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CHARACTERIZATION OF B-LACTAMASES AND MULTIDRUG RESISTANCE MECHANISMS IN ENTEROBACTERALES FROM HOSPITAL EFFLUENTS AND WASTEWATER TREATMENT PLANT

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ABSTRACT

Antimicrobial resistance presents a global challenge to the fight against infections in modern time. It is projected that, close to 2.4 million people are likely to die globally by the year 2050 due to infections linked to antibiotic resistance. Antimicrobials indiscriminately discharged into wastewater promote the emergence of antibiotic resistance, facilitated by selective pressure and transfer of resistance genes. This study aimed to determine the antimicrobial resistance profiles of enteric bacteria from wastewater and to establish the prevalence of plasmid borne β-lactamases and other mechanisms conferring multiresistance. Enteric bacteria, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, and *Citrobacter* species (n = 126) from hospital effluents and proximate wastewater treatment plant in the city of Pecs, Hungary, were assayed for susceptibility to four antimicrobial classes. The β-lactamase encoding genes harbored in plasmids were genotyped and the plasmid DNA was subjected to the next generation sequencing. A multidrug resistance phenotype was found in 72% (n = 58) of *E. coli* isolates, 70% (n = 43) of *Klebsiella* species isolates, and 40% (n = 25) of *Enterobacter* and

Citrobacter species. 86% (n = 50) of *E. coli*, 77% (n = 33) of *Klebsiella* species and 25% (n = 4) of *Citrobacter* species isolates phenotypically expressed extended spectrum β-lactamase (ESBL). ESBL genes, $bla_{\text{CTX-M-27}}$ and $bla_{\text{TEM-1}}$ were found in *E. coli*, while *Klebsiella* species harbored $bla_{\text{CTX-M-15}}$, $bla_{CTX\text{-}M\text{-}30}$, *or* $bla_{\text{SHV-12}}$. Genes coding for aminoglycoside modifying enzymes, adenylyltransferases (*aadA1*, *aadA5)*, phosphotransferases (*aph(6)-1d*, *aph(3")-Ib)*, acetyltransferases (*aac(3)-IIa)*, (*aac(6)-Ib*), sulfonamide/trimethoprim resistant dihydropteroate synthase (*sul*), and dihydrofolate reductase (*dfrA*) were also identified. Mobile genetic elements namely; plasmids and integrons acquired via horizontal gene transfer are vehicles for multiresistance in enteric bacteria from wastewater. Monitoring wastewater from human sources for acquired resistance in clinically important bacteria may provide a cheaper alternative in regions facing challenges that limit clinical surveillance.
Keywords: hospital effluents; wastewater treatment plant; Enterobacterales; β-lactamases; multiresistance

INTRODUCTION

Antimicrobial resistance presents a global challenge to the fight against infections in modern time (Iakovlieva & Bahlai, 2020). Each year more than 670 000 infections are due to antibiotic resistant bacteria in the European Union/European Economic Area (EU/EEA) according to data from the European Antimicrobial Resistance Surveillance Network (EARS-Net), and approximately 33 000 people succumb to these infections (Cassini et al., 2019). It is projected that, close to 2.4 million people are likely to die globally in high-income countries by the year 2050 due to diseases caused by antibiotic resistant microorganisms (UN, 2019). The widespread use of antimicrobials in clinical practice to control infectious diseases, their application in veterinary medicine coupled with the discharge of non-treated pharmaceutical effluent into the environment results in selective pressure which is associated with the emergence and subsequent evolution of bacteria resistant to antibiotics (Islam, 2011).

Members of the order Enterobacterales, which bear similar biochemical and genetic characteristics are ubiquitous and form a major part of gut microbiota (Partridge, 2015). Some of them such as *Klebsiella pneumoniae, Escherichia coli, Proteus, Citrobacter* and *Enterobacter* cause infections including in the urinary tract, bloodstream, and respiratory tract (hospital and health-care associated pneumonia), as well as intestinal and intra-abdominal infections (Pitout & Laupland, 2008; Qin et al., 2008). Enterobacterales exhibit a wide range of resistance attributed to either mutations in chromosomal genes or mobile genes captured from different source species by various mobile genetic elements and transferred to plasmids, which can shuttle between cells and confer or enhance resistance to certain chemical classes

of antimicrobials that are frequently used against multi-drug resistant microorganisms (Partridge, 2015). Fluoroquinolones and β-lactam antibiotics, which include the sub-groups of penicillins, cephalosporins and carbapenems are the most frequently prescribed antibiotics and preferred therapeutic choices against infections caused by members of Enterobacterales (Damoa-Siakwan, 2005). Hospitals and other environments characterized by high amounts of antibiotics are associated with multidrug resistant Gram-negative bacteria that have demonstrated an increasing resistance to those compounds (Hocquet, Muller, & Bertrand, 2016). Antimicrobial resistant genes (ARG), such as genes coding for extended-spectrum β-lactamases (ESBLs) and carbapenemases harbored by Enterobacterales and other Gram-negative bacteria are clinically significant and have been reported from hospital effluents and WWTPs (Haller et al., 2018; Lamba, Graham, & Ahammad, 2017). These genes are typically encoded on plasmids which harbor mobile genetic elements such as transposons or integrons and genes known to encode resistance to other antimicrobial agents (Szczepanowski et al., 2009).

