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ORIGINAL ARTICLE

Diversity of β-lactam resistance genes in gram-negative rods isolated from a municipal wastewater treatment plant

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Abstract

Urban wastewater treatment plants (UWTPs) are considered hot spots for the accumulation and transfer of antibiotic resistant bacteria and their genes. We investigated the prevalence, diversity, and genomic localization of β-lactamase resistance genes in wastewater from different parts of an UWTP, such as raw wastewater, wastewater from biological reactor, and treated wastewater, collected from the UWTP of Warsaw, Poland. In this study, we focused on gram-negative rods, mainly Enterobacteriaceae, and used Multiplex PCR to identify various β-lactamase resistance genes (*bla*). Susceptibility to a number of antibiotics and genomic localization of β-lactamase resistance genes were then determined using disc-diffusion and Southern hybridization methods, respectively. No differences in the frequency of different types of *bla* genes between the sampling points were discerned. Three new variants of β-lactamase genes ($bla_{\text{CMY-157}}$, $bla_{\text{MOX-13}}$, and $bla_{\text{FOX-15}}$) were identified. Four *bla* genes ($bla_{\text{TEM-12}}$, $bla_{\text{TEM-30}}$, $bla_{\text{TEM-47/68}}$, bla_{ACT}) that had never been found in UWTPs were identified in this study. Nine of the identified *bla* genes had been found in the same environment previously. The $bla_{\text{FOX-15}}$ variant on a *Kluyvera* sp. plasmid and bla_{GES} type on *Raoultella* spp. plasmids were observed for the first time in these genera. There was a decrease in the number of multidrug resistant strains in the effluent compared to the influent. Finally, a significantly higher number of cefotaxime and carbapenem unsusceptible strains were detected in the influent than in the effluent. The results strongly support the hypothesis of UWTPs as a hot spot for antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) accumulation and indicate that β-lactamase genes are widely disseminated among gram-negative rods isolated from this environment.

Keywords β-Lactamases · Enterobacteriaceae · Plasmid · Antibiotic resistance gene · Horizontal gene transfer · Wastewater treatment plant

Introduction

Antibiotic resistance genes (ARGs) and antibiotic resistance bacteria (ARB) are considered critical threats to global public health. For this reason, international organizations and actions, including the World Health Organization (WHO), the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the Food and Agriculture Organization of the United Nations (FAO), and NEREUS COST Action ES1403 (http://www.nereus-cost.eu/), carry out and recommend numerous antimicrobial resistance surveillance programs (NEREUS COST Action ES1403 2018; European Committee on Antimicrobial Susceptibility Testing 2018; Food and Agriculture Organization of the United Nations 2018; World Health Organization 2017). An important part of these efforts is to monitor and identify antimicrobial "hot spots," mainly those affected by anthropogenic activities.

Previous studies indicated that wastewater treatment plants (WWTPs) are one of the key reservoirs of both ARB and ARGs (Baquero et al. 2008; Michael et al. 2013). Diverse genes that encode resistance to all major classes of antibiotics have been identified in sewage (Zhang et al. 2009). The efficiency of removing ARGs during the wastewater treatment process depends on the

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treatment technology and the sources of sewage coming to a WWTP (hospital, municipal effluent) (Novo et al. 2013; Barancheshme and Munir 2017; Michael-Kordatou et al. 2018). These genes have been found in both environmental and pathogenic strains. The latter may flow to a WWTP with hospital sewage. While ARGs are considered to be easily acquired by bacteria, they are difficult to eliminate even when the selective pressure is removed (Aminov and Mackie 2007). Under the circumstances, cases of transmission of ARGs between environmental and clinical strains represent a serious threat to public health. Mobile genetic elements (MGEs) were postulated as important vectors for ARG exchange between bacterial strains in WWTP settings (Perry and Wright 2013). Research into the wastewater resistome and the associated “mobilome” is currently underway. The importance of such studies is emphasized by the increasing number of multidrug resistant (MDR) strains of clinically relevant bacteria that are spreading around the world.

Together with tetracyclines, macrolides, and sulfonamides, β-lactams are important antibiotics in human and veterinary medicine. The number and diversity of β-lactamase genes are significantly higher compared to ARGs from other antibiotic classes (NCBI Bioproject PRJNA313047). As the largest group of diverse and specific resistance determinants in bacteria, β-lactamase genes are intensively explored in terms of dissemination mechanisms in the environment. B-lactamases are unique also because of their broad spectrum of activity against β-lactams and—consequently—very high mutation frequency (Gniadkowski 2008; Bush and Jacoby 2010). The Bacterial Antimicrobial Resistance Reference Gene Database (Accession: PRJNA313047; ID: 313047) currently gathers more than 2000 renumbered β-lactamase gene (*bla*) sequences belonging to 35 types and numerous variants. The number continues to grow. B-lactamase resistance genes belonging to the AmpC, ESBL, KPC, and NDM groups have been found in bacteria identified in wastewater (Zhang et al. 2009; Picão et al. 2013; Khan and Parvez 2014; Amador et al. 2015; Varela et al. 2016; Piotrowska et al. 2017). However, our understanding of the diversity of types and variants is still limited.

Investigating these differences at the local level is crucial for assessing the risk of spreading of *bla* and other ARGs and the efficiency of ARG removal from wastewater treatment systems. When such results are analyzed at a global level, the spread and proliferation of ARGs in the environment may be controlled. Consequently, the main aim of our study was to characterize the diversity and genomic localization of a wide range of β-lactamase genes in bacteria that had been isolated from wastewater samples collected from different parts of a conventional activated sludge UWTP, such as raw sewage, wastewater from a biological reactor, and treated wastewater.

