an alarming risk to public health.

As previous studies showed (9, 28) the *E. coli* load in HWW included in this study found to be significantly lower than urban and rural STP inlet loads. This might be related to the dilution of wastewater due to the discharge from the hospital facilities e.g. kitchens/laundries. However, the total ESBLEC rate in HWW was found to be at least 3 fold higher than the rate of uSTP-I and 12 fold more than that of rSTP-I, which does not receive any waste from any hospital settings. Although the effect of hospital waste on STPs' resistant microbiological load is a controversial issue (26), the current study certainly reflects that ESBLEC in HWW is one of the main sources for ESBLEC load in receiving STPs. Thus, the presence of relatively high fraction of ESBLEC strains among *E. coli* might be the reason for the corresponding high ESBLEC load in HWW. Interestingly in this study, HWW strains had lower resistance profiles than strains from urban sewage and higher than rural sewage. Therefore, it can be claimed that HWW might not be a primary source of resistance determinants of urban STPs.

These characteristics might be attributed to the contribution of the remaining component of urban structure; the livestock or industrial area. The lack of strict regulations in antimicrobial usage in developing countries might be a factor promoting resistance of the ecosystem resistome (11). Moreover, the significant increase in the gentamicin resistance ratio after two treatment stages might be explained by the durability of gentamicin resistance mechanisms to the treatment applied in STPs.

The association of the phylogenetic groups with the ability of *E. coli* to cause diseases at specific sites of the human body has been widely reported, and allowed for separation of commensal (A and B1) and pathogenic (B2 and D) strains (35-36). However, in 2013 Clermont's phylogenetic groups were modified and the number of groups increased from four to seven (21). In this study, the majority of ESBLEC strains, especially from HWW, belonged to newly identified Group C which has not been regarded as pathogenic or commensal *E. coli* genotype as yet. Thus, considering the resistant trait and sources of the strains belonging to Group C, it might be postulated that Group C could be a pathogenic group such as B2. In addition to this, taking the antimicrobial

susceptibility rates and very little isolation rates (5%) from HWW into account, Group E might be considered as a commensal/environmental group. However, we did not test these strains for the presence of virulence genes or for their pathogenicity. Further studies containing isolates from different sources need to be conducted to validate these claims.

CTX-M-type ESBL is known as resistance elements in community-onset infection pathogens, where SHV-type genes are usually observed as the resistance elements among nosocomial infection pathogens (37). The detection of CTX-M-type ESBL in STP isolates was in accordance with the previous studies (9, 25). However, interestingly in both STPs, SHV-type ESBL carrying *E. coli* were only isolated after treatment. This difference in the distribution of SHV-type genes among the inlet and the outlet of STPs isolates could be partly explained by the low concentration of pathogens that escaped our detection as well as their durability to the treatment.

In conclusion, the mean ESBLEC rates, being dominantly highest in the HWW, lowest in rSTP, and mediocre in uSTP, provide clues on the ESBLEC load contributions of the putative sources. As rSTP contains only human contribution, it can be considered as a reference of human contribution. uSTP, carrying relatively higher ESBLEC ratio might be enriched in ESBLEC load by the contribution of the most concentrated ESBLEC carrier observed: the HWW, i.e. hospital waste might be one of the main contributors of ESBLEC in urban wastewaters. On the other hand, observing the dominantly highest antimicrobial resistance ratios as well as the highest antimicrobial resistance diversity in uSTP might indicate that another source apart from hospital waste and human waste elevates the resistance profiles, which would possibly be the industrial waste. The same trend also applies for the presence of *intI*1 carriage characteristics. Wastewater treatments impose a selective advantage on ESBLEC strains. Considering that some of these strains survive through the treatment stages, ESBLEC strains as well as resistance genes or new resistance combinations could have a potential to reach clinic via environmental dissemination. In order to control the positive feedback in resistome-mobilome cycle, microbiological treatment such as chlorination or UV treatment should be introduced to the sewage treatment systems.

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Competing Interests
None.

Ethical Approval
Not relevant.

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