ESBLs are β-lactamases that are capable of hydrolyzing broad-spectrum cephalosporins and aztreonam, whose activity is inhibited by clavulanic acid (Paterson & Bonomo, 2005).

More than 300 subtypes of ESBLs have been described and their evolution is believed to originate from common ancestral types: TEM-1, TEM-2, or SHV-1 (Bush & Jacoby, 2010). Mutations occurring in those genes resulted in new β-lactamases which can hydrolyze extended- spectrum cephalosporins and aztreonam (Slama, 2008; Smet et al., 2008). Enterobacterales are also known to express ESBLs different from TEM, or SHV related types, such as CTX-M-type β-lactamases which are encoded by genes captured on transferable plasmids and are among the most widespread ESBLs in Europe (Coque, Baquero, & Canton, 2008). Carbapenems (imipenem, doripenem, ertapenem and meropenem) are the most potent antimicrobials used to manage life-threatening infections caused by multiresistant Gram-negative bacilli and their efficacy has been diminishing since carbapenem-resistant Gram-negative strains have emerged following their extensive use (Codjoe & Donkor, 2018). Carbapenemase producers are resistant to almost all β-lactams and to other classes of antibiotics (Woodford, Wareham, Guerra, & Teale, 2014). Their occurrence in the environmental matrices is increasing with hospital wastewater being reported as a key reservoir of carbapenemase-producing Enterobacterales (Zhang, Lu, & Zong, 2012). Data on the prevalence of β-lactamase producing multiresistant Enterobacterales of clinical importance in wastewater from human sources in southwest Hungary is unavailable since most studies are centered on the clinical environment. This study was aimed at bridging this knowledge gap and was therefore designed to: 1. Isolate enteric bacteria of clinical importance including *Klebsiella* species, *Escherichia coli,* and *Enterobacter* species from urban wastewater covering hospital effluents, municipal wastewater, and WWTP and determine their antimicrobial resistance profiles; 3. Molecular typing and sequencing of plasmid DNA to establish the prevalence of β-lactamase enzymes from the isolates and to unearth other mechanisms conferring multiresistance. In this context, the findings contribute to important knowledge and are applicable in planning effective strategies to minimize the spread of multiresistance in the environment.

MATERIALS AND METHODS

Study Sites and Sample Collection

This study was carried out in the city of Pecs, in southwest Hungary. Wastewater samples were drawn from four hospital wastewater discharge points, H1 (387 beds), H2 (106 beds), H3 (127 beds), and H4 (348 beds), a discharge point of a nursing home for the elderly (NH, 490 beds), municipal wastewater sewer lines (MWW), and a wastewater treatment plant (WWTP) (Figure 1). Effluent samples from the healthcare facilities were collected directly from two separate generation points serving different buildings before joining the main sewer pipe. A 30 mL sample was collected every 15 min by lowering a flask into the wastewater flow over a period of 4 h and the aliquots were pooled together to constitute a 480 mL composite sample in sterile 500 mL glass bottles. Samples from the WWTP were collected from the influent directly behind the grating screen. One grab sample was drawn from the activated sludge reactor and the digested sludge after thermophilic digestion. The municipal wastewater was collected 4 km upstream of the health care facilities (MWW1) and at a second spot upstream of the WWTP (MWW2), and was pooled. Samples were transported on ice to the laboratory and stored at 4 °C, before assaying within 6 h. The WWTP processes wastewater from the central business district, health care facilities, domestic wastewater, and some storm runoff and serves a population equivalent to slightly over 200,000 inhabitants. The study was conducted during 2018–2020.