Materials and methods

Characterization and identification of bacterial isolates

In this study, we analyzed 58 ceftazidime- or meropenem-resistant bacterial strains isolated from a municipal UWTP. All described strains were isolated from raw sewage (influent), wastewater from a biological reactor (bioreactor) or treated wastewater (effluent) from the Czajka UWTP located in Warsaw, Poland. Studied UWTP is a secondary treatment facility—CAS WWTP (Conventional Activated Sludge), which collects domestic, urban, and hospital sewage from Warsaw and suburban area. The strains were isolated in four time periods (September 2011, October 2011, April 2014, and June 2014) from three sampling points (influent, bioreactor and effluent). The isolates were harvested from R2Agar complete medium (Graso Biotech, Poland) supplemented with meropenem or ceftazidime at a final concentration of 4 μg ml^{-1} and 16 μg ml^{-1}, respectively. The antibiotic concentrations were selected based on M100 Clinical and Laboratory Standards Institute guidelines for Enterobacteriaceae (CLSI 2017). In this report, ceftazidime- and meropenem-resistant strains were analyzed. Identification to the genus level was performed based on partial 16S rRNA sequences obtained with the use of common primers (8F and U1492R) and under amplification conditions described elsewhere (James 2010). PCR products of expected size were sequenced at Genomed (Warsaw, Poland). Sequence analysis and assembly were performed using Clone Manager 8 (Sci-Ed Software, USA) and FinchTV chromatogram viewer (Geospiza, USA). Determination to the genus level was performed using a minimum 95% threshold in identity with the relevant nucleotide sequence from non-redundant nucleotide GenBank collection database (nr/nt) using BLAST N tool (Altschul et al. 1990). The approach based on 16S rRNA gene sequencing is widely used for bacterial classification, but it has low phylogenetic power at the species level and poor discriminatory power for some genera, such as Enterobacteriaceae (Janda and Abbott 2007; Srinivasan et al. 2015). In this case, we decided to assign isolates only to the genus level based on 95–100% identity score. All sequences were deposited in GenBank under the following numbers: MF457824-MF457850 and MF457852-MF457885 (Tables 1S–3S).

Detection of β-lactamase genes and integrons

Identification of *bla* genes was performed using PCR amplification methods, mainly Multiplex PCR, as described elsewhere (Perez-Perez and Hanson 2002; Dallenne et al. 2010). In this study, five Multipex reactions were performed using specific primers (Table 4S). Moreover, the identified

Raoultella spp. isolates were screened for the presence of chromosomal *bla* genes, such as bla_{ORN} (characteristic of *R. ornithinolytica*) and bla_{PLA} (characteristic of *R. planticola*), with primers and under reaction conditions described previously (Walckenaer et al. 2008). Additionally, the identified strains were screened for the presence of integrase genes—*intI1*, *intI2*, and *intI3*—using the previously described primers and conditions (Henriques et al. 2006). Genomic DNA of clinical strains that carry selected β-lactam resistance genes, obtained from the National Medicines Institute (Warsaw, Poland) (Table 5S), was used as a positive control. *Escherichia coli* ATCC 25922 strain was used as a negative control in Multiplex PCR reactions. In turn, genomic DNA of *Aeromonas* spp. strains with the confirmed presence of integrase genes was used as a positive control for integrase genes (Piotrowska et al. 2017). Specific PCR products were sequenced to avoid false positive results. Sequence assembly and analysis were carried out with Clone Manager version 8 (Sci-Ed Software, USA) and FinchTV chromatogram viewer (Geospiza, USA). Determination of the *bla* types and variants was performed using a 100% threshold in identity with the relevant amino acid sequence from Lahey Clinic beta-lactamse protein sequence database (no longer exist; data transfer into NCBI Bioproject PRJNA31147) with BLAST X tool (Lahey Clinic 2015; Altschul et al. 1990). All of the protein sequences that had less than 100% identity with sequences from the database were classified as new variants. New variants of *bla* genes were deposited in GenBank under the following numbers: MF795086 (bla_{MOX-13}), MF795087 (bla_{FOX-15}), and MF795088 ($bla_{CMY-157}$). In the case of integrase genes, the amplicons were sequenced and compared with non-redundant protein sequences gathered in the GenBank database (nr) using BLAST X tool.

Antibiotic susceptibility of the resistant strains

The susceptibility profiles of 54 strains to 12 antibiotics were determined using the agar diffusion method and Clinical and Laboratory Standards Institute guidelines M100 for Enterobacteriaceae and *Acinetobacter* spp. (CLSI 2017). The following antibiotics were used: amikacin (AK, 30 μg), gentamicin (CN, 10 μg), chloramphenicol (C, 30 μg), ciprofloxacin (CIP,5 μg), tetracycline (TET, 30 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), cefepime (FEP, 30 μg), aztreonam (AZT, 30 μg), imipenem (IMP, 10 μg), ertapenem (ERT, 10 μg), and meropenem (MEM, 10 μg). According to CLSI 2017 guidelines, there are no *Acinetobacter* spp. breakpoints for aztreonam, ertapenem, and chloramphenicol. This resulted in the exclusion of the three antibiotics from the *Acinetobacter* spp. susceptibility profile testing. Susceptibility profiles for *Pseudomonas* spp. (other than *Pseudomonas aeruginosa*), *Ochrobactrum* spp., and *Shewanella* spp. were not determined, because there are no antibiotic agar diffusion guidelines for these bacteria. Inhibition zones smaller than S (susceptible) and larger than R (resistant) were classified as intermediate resistance (I) and excluded from the resistance percentage calculations. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 strains were used for quality control. The multiple antibiotic resistance index (MAR index) for a given isolate was calculated as the number of antibiotics to which the isolate was resistant (a) divided by the total number of antibiotics against which the isolate was tested (b) (Zhang et al. 2015). The MAR index was obtained based on the results of disc diffusion analysis.

Plasmid isolation and Southern hybridization protocol

To determine the location of β-lactamase resistance genes, plasmid DNA from all strains was isolated. Extrachromosomal DNA was extracted and purified using Plasmid Mini AX Gravity kit (A&A Biotechnology, Poland), according to the manufacturer's instruction. The isolated plasmids were separated by electrophoresis in 0.8% agarose gels, stained with ethidium bromide and visualized using a UV transilluminator. Probes for Southern hybridization were obtained from ARGs PCR products identified in the earlier steps of this work and incubated with digoxigenin (DIG). Separated plasmid DNA was transferred to a nylon membrane (Roche Diagnostics GmbH, Germany) and hybridized with the probes. Preparation of (DIG)-labeled probes, hybridization, and visualization was performed using DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Germany). The PCR products utilized to create the probes were further used as positive controls.

Statistical analysis

Statistical analyses were performed using the lme4 package in R 3.3.3 software (R Core Team, 2017). The frequencies of antibiotic resistance phenotypes and ARGs were compared among strains isolated from influent, bioreactor, and effluent. The chi-square test was used to determine the significance of the distribution of ARGs and phenotypes. In this work, the level of significance was p value < 0.05.

