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Molecular characterization of extended spectrum beta lactamase producing *Escherichia coli* in two different wastewater treatment plants in Hatay Province, Türkiye

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Wastewaters are considered as hotspot for multidrug resistant bacteria and genes, especially for extended spectrum beta lactamase producing *Escherichia coli* (ESBL-EC), which pose significant public health concern. Conventional wastewater treatment plants (WWTPs) are not designed to remove such resistant bacteria before discharge. This study, therefore, aimed to evaluate and compare the performance of two municipal WWTPs in eliminating ESBL-EC in Hatay province, Türkiye. The isolates were further characterized by antimicrobial susceptibility testing, pulsed-field gel electrophoresis (PFGE) and resistance gene analysis. A total of 24 wastewater samples [influent (n = 12) and effluent (n = 12)] were collected from both conventional and advanced biological WWTP. A total of 66 ESBL-EC were isolated and confirmed as ESBL producer. The abundance of ESBL-EC was significantly more prevalent in the influent water samples (mean 3.86 ± 0.11 log cfu/ml), when compared to the effluent wastewater samples (mean 2.20 ± 0.27 log cfu/ml). According to the PFGE results (cut-off > 85%), 16 isolates were clonally related, whereas 49 isolates were singletons and one isolate could not be evaluated. Besides beta-lactam antibiotics, the isolates were highly resistant to tetracycline (63.63%) and fluoroquinolones (43.93%). The bla_{CTX-M} gene was the most frequently detected gene in the isolates (86.36%), among which the $bla_{CTX-M-15}$ type (86.36%) was the most frequently detected. At least two disinfectant resistance genes were also detected in each of the isolates. Even though both WWTP were found to be effective in reducing ESBL-EC counts, not well enough to complete elimination, showing the potential public health risk.

Keywords ESBL, *E. coli*, Wastewater treatment, Antibiotic resistance

Antibiotic resistance (AMR) has been one of the most serious global concerns in recent decades, posing a considerable threat to human health as well as adding financial burden on health-care systems. Antibiotic misuse and overuse are thought to be key contributors to the evolution of resistance strains[1], which has been linked to veterinary, medical, and agricultural settings including aquatic production. Many factors such as geographic location, socioeconomic status, climate, antibiotic consumption, and treatment regimens of infections have been shown to alter the abundance and dissemination of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB)[1,2]. Wastewater treatment plants (WWTP) recently attracted great attention enabling monitorization of hazardous materials including infectious agents. Although wastewater treatment comprises many steps and employ different methods (mechanical, biological, physical, chemical, and physical–chemical), for which technology is constantly improving, it is insufficient for total eradication of antibiotics, ARB, and ARGs[3]. Furthermore, the presence of antibiotics in aquatic niches at sub-inhibitory doses create selective pressure, with the likely promotion of other substances, such as pesticides, detergents, and heavy metals, in the sewage for the co-selection of resistant strains[4]. Zhuang et al. analyzed articles published in PubMed from 1990 to 2020 pertaining to reports of ARGs in the environment, especially in WWTPs, and found that the genes

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encoding beta-lactamases had the highest frequency all around the world[5]. In addition, Bréchet et al. estimated more than 600 billion of extended spectrum beta lactamase producing *Escherichia coli* (ESBL-EC) cells released into the receiving rivers daily in France[6].

ESBLs are enzymes that hydrolyze a broad spectrum of beta lactam antibiotics, which confer resistance to penicillins, 3rd generation cephalosporins and monobactams. To date, many ESBLs have been identified, among which CTX-M-15 type represents the most frequently encountered one[7]. Notably, CTX-M-15 producing *E. coli,* especially those belonging to the ST131, have emerged as a significant cause of urinary and blood infections in humans and is frequently found in wastewater[7,8]. The gene $bla_{\text{CTX-M}}$ is mostly found on plasmids, which were also found to carry other resistance genes conferring resistance to fluoroquinolones, aminoglycosides, and disinfectants, limiting the availability of effective therapeutic alternatives[9,10]. Public health authorities, including the Centers for Disease Control and Prevention, have categorized ESBL-EC as serious and critical threats. The occurrence of ESBL-EC continues to increase globally despite the introduction of infection prevention and control strategies intended to prevent further spread in healthcare settings[11]. For instance, with 59,900 reported deaths worldwide in 2019 linked to 3rd generation cephalosporin-resistant *E. coli* infections, the associated morbidity and mortality probably increased with prevalence [12].