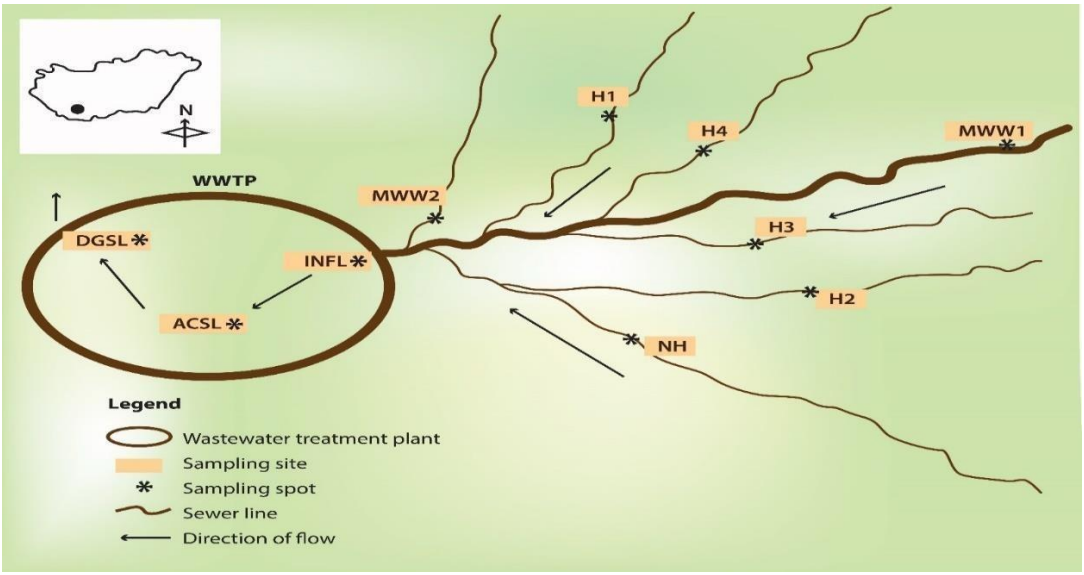

Figure 1. A schematic diagram depicting the sampling locations. H1–H4, hospital wastewater; NH, nursing home; MWW1 and MWW2, municipal wastewater; INFL, influent; ACSL, activated sludge; DGSL, digested sludge; WWTP, wastewater treatment plant.

Characterization of the Bacterial Isolates

On each sampling occasion, up to 5–10 lactose fermenting colonies of presumptive enteric bacteria representative of different colony morphotypes (colony contour, color, or size) were randomly picked from Eosin methylene blue plates. Typical green metallic sheen colonies were suggestive of *E. coli*, large mucoid pinkish colonies characteristic of *Klebsiella* species and pink to purple colonies typical of *Enterobacter* species (Cheesbrough, 1990). The isolates were sub-cultured on nutrient agar, incubated at 35 ± 2 °C for further 18–24 h and were identified with MALDI-TOF MS. Mass spectrometry was performed using a Microflex MALDI Biotyper (Bruker Daltonics, Bremen, Germany) equipment. MALDI Biotyper RTC 3.1 software (Bruker Daltonics, Bremen, Germany), and the MALDI Biotyper Library 3.1 were employed for the spectrum analysis. Score values of ≥ 2.0 were considered reliable identifications (Blondiaux, Gaillot, & Courcol, 2010). For further characterization, the cultures were preserved at −80 °C in nutrient broth supplemented with 20% glycerol.

Antimicrobial Susceptibility Profiles and Phenotypic Detection of β-lactamases

Antimicrobial susceptibility was established using the standardized disk diffusion method on Mueller Hinton agar (Biolabs, Budapest, Hungary) according to EUCAST 2018 guidelines. The standard antibiotic discs belonging to the following classes were used: (1) β-lactams; ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 10 µg), cefotaxime (CTX, 30 µg), cefpodoxime (CPD, 10 µg), cefoxitin (FOX, 30 µg), imipenem (IMP, 10 µg), and meropenem (MEM, 10 µg).

(2) Aminoglycoside; gentamicin (GEN, 10 µg), (3) fluoroquinolone; ciprofloxacin (CIP, 5 µg), and (4) sulfonamide; sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 µg) (Oxoid, Wesel, Germany). Quality control was performed using

E. coli ATCC 25922, *Klebsiella pneumoniae* ATCC 180112, and *Enterobacter cloacae* ATCC 180083 as wild type negative controls while *E. coli* ATCC 151006, *Klebsiella pneumoniae* ATCC 180111, and *Enterobacter cloacae* ATCC 161002 were control strains with known resistance phenotype. Inoculum's concentration was standardized to

0.5 McFarland turbidity, and the plates were incubated for 18–20 h at 35 °C and evaluated for the formation of inhibition zones. The zone diameters were interpreted based on the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints, version