Results

Genus-level diversity of the identified strains

Of 58 strains that had been chosen for this study, 26 were found in raw wastewater, 23 were detected in a bioreactor, and nine were identified in treated wastewater (Tables 1S–3S). Bacteria of the Enterobacteriaceae family predominated at each sampling point, and their number was 24, 20, and 7 in raw wastewater, bioreactor, and treated wastewater, respectively. Based on 16S rRNA sequencing results, the identified genera of this family most likely belonged to *Citrobacter* spp., *Raoultella* spp., *Kluyvera* spp., *Enterobacter* spp., *Klebsiella* spp. *Escherichia* spp., and *Serratia* sp. The most frequent were *Raoultella* spp. (17 strains) and *Citrobacter* spp. (11 strains). Moreover, only three genera and one species were observed at each of the three sampling points, i.e., *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp., and *Escherichia* spp.

Other isolated strains belonged to *Pseudomonas* spp., *Shewanella* sp., *Ochrobactrum* sp., and *Acinetobacter* spp. and were the minority. *Pseudomonas* spp. were found in raw wastewater (one strain) and bioreactor (two strains). The remaining genera were identified at only one WWTP sampling point.

β-Lactamase resistance determinants

The main part of this study was the identification of the wide range of β-lactamase resistance genes (*bla*) within the selected strains. As a result of our investigation, 128 *bla* genes were identified among the studied isolates (Table 1). From among 24 *bla* gene types that were chosen for molecular screening, the following 13 types were identified: bla_{TEM}, bla_{OXA}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{CMY}, bla_{MOX}, bla_{FOX}, bla_{ACT}, bla_{GES}, bla_{PER}, bla_{VIM}, bla_{KPC}, and bla_{ORN}. Bla_{TEM} genes were found in 33 strains and were the most frequent *bla* gene types recovered from all strains. Genes that were found at all three sampling points belonged to bla_{TEM}, bla_{OXA}, bla_{SHV}, bla_{CMY}, and bla_{VIM} types with the predominance of bla_{TEM} (Fig. 1). Statistical analysis showed there were no significant differences in the number of each *bla* gene type between the sampling points ($p < 0.05$).

Genes of bla_{TEM} type were found in all identified bacterial genera besides *Pseudomonas* spp. and *Acinetobacter* spp. (Tables 1S–3S). Using the chosen pair of primers, in most of the sequenced bla_{TEM} amplicons, we were unable to unambiguously determine any specific variant of this gene. However, in two isolates from the influent and one from the effluent, we identified three bla_{TEM} variants based on the obtained partial sequences. BLASTx alignment of bla_{TEM} sequence from *Shewanella* sp. 192 showed 100% identity to the sequence of TEM-12 β-lactamase variant (GenBank Protein accession number AAA25053.1, GenBank *bla* gene accession number M88143). Analogously, bla_{TEM} sequences from *Escherichia* sp. 149 and *Enterobacter* sp. 480 showed 100% identity to TEM-30 (CAD24670.1, AJ437107) and TEM-116 (AAB39956.1, U36911) sequences, respectively. Moreover, in *Raoultella* sp. 7.42 strain isolated from influent, the BLASTx alignment of bla_{TEM} sequence showed a 100% identity to the sequences of two β-lactamase variants: TEM-47 (CAA71322.1, Y10279) and TEM-68 (CAB92324.1, AJ239002), whose variable amino acids are located outside the translated PCR product.

Table 1 Number of β-lactamase resistance and integrase gene variants and types among strains isolated from UWTP with division into three sampling points

ARG type	Influent	Bioreactor	Effluent	Summary
bla_{TEM}	*13*	*17*	*3*	*33*
$bla_{TEM\text{-}1\text{-}like}$	10	17	2	29
$bla_{TEM\text{-}12}$	1	0	0	1
$bla_{TEM\text{-}30}$	1	0	0	1
$bla_{TEM\text{-}47/68}$	1	0	0	1
$bla_{TEM\text{-}116}$	0	0	1	1
bla_{OXA}	*9*	*3*	*1*	*13*
bla_{SHV}	*5*	*1*	*3*	*9*
$bla_{SHV\text{-}11\text{-}like}$	0	1	0	1
$bla_{SHV\text{-}12\text{-}like}$	5	0	3	8
$bla_{CTX\text{-}M}$	*14*	*0*	*1*	*15*
$bla_{CTX\text{-}M\text{-}15\text{-}like}$	10	0	1	11
$bla_{CTX\text{-}M\text{-}1\text{-}like}$	1	0	0	1
$bla_{CTX\text{-}M\text{-}3\text{-}like}$	2	0	0	2
$bla_{CTX\text{-}M\text{-}27\text{-}like}$	1	0	0	1
bla_{GES}	*2*	*11*	*0*	*13*
bla_{PER}	*1*	*0*	*0*	*1*
$bla_{PER\text{-}1/5}$	1	0	0	1
bla_{CMY}	*6*	*2*	*1*	*9*
$bla_{CMY\text{-}4}$	1	0	0	1
$bla_{CMY\text{-}39}$	0	0	1	1
$bla_{CMY\text{-}40}$	0	1	0	1
$bla_{CMY\text{-}139}$	0	1	0	1
$bla_{CMY\text{-}157}$	2	0	0	2
$bla_{CMY\text{-}42/146/145}$	1	0	0	1
$bla_{CMY\text{-}2\text{-}like}$	1	0	0	1
$bla_{CMY\text{-}65/75/89/113}$	1	0	0	1
bla_{MOX}	*0*	*0*	*1*	*1*
$bla_{MOX\text{-}13}$	0	0	1	1
bla_{FOX}	*0*	*0*	*1*	*1*
$bla_{FOX\text{-}15}$	0	0	1	1
bla_{ACT}	*0*	*1*	*0*	*1*
bla_{VIM}	*1*	*3*	*3*	*7*
$bla_{VIM\text{-}1\text{-}like}$	0	1	3	4
$bla_{VIM\text{-}2\text{-}like}$	1	2	0	3
bla_{KPC}	*0*	*12*	*1*	*13*
$bla_{KPC\text{-}2\text{-}like}$	0	12	1	13
bla_{ORN}	*3*	*10*	*0*	*12*
intI	*18*	*13*	*9*	*40*
intII	*7*	*0*	*0*	*7*
intIII	*1*	*1*	*2*	*4*