Many studies have been conducted on the diversity and composition of the bacterial communities in WWTPs (reviewed in[1]) and there have been reports of ESBL-EC bacteria in wastewater and rivers in Türkiye[13,14]. However, a comprehensive comparison of bacterial communities and AMGs in WWTPs treating different types of wastewaters is needed for the development of efficient and consistent treatment processes. Accordingly, the main objective of this study was to examine and compare the performance of two municipal WWTPs in the removal of ESBL-EC. These WWTPs differs in their fundamental modes of operation, for which the one operates under a conventional treatment process, while the other employs advanced biological processes, that embodies novel biological approaches, harnessing through newly identified microbial strains with enhanced nitrogen or phosphorus removal capabilities via enrichment or cultivation of specific microbial species[15]. The present study further aimed to investigate the molecular characteristics of ESBL-EC by determining antimicrobial susceptibility, ARGs including disinfectant and providing epidemiological relatedness.

Material and methods

Wastewater treatment plants and sample collection

Sampling was carried out from March to May in 2021 in two different WWTPs (Iskenderun advanced biological treatment and Antakya conventional treatment) in Hatay province, for which detailed information are provided below (Table 1). A total 24 water samples (250 mL each) were collected, comprising six influent and six effluent samples from each of the two treatment plants.

ESBL-EC count and bacterial isolation

For ESBL-EC enumeration, water samples were serially diluted (1/10) and cultured on Tryptone-bile X glucuronide agar (TBX) medium (Merck, Germany) containing ceftazidime [2 µg/ml, above the epidemiological cut-off value established by the European Committee on Antimicrobial Susceptibility Testing (https://mic.eucast.org/search/)]. After 24 h of incubation at 37 °C, blue-green colonies showing typical *E. coli* characteristics were counted in all petri dishes. For each sample, suspicious blue-green *E. coli* colonies (1–5 colonies) were selected and inoculated on blood agar (Merck, Germany) for purification. Finally, all bacterial isolates were stored at -80 °C in Brain Heart Infusion Broth with 20% glycerol.

Species level identification

Species level identification of the isolates was performed by polymerase chain reaction (PCR) using the *usp*A (universal stress protein A) gene specific for *E. coli*[16]. The primers used for the *usp*A gene region were F-5' CCG ATACGCTGCCAATCAGT and R-5' ACGCAGACCGTAGGCCAGT. *E. coli* ATCC 25,922 DNA was utilized as the positive control, while DNA and RNA-free water was utilized as the negative control.

Antibiotic susceptibility testing and ESBL confirmation

Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method on Mueller–Hinton agar, in accordance with Clinical Laboratory Standards Institute (CLSI) guidelines[17] for the following antibiotics: amoxicillin + clavulanic acid (20 + 10 µg) cefoxitin (30 µg), cefotaxime (30 µg), cepefime (30 µg), cefuroxime (30 µg), cephalothin (30 µg), ceftiofur (30 µg), aztreonam (30 µg), ampicillin (10 µg), imipenem (10 µg), tetracycline (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), and fosfomycin (200 µg). The plates were incubated at 37 °C for 24 h. *E. coli* ATCC 25,922 was used as a reference strain.

	Facility type	Current capacity (m^3/day)	Amount of wastewater treated/ Discharged (m^3/sec)	Discharge point	Service provided by population	Formed by AAT mud amount presence (tons/day)
Iskenderun WWTP	Advanced biological (Phosphor eliminated)	86.400	0.41	Mediterranean sea	438.030	8.03
Antakya WWTP	Conventional (Trickle filter)	28.800	0.1	Asi river	160.220	0.49

Table 1. The status of urban wastewater treatment plants (Hatay Metropolitan Municipality, 2020).