8.1, 2018 (EUCAST, 2018). Multidrug resistance among the strains was defined as resistance to three or more antibiotic classes. A combined disk test was used to screen for the production of extended-spectrum β-lactamase. Cefotaxime (CTX 30 µg) and cefpodoxime (CPD 10 µg) (Oxoid, Wesel, Germany) disks were placed next to the disks with cefotaxime/clavulanic acid (CTC, 30/10 µg, CTC, 40) and cefpodoxime/clavulanic acid (10/10 µg, CD, 01). Similarly, carbapenem resistant isolates were screened for metallo-β-lactamase (MBL) production using a combined disk of imipenem/ethylene diamine tetraacetic acid (IMP 10 µg / EDTA 292 µg—IEL 292) (Oxoid, Wesel, Germany) on Mueller Hinton agar plates (Biolabs, Budapest, Hungary) with an inoculum of 0.5 McFarland. An increase in the inhibition zone size to ≥ 5 mm with the combined disks compared

to the disk of cephalosporin/carbapenem alone was considered a positive test for β-lactamase production (Oduro- Mensah et al., 2016).

Molecular Typing of ESBL and Carbapenemases

Plasmid DNA was isolated by the alkaline lysis method using the Monarch plasmid DNA miniprep kit according to the manufacturer's instructions (New England Biolabs T1010, Ipswich, Massachusetts, USA). DNA was isolated from freshly grown pure colonies transferred into Luria Bertani broth and incubated in an orbital shaker at 35 °C and 200 rpm for 12–16 h. All the centrifugation steps were carried out at 16,000 x g. DNA concentration and purity were determined using a Nanodrop spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, USA), and stored at –20 °C for subsequent PCR amplification. PCR reactions for selected genes belonging to *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM,}}$ and *bla*$_{\text{NDM}}$ families were performed in a final volume of 25 µL containing 12.5 µL DreamTaq PCR master mix (2x) composed of Dream Taq DNA polymerase, optimized 2x Dream Taq buffer, 4.0 mM, $MgCL_2$, 0.4 mM each of dATP, dCTP, dGTP, and dTTP (Thermo Scientific, Waltham, Massachusetts, USA),
1.0 µM, Forward primer, 1.0 µM, Reverse primer, 1 µL template DNA, and by addition of nuclease free water. A conventional PCR assay was used and the amplification thermal profile was applied as follows: Initial denaturation at 95 °C for 2 min, 35 times repeated cycle of 95 °C for 30 s, 30 s at the appropriate primer annealing temperature for the specific primer, primer extension at 72 °C for 1 min, and final elongation at 72 °C for 10 min, with a holding step at 4 °C. Primers used and their corresponding annealing temperatures are as shown in Table 1. Gly238→Ser mutation associated with the hydrolysis of third-generation cephalosporins was identified through digestion of *bla*$_{\text{SHV}}$ PCR product with *NheI* (New England Biolabs). Colony PCR was performed using OneTaq quick load Mastermix to determine the presence of chromosomally encoded metallo-β-lactamases among carbapenem resistant isolates. Individual colonies were dipped into the reaction tubes containing 25 µL One Taq master mix (New England Biolabs, Budapest, Hungary) PCR primers and nuclease free water. Thermal cycling conditions were initial denaturation at 94 °C for 2 min, 35 times repeated cycle of 94 °C for 30 s, 30 s at the appropriate primer annealing temperature for the specific primer, primer extension at 68 °C for 1 min, and final elongation at 68 °C for 10 min, with a holding step at 4 °C. Post PCR analysis was performed in 1.5% (w/v) agarose gel stained with 2 µL serva DNA stain G (Bio-Connect, Begonialaan, Netherlands). Then, 5 µL of each DNA sample was mixed with 2 µL of 6x loading dye and electrophoresed in 1x Tris—EDTA (TAE) buffer at 100 V for 1 h and visualized under an ultraviolet transilluminator. Next, 10 µL of either phage Lambda DNA digested with *EcoRI/HindIII* and a low range molecular weight marker (Thermo Scientific, Waltham, Massachusetts, USA) or both, in some cases, were included in each run as DNA size markers. In each PCR run, a positive control consisting of a clinical isolate of a confirmed reference strain was included for each genotype. The control strains were well characterized clinical isolates kindly provided by the microbiology laboratory of the University of Pecs Medical School (Melegh et al., 2014). For the robust colony PCR, 10 µL of each reaction was directly loaded onto an agarose gel alongside a PCR product from an appropriate reference strain and a DNA ladder.

Table 1. Sequences, annealing temperature, and expected product sizes of primer pairs targeting the specified β-lactamase genes.