Total number of beta-lactamase and integrase gene types were marked in italics

The remaining bla_{TEM} sequences were 100% identical to those of TEM-1 (AAB59737.1, J01749) and many different β-lactamase variants that have their variable regions outside the

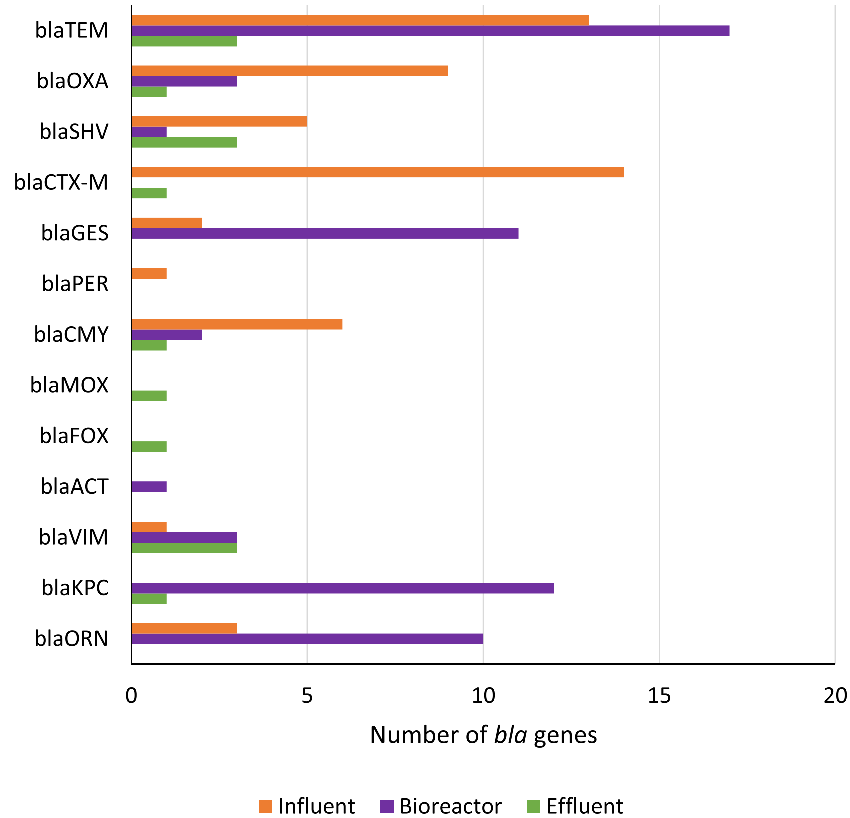

Fig. 1 Number of *bla* genes of different types identified in bacteria from influent (orange), bioreactor (violet), and effluent (green)

obtained PCR products. In these cases, the remaining variants were named $bla_{\text{TEM-1-like}}$.

As for the bla_{OXA} genes, they were found only within Enterobacteriaceae strains, including nine strains from the influent, three from the bioreactor, and one from the effluent. However, all identified bla_{OXA} sequences shared 100% similarity with the sequences of OXA-1 (AAA91586.2, J02967), OXA-16 (AF043100.1, AF043100), OXA-113 (ABW70410.1, EF653400), and OXA-320 (AGR55864.1, KF151169) variants of this β-lactamase. Again, the determination of bla_{OXA} variant of the gene based on the obtained partial sequence was impossible.

In the case of bla_{SHV} genes, all nine sequences were identified within the Enterobacteriaceae family, with five isolates from the influent, one from the bioreactor, and three from the effluent. Three bla_{SHV} genes were found in *Klebsiella* spp. and are intrinsic in this genus. All bla_{SHV} gene sequences from the influent and effluent were 100% identical to the sequences of SHV-12 (CAI76927.1, AJ920369) and a couple different β-lactamase variants. However, one *Klebsiella* sp. T12 strain from the bioreactor carried bla_{SHV} whose sequences were 100% identical to those of SHV-11 (CAA66729.1, X98101) and a couple of different SHV β-lactamases with the same predicted amino acid sequence of the fragment of the sequence that was obtained in this study. These genes will be further referred to as $bla_{\text{SHV-11-like}}$ and $bla_{\text{SHV-12-like}}$.

$Bla_{\text{CTX-M}}$ was another numerous group of *bla* genes that were identified only in Enterobacteriaceae strains with a predominance in raw wastewater (14 strains). $Bla_{\text{CTX-M}}$ was also found in one *Escherichia* sp. T24 strain from the effluent. In ten identified strains, $bla_{\text{CTX-M}}$ gene sequences were 100% identical to those of CTX-M-15 (AAL02126.1, AY044436), CTX-M-28 (CAD70280.1, AJ549244), and a couple different variants, which made the final determination of this variant impossible. One *Escherichia* sp. 139 strain had a $bla_{\text{CTX-M}}$ gene whose sequence was 100% identical to those of CTX-M-1 (CAA63262.1, X92506), CTX-M-61 (ABN09669.1, EF219142), and CTX-M-138 (WP_070064534.1, NG_051737.1). This was a different variant of the $bla_{\text{CTX-M}}$ gene than the previous 12, but again, it could not be fully determined. Finally, there were two strains of *Kluyvera* sp. 102 and *Klebsiella* sp. 133 in which $bla_{\text{CTX-M}}$ gene sequences were 100% identical to those of CTX-M-3 (CAA71321.1, Y10278), CTX-M-22 (AAL86924.1, AY080894), CTX-M-66 (ABQ45409.1, EF576988), and CTX-M-162 (AKO63213.1, KP681697). Again, determination of the variant from this part of the gene was impossible. All the foregoing CTX-M β-lactamases belonged to the group 1 CTX-M enzymes (Bonnet 2004). However, in one *Escherichia* sp. 6.42 strain, there was a $bla_{\text{CTX-M}}$ gene that encoded a β-lactamase with 100% sequence identity to CTX-M-27 (AAO61597.1, AY156923), CTX-M-98 (ADO17948.1, HM755448), and a couple of different variants that belonged to group nine of this type of enzymes.