Confirmation of ESBL production was done according to CLSI criteria via using double disk synergy and disk combination methods[17].

Pulsed field gel electrophoresis (PFGE) and phylogenetic subtypes of ESBL-EC isolates

Clonal relationship of ESBL-EC isolates from wastewater were determined by PFGE using *Xba*I enzyme. The protocol specified by PulseNet (www.cdc.gov/pulsenet) was used for PFGE analysis. Band profiles analysis was performed using BioNumerics software 7.6 (Applied Maths, Gent, Belgium). Phylogenetic types of *E. coli* isolates (A, B1, B2, C, D, E and F) were determined by multiplex PCR using the protocol reported by Clermont et al.[18]. The primers (*chuA*, *yja*A, *TspE4C2*, *ArpAgpE*, *trpAgpC1* and *ace*K) were used and phylogenetic typing of the isolates was determined according to the combinations described by Clermont et al.[18].

Detection of antibiotic resistance genes

ESBL-EC isolates were screened for the presence of the bla_{SHV}, bla_{CMY-2}, bla_{CTX-M} and bla_{TEM} genes by multiplex PCR using primers reported by Ahmed et al.[19]. DNA sequencing of amplicons was carried out by Sanger sequencing (Metsantek, Türkiye). The sequencing results were compared to those available in GenBank using the BLAST tool (http://blast.ncbi.nlm.nih.gov/) to identify subtypes. Quinolone resistance genes (*aac(6)-Ib*, *qnrA*, *qnrB*, *qnr*C and *qnrD*) were screened by using PCR as reported by Cavaco et al.[20]. Disinfectant resistance genes (*qacEΔ1*, *qacF/H/I*, *emrE*, *mdfA*, *sugE(c)*, *sugE(p)*, *ydgE*, *ydgF* and *oqxA*) were also investigated by PCR in all isolates as reported by Zou et al.[21] and Deus et al.[22].

Statistical analysis

The data obtained in this study were analyzed by the SPSS 22.0 program and the effects of regions (Antakya and Iskenderun), point (influent and effluent) and their interactions in terms of CREC loads were analyzed by two-way analysis of variance. Multiple comparison tests with Bonferroni correction were applied in cases where significance was found. Significance level was taken as 0.05.

Results

ESBL-EC number and isolation

ESBL-EC was identified in 23 of the 24 collected water samples, except one effluent sample from advanced biological treatment plant. The total number of ESBL-EC in the influent water samples from two WWTPs was between 3.74 ± 0.16 and 3.97 ± 0.16 log cfu/ml (mean 3.86 ± 0.11 log cfu/ml), while this number was between 1.44 ± 0.35 and 2.71 ± 0.20 log cfu/ml (mean 2.20 ± 0.27 log cfu/ml) in the effluent water samples from two sides (Table 2). There was no statistically significant difference for the ESBL-EC counts in influent water samples in both facilities ($p > 0.05$), while effluent water samples from advanced biological treatment plant had lower number of ESBL-EC compared to these from conventional treatment ($p < 0.05$; Table 2).

A total of 66 suspected *E. coli* isolates were obtained on TBX agar and were confirmed by PCR using *E. coli* specific *usp*A gene. Thirty-seven isolates were obtained from conventional treatment plant (influent water 18 isolates and effluent 19 isolates) and 29 isolates were obtained from advanced biological treatment plant (influent water 21 isolates and effluent 8 isolates).