Gene	Sequence (5′-3′)	Product Size (bp)	Annealing Temp (°C)	References
CTX-M-F CTX-M-R	TTTGCGATGTGCAGTACCAGTAA CGATATCGTTGGTGGTGCCATA	544	51	(Edelstein, Pimkin, Palagin, Edelstein, & Stratchounski, 2003)
SHV-F SHV-R	ATGCGTTATATTCGCCTGTG GTTAGCGTTGCCAGTGCTCG	865	49	(Wiegand, Geiss, Mack, Stürenburg, & Seifert, 2007)
TEM-F TEM-R	GCGGAACCCCTATTTG ACCATTGCTTAATCAGTGAG	963	56	(Olesen, Hasman, & Møller Aarestrup, 2004)
IMP-F IMP-R	GGAATAGAGTGGCTTAAYT TCGGTTTAAYAAAACAACCACC	232	52	(Poirel, Walsh, Cuvillier, & Nordmann, 2011)
KPC-F KPC-R	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	798	52	(Poirel et al., 2011)
NDM-F NDM-R	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	621	52	(Poirel et al., 2011)
OXA-48-F OXA-48-R	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438	60	(Poirel et al., 2011)
VIM-F VIM-R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	52	(Poirel et al., 2011)

Plasmid DNA Library Preparation and Sequencing

Selected isolates (*E. coli* n=10, *K. pneumoniae* n=9, *K. oxytoca* n=1, *C. freundii* n=1) were subjected to NGS sequencing. The selection was based on antimicrobial susceptibility profiles and site of isolation. The library for NGS sequencing was prepared using Swift 2S Turbo DNA Library Kits (Swift Biosciences, Ann Arbor, Michigan, United States). Briefly, 100 ng genomic DNA was fragmented, end prepped, and adapter ligated. Magnetic bead size selection was performed to select 250-300 bp insert size fragments, followed by the library amplification according to the manufacturer's instructions. The quality of the library was checked on the 4200 TapeSation System using D1000 Screen Tape (Agilent Technologies, Palo Alto, California, USA) and the quantity was measured on Qubit 3.0. (Thermo Scientific, Waltham, Massachusetts, USA). Illumina sequencing was performed on the NovaSeq 6000 instrument (Illumina, San Diego, California, USA) with a 2x151 run configuration. Quality control (QC), trimming, and filtering of 150 pb paired-end raw reads were performed in the preprocessing step. The QC analysis was performed with FastQC (Andrews et al., 2010). The Phred-like quality scores (Qscores) were set to >30. Poor quality reads, adapters at the ends of reads, limited skewing at the ends of reads were eliminated by using Timmomatic (Bolger, Lohse, & Usadel, 2014). Since data contained genomic DNA debris, identification of plasmid-derived contigs was performed after de novo assembly of cleaned reads. For plasmid identification, genes characteristically encoded in plasmids for each strain were determined based on literature and by aligning them for the contigs using locally the Blast+ (Altschul, Gish, Miller, Myers, & Lipman, 1990). Prokaryotic gene finding was performed by Glimmer using the Bacterial, Archaeal, and Plant Plastid Code. Glimmer uses

Interpolated Markov Models (IMMs) to identify the coding regions and to distinguish them from non-coding DNA, which enabled identified genes to be annotated (Delcher, Harmon, Kasif, White, & Salzberg, 1999). Functional annotation and Gene Ontology (GO) analysis were carried out using OmixBox.Biobam as follows: sequences were blasted against the NCBI nr (non-redundant) database (taxID: 2Bacteria), applying blastn configuration locally. To retrieve GO terms associated with the 10 Hits obtained by the Blast search GO mapping and annotation were performed. GeneBank identifiers (gi), the primary blast Hit ids, were used to retrieve UniProt IDs making use of a mapping file from PIR (Non-redundant Reference Protein Database), including PSD, UniProt, Swiss-Prot, TrEMBL, RefSeq, GenPept, and PDB. Accessions were searched directly in the dbxref table of the GO database. BLAST result accessions were searched directly in the gene-product table of the GO database; GO annotations were specified according to GO terms: molecular function, cellular component and biological process (Götz et al., 2008). For detection of antimicrobial resistance genes and identification of plasmid incompatibility groups ResFinder 4.1 and plasmidFinder 2.1 were used (Bortolaia et al., 2020; Carattoli et al., 2014). Each contiq of all isolates was aligned with MUMmer 4.0 in order to identify similar regions (Marçais et al., 2018). MUM indices were calculated pairwise and the resulting distance matrix was used to cluster the isolates with neighbor joining method (Deloger, El Karoui, & Petit, 2009). Visualization of clusters was done with splits 4 (Huson & Bryant, 2006).