In the case of bla_{GES} sequences, it was impossible to determine the gene variant based on the obtained partial sequences. However, all 13 bla_{GES} sequences were 100% identical to those of GES-1 (AAF27723.1, AF156486) and some other GES β-lactamases variants. Most of the bla_{GES} genes were identified within *Raoultella* spp. strains (10 from the bioreactor and one from the influent). In addition, two more bla_{GES} were found: one in *Klebsiella* spp. 128 strain from the influent and the other in *Enterobacter* spp. 291 strain from the bioreactor.

Moreover, bla_{PER} gene was found in only a single *Shewanella* spp. 192 strain, which was isolated from the influent. BLASTx alignment showed its 100% sequence identity to the sequences of two variants of this type, i.e. PER-1 (CAA79968.1, Z21957) and PER-5 (ACN22483.1, FJ627180). To precisely determine bla_{PER} variant, sequencing of a larger part of the gene is needed.

Within the searched *ampC* genes, only bla_{CMY}, bla_{MOX}, bla_{FOX}, and bla_{ACT} types were identified. In one *Citrobacter* sp. 403 strain, isolated from the effluent, a new variant of $bla_{MOX\text{-}13}$ gene was identified (MF795086). Amino acid sequence was 99% identical to six unclassified sequences from class C β-lactamases of CMY-1/MOX family from *Aeromonas* spp. (WP_052815126.1, WP_042880807.1, WP_041215611.1, KEP89560.1, OJW64298.1, WP_045525552.1). The most closely related MOX variant was MOX-12 with 98% sequence identity (WP_043155783.1) to the new variant with seven amino acid changes (Fig. 1S). Moreover, a new β-lactamase gene variant—$bla_{FOX\text{-}15}$ (MF795087)—was found in *Kluyvera* sp. 453 isolated from the effluent. In this case, the amino acid sequence was 97% identical to that of FOX-2 variant (WP_032489067.1). Differences in 12 amino acids were identified between the two sequences (Fig. 2S). Another AmpC *bla* gene belonged to bla_{ACT} type and was found in *Enterobacter* sp. 327 isolated from the bioreactor. However, bla_{ACT} genes are intrinsic to *Enterobacter* spp. This β-lactamase determinant showed 100% sequence identity to many different ACT variants, e.g., ACT-21 (AHA80106.1, KF526118) and ACT-23 (AGU38146.1, KF515536). However, it was impossible to unambiguously determine the variant.

The most numerous and diverse *ampC* gene type was bla_{CMY}, which was observed in eight strains belonging mostly to *Citrobacter* spp., to which bla_{CMY} is intrinsic. The identified *bla* genes encoded eight different amino acid sequences with one new variant of a CMY family β-lactamase. Based on the obtained sequences, the following four variants of CMY β-lactamases were unambiguously determined: CMY-139 (AMK49571.1, KU641016.1) from *Citrobacter* sp. T10, CMY-40 (EU515251, EU515251.1) from *Citrobacter* sp. 323A, CMY-39 (BAF95726.1, AB372224) from *Citrobacter* sp. 424, and CMY-4 (CAA75402.1, Y15130) from *Citrobacter* sp. 106. Three strains from raw wastewater carried bla_{CMY} whose sequences were 100% identical to the sequences of more than one CMY variant. The bla_{CMY} sequence from *Escherichia* sp.129 was identical to the sequences of CMY-42 (ADM21467.1, HM146927), CMY-145 (WP_075985684.1, NG_052649.1), and CMY-146 (WP_075985683.1, NG_052648.1). The bla_{CMY} sequence from *Citrobacter* sp. 122 was identical to the sequences of CMY-2 (CAA62957.1, X91840), CMY-53 (ADQ38362.1, HQ336940), and a couple of other variants. The bla_{CMY} sequence from *Citrobacter* sp. 136 was identical to the sequences of CMY-65 (AEI52842.1, JF780936), CMY-75 (AFK73434.1, JQ733572), CMY-89 (WP_063859891.1, NG_048886.1), and CMY-113 (AIT76089.1, KM087836). Finally, in *Citrobacter* sp. 101 strain, a new variant of $bla_{CMY\text{-}157}$ gene (MF795088) was identified and its amino acid sequence shared 99% identity with two unclassified CMY family β-lactamases from *Citrobacter* spp. (WP_061067088.1, WP_048212911.1) (Fig. 3S). However, compared to one of the closest variants of CMY family β-lactamase—CMY-34 (ABN51006.1, EF394370.1)—at the sequence level, CMY-157 differed in 25 amino acids. This major change indicates the need for a further phenotypical analysis of this variant.

Furthermore, bla_{VIM} genes, which encode VIM metallo-β-lactamase, were identified in seven strains from all three sampling points. It was impossible to determine the variants unambiguously, but two identical amino acid sequences were observed. In *Pseudomonas* sp. 164 strain from the influent, and two strains from the bioreactor (*Citrobacter* sp. 383 and *Pseudomonas* sp. 378), the observed bla_{VIM} sequences were 100% identical to those of VIM-2 (AAF61483.1, AF191564) and different variants carrying an identical amino acid sequence within this fragment of the protein. A second group of four strains (one from the bioreactor and three from the effluent) carried bla_{VIM} whose amino acid sequences were 100% identical to those of VIM-1 β-lactamase (CAB46686.1, Y18050), VIM-4 (AAN04257.1, AY135661.1), and different variants with identical amino acid sequence of this fragment.

Also, *bla* genes that belong to the bla_{KPC} type were found in 13 strains, mostly from the bioreactor. All bla_{KPC} genes observed in the bioreactor were found in *Raoultella* spp. strains, and sequence alignment using BLASTx showed 100% identity with KPC-2 (AAK70220.1, AY034847), KPC-3 (AAL05630.1, AF395881), and a couple of different variants. One *Citrobacter* sp. 424 strain from the effluent also possessed a bla_{KPC} gene with the same amino acid sequence as in β-lactamases from the bioreactor.

The last group of identified *bla* genes belonged to the bla_{ORN} type of chromosomal β-lactamases characteristic of *Raoultella ornithinolytica*. All genes were found in *Raoultella* spp., including three strains from the raw wastewater and ten from the bioreactor.