Determination of antibiotic resistance status and detection of ESBL-EC

As expected, all CREC isolates were found to be resistant to ampicillin and cephalothin and all CREC isolates were found to be ESBL positive according to double disk synergy and disc combination method. In addition, 65 isolates (98.48%) were resistant to cefotaxime and cefuroxime, 31 isolates (46.96%) to amoxicillin clavulanic acid and nine isolates (13.63%) to cefoxitin. While 60 isolates (90.90%) were resistant to ceftiofur, 38 isolates (57.57%) were resistant to cefepime. Among the monobactam group, 59 isolates (89.39%) were resistant to aztreonam. Among the aminoglycoside group of antibiotics, 12 isolates (18.18%) were resistant to gentamycin. Additionally, 20 isolates (30.3%) were resistant to levofloxacin, while 29 isolates (43.93%) were resistant to ciprofloxacin. Forty-two isolates (63.63%) were resistant to tetracycline antibiotic. All isolates were found to be susceptible to imipenem.

PFGE results and Phylogenetic grouping

No bands were obtained from one isolate in PFGE analysis and therefore this isolate could not be further evaluated. Upon analysis (cut-off value > 85%) of band profiles for 65 isolates, it was observed that 16 isolates were clonally related and 49 isolates were not (Fig. 1). The clusters that were clonally related contained two isolates and these isolates were mostly from the same wastewater plant. Notably, some strains (Ww21.25/Ww21.74 and Ww21.17/Ww21.20) isolated from both influent and effluent water samples within the same plant showed the

WWTPs	Influent	Effluent
Conventional treatment plant	3.74 ± 0.16 [a]	2.71 ± 0.20 [b, A]
Advanced biological treatment	3.97 ± 0.16 [a]	1.44 ± 0.35 [b, B]
Mean	3.86 ± 0.11	2.20 ± 0.27

Table 2. ESBL-EC in the influent and effluent waters of Antakya and Iskenderun WWTPs count (log cfu/ml). a,b: Lowercase letters in the same row indicate statistical difference between points ($p < 0.05$), A,B: Capital letters in the same column indicate statistical difference between regions ($p < 0.05$).