Statistical Analysis

A descriptive statistical analysis (mean, range, and percentage) was performed using Microsoft Excel 2013 (Redmond, WA, USA, Microsoft Corp.). OriginPro version 2016 (Northampton, Massachusetts, USA, OriginLab Corp.) was used for plotting and analysis. Shapiro–Wilk tests were performed to check the normality of variables, while one-way analysis of variance (ANOVA) was used to compare resistance rates among sampling locations. Pairwise t-test was performed to determine differences in resistance rates between hospitals and WWTP. A correlation matrix was used

to examine the relationship between β and non-β-lactam antibiotic resistance. P values ≤ 0.05 were considered statistically significant.

RESULTS

Characterization of Antimicrobial Resistant Enterobacterales

A total of 126 isolates were recovered from the samples and identified with MALDI-TOF MS. The isolates belonged to *E. coli,* 46% (n = 58), *Klebsiella pneumoniae,* 20.6% (n = 26), *Klebsiella oxytoca,* 13.5% (n = 17), *Enterobacter cloacae,* 7.1% (n = 9), *Citrobacter freundii,* 11.11% (n = 14), *Citrobacter braakii,* 0.8% (n = 1), and *Citrobacter amalonaticus,* 0.8% (n = 1). The isolates were obtained from the following samples: 63.49% (80 strains) from the hospital effluents, 8.7% (11 strains) from nursing home, 20.6% (26 strains) from wastewater treatment plant, and 7.1% (9 strains) from municipal wastewater. Other isolates identified as not belonging to the Enterobacterales (*Stenotrophomonas maltophilia* n = 19, *Elizabethkingia meningoseptica* n = 7, *Elizabethkingia miricola* n = 6, and *Acinetobacter junii* n = 1) were not of interest for this study and were excluded from the subsequent analysis.

Antimicrobial Susceptibility Profiles and Multiple Antibiotic Resistance Indices

The enteric bacteria demonstrated variable susceptibility to the tested antibiotics, with isolates from the hospital effluents and the nursing home showing a relatively higher resistance rate than isolates from the WWTP and the municipal wastewater (Table 2). The multiple antibiotic resistance index (MAR index) for an isolate was calculated as *a/b* where *a* is the number of antibiotics to which an isolate was resistant, and *b* is the total number of antibiotics against which the isolate was tested. The MAR index for a site was calculated as *a/(b*c)* where *a* is the aggregate antibiotics resistance score of all isolates from a sample, *b* is the total number of antibiotics tested and *c* is the number of isolates from sample. Isolates from H3 had the highest resistance rate (MAR index 0.683) among the hospital

effluents. Those from the digested sludge were the most resistant (MAR index 0.560) among the wastewater treatment plant isolates, while municipal wastewater had the least resistant isolates (MAR index 0.444). *E. coli* demonstrated the highest MAR index (0.65) among the four genera, while *Citrobacter* spp. showed the lowest MAR index (0.39) (Table 3). A high prevalence of resistance (>80%) was observed for the third generation cephalosporins (3GCs) ceftriaxone (CRO), ceftazidime (CAZ), cefotaxime (CTX), and cefpodoxime (CPD), while significantly lower resistance rates were measured for carbapenems, imipenem, and meropenem (IMP and MEM) compared to the other antibiotics. From H1 and H2 samples, resistance to IMP was found in 20% and 8% of *Klebsiella* and *E. coli* isolates, respectively, and 1 (4%) *Klebsiella* isolate from H1 was resistant to MEM. Gentamicin (GEN) resistance was the least frequent among the three non-β-lactams.

Table 2. The antimicrobial susceptibility of enteric bacteria in percentage in the samples from the various sites.

		Antimicrobial Susceptibility %										
Source	**n = 126**	**CRO**	**CAZ**	**CTX**	**CPD**	**FOX**	**IMP**	**MEM**	**SXT**	**GEN**	**CIP**	**MAR Index**
H1	27	100	81	100	100	19	20	4	57	44	78	0.596
H2	25	96	80	96	92	24	8	0	48	60	88	0.592
H3	6	100	100	100	100	50	0	0	100	33	100	0.683
H4	22	96	82	91	91	33	0	0	41	27	68	0.532
NH	11	100	100	92	82	18	0	0	91	27	82	0.591
INFL	9	100	100	78	67	22	0	0	56	22	56	0.500
ACSL	12	92	75	83	92	8	0	0	75	18	67	0.508
DGSL	5	100	100	100	100	40	0	0	40	40	40	0.560
MWW	9	100	78	100	100	33	0	0	11	11	22	0.444

Antimicrobial agents; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; CPD, cefpodoxime; FOX, cefoxitin; IPM, imipenem; MEM, meropenem; SXT, sulfamethoxazole/trimethoprim; GEN, gentamicin; CIP, ciprofloxacin; n, number of isolates. MAR index, multiple antibiotic resistance index. **Study sites:** H1–H4, hospital effluent; NH, nursing home; INFL, influent; ACSL, activated sludge; DGSL, digested sludge; MWW, municipal wastewate.