Besides the identification of β-lactamase resistance genes, also the presence of I, II, and III type integrase genes was determined. As a result, all three types of integrase genes—*intI1*, *intI2*, and *intI3*—were observed with the highest representation of *intI1* (66% of strains) (Table 1). Only *intI1* and *intI3* types were found at all three sampling points. The *IntI2* genes were identified in only seven strains from the influent, but the strains belonged to various genera, i.e., *Escherichia* sp., *Enterobacter* sp., *Klebsiella* sp., and *Raoultella* spp.

Antibiotic susceptibility profiles

Among 53 strains whose antibiotic susceptibility profiles were determined, unsusceptibility to third generation cephalosporins, such as ceftazidime (91%) and cefotaxime (85%), was the most abundant (Table 2). Numerous strains were also unsusceptible to aztreonam (81%). On the other hand, only 6% of strains were unsusceptible to amikacin, which was the lowest percentage of resistance among all the antibiotics tested. Multidrug resistance strains represented 41% of all the studied strains. The MAR index showed that each strain was unsusceptible to from three to ten antibiotics simultaneously, with the highest percentage (24%) of strains unsusceptible to six antibiotics (Tables 1S–3S). Based on statistical analysis results, there were significantly more strains unsusceptible to cefotaxime in the effluent than in the influent ($p < 0.05$). Furthermore, strains unsusceptible to all three carbapenems (ertapenem, imipenem, and meropenem) were significantly more abundant in the bioreactor than in the influent ($p < 0.05$). Finally, there was a statistically significant ($p < 0.05$) reduction in the number of MDR strains between the influent and the effluent, from 48 to 44% (Table 2).

Genomic localization of β-lactamase resistance genes

Extrachromosomal replicons were isolated from 79% of all the studied strains (46 out of 58), including 21 strains from the influent, 18 strains from the bioreactor, and seven strains from the effluent. Most of the strains isolated from the three sampling points appeared to have more than one extrachromosomal replicon. The number of different plasmid profiles identified in the influent, bioreactor, and effluent samples was 21, 12, and 7, respectively, indicating unique plasmid profiles in most of the strains. In the bioreactor, there were four different profiles, which were found in *Raoultella* spp. strains: the first profile in *Raoultella* sp. 210C, *Raoultella* sp. 213C, *Raoultella* sp. 382A, and *Raoultella* sp. 293; the second in *Raoultella* sp. 274B, *Raoultella* sp. 376, and *Raoultella* sp. 286; the third in *Raoultella* sp. 385A, *Raoultella* sp. 299A, and *Raoultella* sp. 328; and the fourth in *Raoultella* sp. 228A (Fig. 4S).

The results of Southern blot hybridization indicated that most of the identified *bla* genes were located on chromosomes. However, several bla_{GES} and one bla_{FOX} gene were detected in extrachromosomal DNA. Firstly, one $bla_{\mathrm{FOX\text{-}15}}$ gene from *Kluyvera* sp. 435 from the effluent was observed on a plasmid. Moreover, bla_{GES} genes were identified in ten *Raoultella* spp. plasmids from bioreactor samples (Table 3). Besides four plasmid profiles of these strains, the bla_{GES} signal was detected on one particular plasmid band that these strains have in common (Fig. 4S).

Discussion and conclusions

Antibiotic resistant bacteria and antibiotic resistance genes are heavily discharged into municipal sewage systems with wastewater of different origins (Kümmerer 2009). Therefore, ARGs from all main groups, including tetracycline, aminoglycoside, quinolone, and β-lactam resistance genes, have been found in this environment (Zhang et al. 2009). Moreover, wastewater microbiota is mainly composed of bacteria of human origin, including commensal and pathogenic strains. Antibiotic resistance genes in WWTPs have been identified among various pathogenic bacteria, e.g., *Pseudomonas* spp., Enterobacteriaceae, *Staphylococcus* spp., enterococci, and *Aeromonas* spp. (Igbinosa and Okoh 2012; Picão et al. 2013; Varela et al. 2016; Oravcova et al. 2017; Ben Said et al. 2017). With this in mind, we decided to identify β-lactamase profiles and analyze the fate of these ARGs in bacteria isolated from samples collected from UWTP. We also sought to compare these results with those of previous studies.

Based on the available literature, seven of the *bla* genes identified in this study were found for the first time in bacteria isolated from a WWTP. These were as follows: $bla_{\mathrm{TEM\text{-}12}}$, $bla_{\mathrm{TEM\text{-}30}}$, $bla_{\mathrm{TEM\text{-}47/68}}$, and bla_{ACT} and within them there were three

Table 2 Percentage (%) of phenotypically unsusceptible strains of Enterobacteriaceae and *Acinetobacter* sp. isolated from influent, bioreactor and effluent of UWTP

Sampling point	CTX	CAZ	FEP	ATM	ERT	IMP	MEM	CIP	TE	C	CN	AK	MDR > 3
Influent	72 a	88 a	48 a	80 a	0 a	0 a	0 a	16 a	44 a	28 a	32 a	4 a	48 a
Bioreactor	95 ab	90 a	35 a	90 a	55 b	50 b	65 b	20 a	35 a	15 a	15 a	0 a	30 b
Effluent	100 b	100 a	67 a	67 a	22 ab	44 ab	22 ab	22 a	44 a	11 a	22 a	22 a	44 b
Summary	87	91	46	81	24	26	28	19	41	20	24	6	41

Values within a column marked with different letters are significantly different ($p < 0.05$)

CTX cefotaxime, *CAZ* ceftazidime, *FEP* cefepime, *AZT* aztreonam, *ERT* ertapenem, *IMP* imipenem, *MEM* meropenem, *CIP* ciprofloxacin, *TET* tetracycline, *C* chloramphenicol, *CN* gentamicin, *AK* amikacin, *MDR* multidrug resistance strains