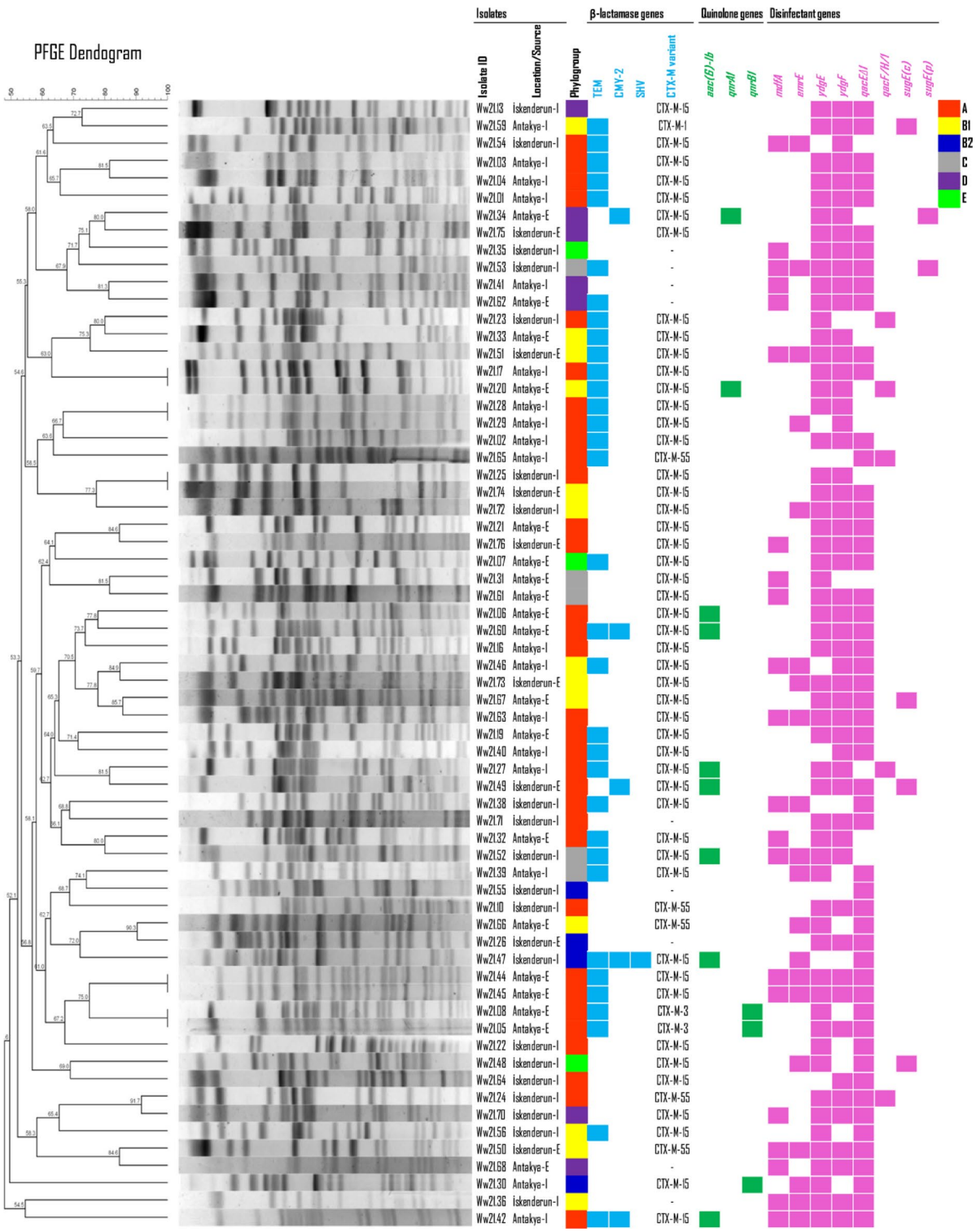


Fig. 1. PFGE analysis of ESBL producing *E. coli* isolates and detailed resistance data for ESBL-EC. Filled and empty square means presence and absence of the gene, respectively.

same PFGE profile, suggesting poor removal efficiency for AMB. Additionally, only two isolates (Ww21.66 and Ww21.26) originated from wastewater plants of different cities displayed similar banding pattern.

According to the combinations described by Clermont et al.[18], 33 (50%) ESBL-EC isolates belonged to group A, 13 (19.69%) to group B1, eight (12.12%) to group D, five (7.57%) to group C, four (6.06%) to group B2 and three (4.54%) to group E.

Determination of beta-lactamase types and subtypes

It was determined that 86.36% (n = 57) of the isolates carried the bla_{CTX-M} gene. 85.96% (49/57) of the isolates were found to contain $bla_{CTX-M-15}$, 8.77% (n = 5) $bla_{CTX-M-55}$ and 1.75% (n = 1) $bla_{CTX-M-1}$ (Fig. 1). Additionally, 3.5% (n = 2) of the isolates contained the $bla_{CTX-M-3}$ gene. The bla_{TEM} gene was found in 54.4% (n = 36) and the bla_{CMY-2} gene in 7.57% (n = 5) of the isolates. However, no bla_{SHV} gene was detected in any isolate.

Findings on quinolone and disinfectant resistance genes

Five quinolone resistance genes (*qnr*A1, *qnr*B1, *qnr*C, *qnr*D and *aac(6)-Ib*) were analyzed and the *aac(6)-Ib*, *qnr*B1 and *qnr*A1 genes were found in 10.60% (n = 7), 4.54% (n = 3) and 3.03% (n = 2) of the isolates, respectively (Fig. 1). However, the *qnr*C and *qnr*D genes were not detected in any of the isolates.

Eight of nine disinfectant resistance genes (*qacEΔ1*, *qacF/H/I*, *emr*E, *mdf*A, *sug*E(c), *sugE(p)*, *ydg*E, *ydg*F and *oqxA*) were detected in the isolates (except for the *oqx*A gene) (Fig. 1). The distribution of these genes in CREC isolates were *ydgE* (84.84%; n = 56), *qacEΔ1* (81.81%; n = 54), *ydgF* (80.30%; n = 53), *mdfA* (33.33%; n = 22), *emrE* (30.30%; n = 20), *qacF/H/I* (7.57%; n = 5), *sugE(c)* (6.06%; n = 4), and *sugE(p)* (3.03%; n = 2).