Table 3. Antimicrobial susceptibility of each genus in percentage and their multiple antibiotic resistance indices (n
= 126).

Isolates	***E. coli* (n = 58)**		***Klebsiella* spp (n = 43)**		***E. cloacae* (n = 9)**		***Citrobacter* spp (n = 16)**	
Antimicrobial Agent	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**
CRO	58 (100)	0 (0)	42 (98)	1 (2)	9 (100)	0 (0)	14 (86)	2 (13)
CAZ	51 (88)	7 (13)	31 (72)	12 (28)	9 (100)	0 (0)	16 (100)	0 (0)
CTX	58 (100)	0 (0)	40 (93)	3 (7)	7 (78)	2 (22)	11 (69.)	5 (31)
CPD	58 (100)	0 (0)	38 (88)	5 (12)	8 (89)	1 (11)	14 (88)	2 (13)
FOX	2 (3)	56 (97)	5 (12)	38 (88)	9 (100)	0 (0)	15 (94)	1 (6)
IMP	2 (3)	56 (97)	6 (14)	37 (86)	0 (0)	9 (100)	0 (0)	16 (100)
MEM	0 (0)	58 (100)	1 (2)	42 (98)	0 (0)	9 (100)	0 (0)	16 (100)
SXT	43 (74)	15 (26)	16 (37)	27 (63)	5 (56)	4 (44)	2 (13)	14 (86)
GEN	15 (26)	43 (74)	24 (56)	19 (44)	0 (0)	9 (100)	5 (31)	11 (69)
CIP	46 79)	12 (21)	29 (67)	14 (33)	5 (56)	4 (44)	7 (44)	9 (56)
MAR index	0.646		0.551		0.555		0.394	

Antimicrobial agents; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; CPD,

cefpodoxime; FOX, cefoxitin; IPM, imipenem; MEM, meropenem; SXT, culfamethoxazole/trimethoprim; GEN, gentamicin: CIP, ciprofloxacin; R, resistant; S, susceptible; MAR index, multiple antibiotic resistance index.

The resistance rates between β-lactams (ceftriaxone, ceftazidime, cefotaxime, cefpodoxime, cefoxitin, imipenem, and meropenem) and the non-β-lactams (sulfamethoxazole/trimethoprim, gentamicin, and ciprofloxacin) antibiotics were not significantly different (p = 0.8550). However, a positive correlation was found between resistance in the two groups. Ceftriaxone resistance was positively correlated to SXT and CIP, ceftazidime resistance to SXT, cefotaxime to GEN, SXT and CIP, and cefpodoxime resistance to SXT and CIP. Notably, the *Enterobacter cloacae* and the *Citrobacter* spp. isolates were resistant to cefoxitin (a cephamycin-second generation cephalosporin), unlike the other genera. Figure 2a,b,c illustrate the antibiotic resistance patterns of *E. coli*, *K. pneumoniae*, and *K. oxytoca*, *E. cloacae*, and *Citrobacter* species isolates in the hospitals, nursing home, wastewater treatment plant, and municipal wastewater samples.