Table 3 Characterization of *Raoultella* spp. strains isolated from bioreactor

Strain	Identification	Antibiotic resistance phenotype	β-Lactamase genes	Integrase gene	Plasmid replicon	Plasmid localization of ARG
210C	*Raoultella* sp. (MF457856)	CTX, CAZ, ATM, ERT, IMP, MEM	$bla_{TEM-1-like}$, bla_{GES}, $bla_{KPC-2-like}$, bla_{ORN}	–	+	bla_{GES}
213C	*Raoultella* sp. (MF457857)	CTX, CAZ, FEP, ATM, ERT, IMP, MEM, CIP	$bla_{TEM-1-like}$, bla_{GES}, $bla_{KPC-2-like}$, bla_{ORN}	*intI1*	+	bla_{GES}
228A	*Raoultella* sp. (MF457858)	CTX, CAZ, FEP, ATM, ERT, IMP, MEM	$bla_{TEM-1-like}$, $bla_{KPC-2-like}$	–	+	–
274B	*Raoultella* sp. (MF457859)	CTX, CAZ, ATM, ERT, IMP, MEM	$bla_{TEM-1-like}$, bla_{GES}, $bla_{KPC-2-like}$, bla_{ORN}	–	+	bla_{GES}
328	*Raoultella* sp. (MF457860)	CTX, CAZ, ATM, MEM	$bla_{TEM-1-like}$, bla_{GES}, $bla_{KPC-2-like}$, bla_{ORN}	–	+	bla_{GES}
376	*Raoultella* sp. (MF457861)	CTX, CAZ, ATM, ERT, IMP, MEM	$bla_{TEM-1-like}$, bla_{GES}, $bla_{KPC-2-like}$, bla_{ORN}	–	+	bla_{GES}
382A	*Raoultella* sp. (MF457862)	CTX, CAZ, ATM, ERT, IMP, MEM, TET, C	$bla_{TEM-1-like}$, bla_{GES}, $bla_{KPC-2-like}$, bla_{ORN}	*intI1*	+	bla_{GES}
385A	*Raoultella* sp. (MF457863)	CTX, CAZ, ATM, ERT, MEM	$bla_{TEM-1-like}$, bla_{GES}, $bla_{KPC-2-like}$, bla_{ORN}	–	+	bla_{GES}
286	*Raoultella* sp. (MF457864)	CTX, CAZ, ATM, ERT, IMP, MEM	$bla_{TEM-1-like}$, bla_{GES}, $bla_{KPC-2-like}$, bla_{ORN}	–	+	bla_{GES}
293	*Raoultella* sp. (MF457865)	CTX, CAZ, FEP, ATM, ERT, IMP, MEM, TET	$bla_{TEM-1-like}$, bla_{GES}, $bla_{KPC-2-like}$, bla_{ORN}	*intI1*	+	bla_{GES}
299A	*Raoultella* sp. (MF457866)	CTX, CAZ, ATM, MEM	$bla_{TEM-1-like}$, bla_{GES}, $bla_{KPC-2-like}$, bla_{ORN}	–	+	bla_{GES}

new variants: $bla_{CMY-157}$, bla_{MOX-13}, and bla_{FOX-15}. What is worth noting is that bla_{TEM-12} was previously detected in numerous clinical isolates of Enterobacteriaceae, e.g., *Escherichia coli*, *Klebsiella* spp., or *Enterobacter* spp. (Sturm et al. 2010; Perilli et al. 2011). In our study, bla_{TEM-12} was found in *Shewanella* spp., which is the first case of this variant in this species ever. Another variant, bla_{TEM-30}, was also found in clinical strains of Enterobacteriaceae, with high frequency of isolates in Spain (Martín et al. 2010; Ortega et al. 2012) and minor identifications in Israel, the UK (NZ_FLMS01000013.1), Sweden (NZ_KE701793.1), Portugal (NG_050260.1), Vietnam (NZ_CZLO01000043.1), the USA, and Poland (Bradford et al. 2004; Empel et al. 2008; Leavitt et al. 2009).

Both bla_{TEM-47} and bla_{TEM-68} were previously identified mostly in clinical strains of Enterobacteriaceae in Polish hospitals (Gniadkowski et al. 1998; Baraniak et al. 2005; Robin et al. 2012). The bla_{TEM-68} variant probably evolved from bla_{TEM-47}, and both are not very frequent in Poland. Outside this area, there was a report from Canada, where bla_{TEM-47} was found in *Streptococcus dysgalactiae* (Vélez et al. 2017). To the best of our knowledge, this study reports the first isolate carrying a $bla_{TEM-47/68}$ variant found outside the clinical setting in Poland.

Fifty-three variants of ACT β-lactamase have been identified so far in numerous bacterial species. However, little is known about the dissemination of this β-lactamase in an aqueous environment. Amador et al. (Amador et al. 2015) found bla_{EBC} gene, including bla_{ACT}, the most prevalent gene of the AmpC β-lactamase type (38.9%). There was also a report on bla_{ACT-13} gene that was found in *Enterobacter asburiae* isolated from a water distribution network (Manageiro et al. 2014). Hence, our identification of bla_{ACT} in *Enterobacter* sp. is the first isolation of this β-lactamase gene from a wastewater environment.

All the new *bla* variants (bla_{FOX-15}, bla_{MOX-13}, and $bla_{CMY-157}$) belong to class C serine β-lactamases—AmpC—and have motifs characteristic of this class, such as SXSK motif (typical of AmpC active site), YXN motif (characteristic of the C class), and KTG domain (Ghuysen 1991). *The* bla_{FOX-15} and bla_{MOX-13} variants are more similar to the remaining variants of the FOX and MOX types of β-lactamases, respectively, than is $bla_{CMY-157}$ to the CMY type. In the CMY-157 sequence, we found 12 unique amino acid differences compared to the sequences of other CMY variants (Fig. 3S). However, the sequence of a putative signal peptide (first 20 aa) is highly similar to the consensus sequence of CMY family. To conclusively confirm that CMY-157 is a member of the CMY family, additional phenotypical tests are needed. The identification of bla_{FOX-15} on a small plasmid in *Kluyvera* sp. is—to our knowledge—the first observation of bla_{FOX} in this genus.

The case of $bla_{TEM-116}$, which was recently widely discussed by a couple of researchers (Jacoby and Bush 2016; Pleiss and Zeil 2016; Furlan et al. 2017), is also worth noting. The problem with this variant concerns possible false-positive results caused by contamination of a PCR reaction with a commercial *Taq* polymerase. The $bla_{TEM-116}$ variant was developed synthetically and cloned into pUC vectors as a selection gene (selection with ampicillin). Production of *Taq* polymerase is based on vectors that contain the $bla_{TEM-116}$ gene, which could be the cause of false-positive identifications of this variant in the clinical or natural environment. However, the $bla_{TEM-116}$ variant was recently identified in numerous genome sequences from the natural environment (NZ_ADUV01000033.1, NZ_NDGT01000042, NZ_AKVH01000020.1, NZ_LATZ01000118.1). Consequently, sequencing of a PCR product is—in our opinion—insufficient to prove the presence of $bla_{TEM-116}$ in natural habitats. Only sequencing of a whole genome or a metagenome approach could produce conclusive results.