Discussion

Several studies have reported the occurrence of ESBL-EC as a common public health related bacteria in the sewage and wastewater, indicating likely environmental and human health issues[1,13]. Results of this study presented high counts of ESBL-EC (3.74–3.97 log cfu/ml) in the incoming water of two cities. Adegoke et al. found similar values (1.8–4.8 log cfu/ml) in their study on a WWTP in South Africa[23]. Bréchet et al. also monitored urban wastewater samples in France and found ESBL-EC at concentration of 2.87 log cfu/ml[6]. Conversely, ESBL-EC was detected at higher concentration (approximately 5.34 log cfu/ml) in the influent water samples in another city in Türkiye[14]. It has been stated previously that the number of bacteria depends on the factors including the nature of the influent. For example, hospital wastewater has been reported to be heavily contaminated with ESBL-EC[6]. When the counts of CREC were determined in the effluent water samples of both plants, CREC number were found to range from 1.44 to 2.71 log cfu/ml in this study. The most substantial reduction (over 2 log cfu/ml vs 1 log cfu/ml) was obtained in the effluents collected from advanced biological treatment when compared conventional treatment plant, indicating the importance of treatment processes. Gündoğdu et al. also reported reductions of more than 2 log cfu/ml of ESBL-EC with conventional treatment[14]. Bréchet et al. reported the biological content degradation with chlorination procedure as effective method in WWTPs, removing 94% ESBL-EC[6].

According to the phylogenetic classification, the most of the CREC isolates (n = 54) were in groups A, B1 and D. As mentioned in different studies on ESBL-EC, phylogenetic groups of *E. coli* strains vary depending on the sources and geographical regions[24]. Some studies have suggested that group A is the group that adapts best to different environments[25]. In a study conducted in Japan, Xie et al. showed that antibiotic-resistant phylogroup B2 isolates have a high survivability in treated wastewater, and these strains were found to be the most predominant strains in wastewater samples collected from both treatment plants in different areas of the same city[26]. There are major public health concerns regarding the survival of the phylogroup B2 strains in the treated wastewater. Extra-intestinal *E. coli* strains primarily fall into groups B2 and D, whereas intestinal pathogenic strains of the bacteria have been linked to groups A, B1, and E[27]. The diversity of phylogroups in this study is not surprising given that the wastewater treatment plant receives waste from a wide variety of sources, including environmental and biological wastes.

When the band profiles of isolates were analyzed in PFGE analysis, most of the isolates were not linked and a homogeneous distribution was observed. Since the wastewater coming to the WWTP comes from different sources, the results are not surprising. Similarly, a study done in Colombia in 2019 also revealed that ESBL-EC isolates were not clonally related according to PFGE analysis results[28].

ESBL-EC isolates obtained in this study were found to be also resistant to tetracycline at high rate (63.63%), which is consistent with previous studies from different countries[8,29]. In addition, 43.93% and 30.3% of ESBL-EC were also found to be resistant to ciprofloxacin, and levofloxacin, respectively, in the current study. However, in previous study, much lower resistance rate (42%) was obtained for ciprofloxacin resistance among ESBL-EC isolates from WWTP and Orontes River in the same city[13].