Moreover, three types of integrase genes were found in this study, but only *intI1* and *intI3* were identified at all three sampling points. While *intI2* genes were also found, they were detected only in raw wastewater samples. Based on literature data, *intI2* is less frequently observed in wastewater samples than *intI1* (Moura et al. 2007; Su et al. 2014). That relationship was also observed in our study.

In this study, an interesting group of highly resistant *Raoultella* spp. strains from a bioreactor was observed (Table 3). *Raoultella* spp. are gram-negative aerobic rods belonging to the Enterobacteriaceae family. They typically inhabit natural environments but were also recognized as opportunistic human pathogens (Sękowska 2017). These bacteria are naturally resistant to aminopenicillins because of the possession of chromosomal, class A β-lactamase genes (Walckenaer et al. 2004). Additionally, many different β-lactamase genes have been reported in this genus so far, including bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{IMP}, bla_{KPC}, bla_{NDM}, bla_{OXA}, and bla_{OXA-48} (Zurfluh et al. 2013; Sun et al. 2015; Demiray et al. 2016; Yao et al. 2017). In this study, bla_{TEM}, bla_{GES}, bla_{KPC}, and bla_{ORN} genes characteristic of *R. ornithinolytica* were identified in all of the strains with the exception of one (228A). The results of molecular determinants analysis were also convergent with those of phenotypical tests, where all *Raoultella* spp. strains were unsusceptible to third-generation cephalosporins (ceftazidime and cefotaxime), aztreonam (monobactam), and meropenem (carbapenem). Moreover, this is the first identification of bla_{GES} in *Raoultella* spp. and, additionally, in ten of the isolated strains, this gene has been located on small plasmids (~ 9 kb). B-lactamase genes had been identified on a couple of *Raoultella* spp. big plasmids and megaplasmids before (bla_{IMP-4} and bla_{KPC-2} on 450 kb plasmid, bla_{NDM-1} on 41 kb and 100 kb plasmids, bla_{OXA-48} on 63 kb or bla_{KPC-2} 40 kb plasmid) (Sun et al. 2015; Zheng et al. 2015; Yao et al. 2017). However, this is the first observation of a *bla* gene on a very small plasmid in *Raoultella* spp.

Numerous β-lactamases whose genes (*bla*) were identified among the isolated strains belonged to all four principal classes of these enzymes: A, B, C, and D according to the Ambler classification (Bush and Jacoby 2010). Of the identified *bla* genes, only $bla_{TEM-1-like}$ and bla_{OXA} were found at all three sampling points (raw wastewater, bioreactor, and effluent). However, statistical analysis results indicated no significant differences in the frequency of *bla* genes between samples from different parts of the WWTP. Similar results were observed in a previous study for *Aeromonas* spp. (Piotrowska et al. 2017), in which eight *bla* class A genes ($bla_{TEM-1-like}$, $bla_{SHV-11-like}$, $bla_{SHV-12-like}$, $bla_{CTX-M-15-like}$, $bla_{CTX-M-27-like}$, bla_{GES}, $bla_{PER1/5}$, $bla_{KPC2-like}$) and one class D gene (bla_{OXA}) were found. Such a result suggests the possible transmission of the mentioned *bla* genes between these groups of bacteria. However, in this study, results of antibiotic susceptibility testing indicated significantly fewer MDR isolates (43%) than in the *Aeromonas* spp. study (68%) (Piotrowska et al. 2017). The main reason for that was a decrease in the susceptibility to antibiotics other than the β-lactams, i.e., ciprofloxacin, chloramphenicol, and amikacin. At the same time, significantly more bacteria were unsusceptible to most of the β-lactams (besides cefepime) and at least 60% of strains were unsusceptible to cephalosporins and monobactam. This result indicates that β-lactam resistance in the studied WWTP is more likely maintained among gram-negative rods than in *Aeromonas* spp. Differences between phenotypical and molecular results could be explained by the presence of molecular determinants that were not found among the isolated strains, both specific ones (various β-lactamases) or those with a wider range of specificity (efflux-pumps).

In conclusion, β-lactamase genes are widely disseminated among potentially pathogenic gram-negative rods isolated from a WWTP, which survive the wastewater treatment process and are released into the aquatic environment. The finding of 116 genes that encode enzymes belonging to 12 β-lactamase types confirms this statement, especially since three new variants of β-lactamase genes ($bla_{CMY-157}$, bla_{MOX-13}, and bla_{FOX-15}) were identified. Moreover, the identification of four *bla* genes that have never been found in a WWTP before (bla_{TEM-12}, bla_{TEM-30}, $bla_{TEM-47/68}$, bla_{ACT}) reveals hazardous potential and continuous variability of this environment. Importantly, nine *bla* genes were previously found in the same environment in *Aeromonas* spp. ($bla_{TEM-1-like}$, $bla_{SHV-11-like}$, $bla_{SHV-12-like}$, $bla_{CTX-M-15-like}$, $bla_{CTX-M-27-like}$, bla_{GES}, $bla_{PER1/5}$, $bla_{KPC-2-like}$, bla_{OXA}), which indirectly indicates the possible dissemination of *bla* genes between different bacterial genera. The identification of bla_{FOX-15} and bla_{GES} within mobile genetic elements (small plasmids) is alarming. Whereas plasmid contribution to horizontal gene transfer (HGT) is undeniable, evaluation of the scale of this process and characterization of mobilomes in particular environments and bacterial hosts are important for understanding HGT mechanisms. Culture-based approach employed in this study enabled us to demonstrate the occurrence of small plasmids in *Kluyera* spp. and *Raoultella* spp., which was the first observation of bla_{FOX-15} and bla_{GES} genes within these genera. This result shows the correlation that is missing in a metagenomic approach. Finally, this approach shows that besides the decrease in the number of MDR strains in the effluent compared to the influent and the significantly higher number of cefotaxime and carbapenem unsusceptible strains in the influent, no differences in the frequency of diverse types of *bla* genes between the isolation points were found. This is another disturbing observation that supports the hypothesis of WWTPs as hot spots for ARG and ARB dissemination.

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Compliance with ethical standards

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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