The bla_{CTX-M} group, found in 86.36% of the isolates, was the most common ESBL gene among our isolates. Brecket et al. found that most of the urban wastewater isolates (215 of 243 [88%]) produced bla_{CTX-M} type ESBLs. $bla_{CTX-M-1}$ was the most common bla_{CTX-M} type enzyme (50%), followed by $bla_{CTX-M-15}$ (25%) and $bla_{CTX-M-14}$ (10%)[6]. ESBLs are unevenly distributed in Europe, as previous studies have reported that $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ are the most common ESBLs among ESBL-EC[30–32]. As in our study, among ESBLs, $bla_{CTX-M-15}$ was found to be the most common type of enzyme in wastewater, as reported by studies from Türkiye and various countries[8,14,28]. For example, Amos et al. discovered that the majority ESBLs observed in *E. coli* from an English river were of the $bla_{CTX-M-15}$ type, and this was associated with the high abundance of this type in the wastewater from a WWTP[32]. Similar findings about the widespread distribution of bla_{CTX-M} enzyme were also found by Blaak et al.[33], with $bla_{CTX-M-15}$ being the most prevalent kind in Dutch recreational waters[33].

Kim et al. have reported that quinolone resistance genes are also carried along with bla_{CTX-M} genes[34] and in 18.46% (n = 18) of the samples, one of the quinolone resistance genes and bla_{CTX-M} were found together. This can be explained by both beta-lactams and fluoroquinolones stimulate the transcription of *qnr*B genes, and the existence of *qnr* is strongly associated with the use of cephalosporins in the past. When antibiotic selection pressure is present, co-localization of *qnr*B with ESBL may be advantageous for bacterial isolates[34].

Several efflux pump genes, particularly *ydgE* and *ydgF*, were commonly detected in the ESBL-EC in the current study. Of the 60 ESBL-EC isolates from chicken meat, *ydgE*, *ydgF*, *sugE(c)*, and *mdfA* were found in 100% of isolates but they did not detect the *oqxA* gene[10]. It was also notable that the *qacEΔ1* gene was present

in 81% of the isolates in our study. However, Sahin et al. reported much lower (20%) rate among ESBL-EC from chicken meat[10]. Likewise, when Zou et al. examined QAC resistance genes in *E. coli* isolated from the retail meats, they found that *ydgE* and *ydgF* had 100% detection rates, with *mdfA* (99.5%), *qacEΔ1* (23%), and *qacG* (0.4%) having the next highest detection rates[21]. Chromosomally encoded *sugE(c)* mediates strong resistance to QAC disinfectant[21] and was not very common in this study. *qacEΔ1* was found in all samples of ESBL-producing *Klebsiella pneumoniae* isolates obtained from the influent and effluent of three WWTPs in Romania[35] and in a study of ARGs in sewage sludge, *qacEΔ1* was one of the five most abundant ARG transcripts[36]. In our study, the *qacEΔ1* gene was detected at a high rate (81.81%), indicating the selection of this kind of resistance in wastewater, most likely as a result of the strong selection pressure that disinfectants impose. The prevalence of the genes *ydgE*/*ydgF* (83–100%), *mdfA* (86–100%), and *qac* (0–18%) in *E. coli* isolates from various sources has also been observed by a number of studies[21,22,37,38].

This study has potential limitations such as the lack of quantitative measurements of antibiotic concentrations in wastewater and the absence of real-time PCR analysis for the ESBL gene quantification. Furthermore, more in-depth genomic characterization using whole-genome sequencing and comparative analysis with previously identified dominant clones from human infections were not performed. However, these limitations could be addressed in future investigations with advance molecular techniques to provide detailed insights into the dynamics of ESBL-EC in wastewater environments.

Conclusions

The results of this study showed that treatment process of wastewater reduce the ESBL-EC, however significant number of bacteria still release into the receiving water bodies (river and sea). It is also noteworthy that the biological wastewater treatment plant reduced ESBL-EC logarithmically more than the conventional wastewater treatment plant. Importantly, the one with advanced biological treatment has more than 2 log cfu/ml reduction. The $bla_{\text{CTX-M-15}}$ gene was the most prevalent type of ESBL in both of WWTPs and is an important finding for public health. According to our findings, there is a need to monitor antibiotic-resistant microorganisms in WWTPs and processes with higher treatment power are needed.

Data availability

The data support the findings of this study are available from the authors upon reasonable request (ckurekci@hotmail.com).

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Author contributions

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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