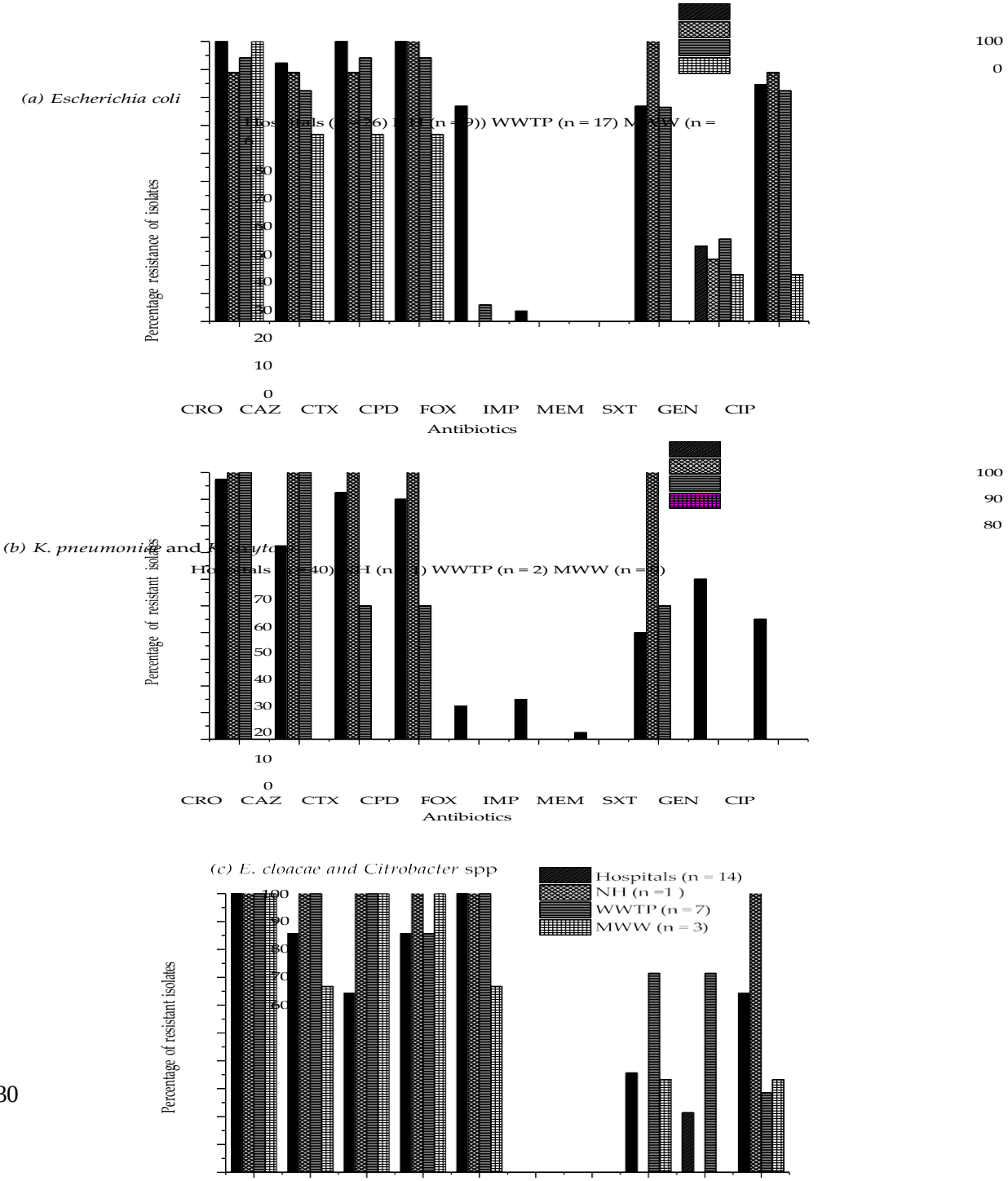

Figure 2. Antimicrobial resistance percentage of (**a**) *E. coli*, (**b**) *Klebsiella pneumoniae*, and *K. oxytoca*, (**c**) *Enterobacter cloacae*, and *Citrobacter* species isolates from four hospital effluents, nursing home, wastewater treatment plant, and municipal wastewater to ten different antibiotics. CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; CPD, cefpodoxime; FOX, cefoxitin; IPM, imipenem; MEM, meropenem; SXT, sulfamethoxazole/trimethoprim; GEN, gentamicin; CIP, ciprofloxacin. **Study sites:** NH, nursing home; WWTP, wastewater treatment plant; MWW, municipal wastewater. The absence of a bar indicates that no resistance was observed.

Phenotypic Expression of β-lactamases

Combined disc test of two different antibiotics and their β-lactamase inhibitor combinations were used to classify the isolates as extended-spectrum β-lactamase (ESBL) positive. Cefotaxime/clavulanic (CTC 40) and cefpodoxime/clavulanic acid (CD 01) markers defined 87 isolates (69.05%) as ESBL producers. 62.07% (n = 54), 25.3% (n = 22), and 6.9% (n = 6) of ESBL-positive isolates originated from hospital effluents, wastewater treatment plant, and municipal wastewater, respectively. All *Enterobacter cloacae* isolates were confirmed to be non-ESBL- producing and showed 100% resistance to cefoxitin, which is associated with AmpC cephalosporinase activity. The isolates resistant to imipenem and/or meropenem were confirmed to be metallo-β-lactamase negative by phenotypic test.

Molecular Characterization of ESBL and Carbapenemase Genes

The ESBL-positive isolates were confirmed to harbor $bla_{\text{CTX-M}}$ (100%) and bla_{TEM} 72.4% (n = 63) with PCR. The *NheI* digestion of the bla_{SHV} PCR product indicating the GLy238 → Ser mutation was positive in 17.2% (n = 15) of the samples (Table 4). Additionally, 69% (60 out of 87) of the isolates harbored both $bla_{\text{CTX-M}}$ and bla_{TEM}. This co-occurrence of $bla_{\text{CTX-M}}$ and bla_{TEM} was observed in *E. coli* (62%), *Klebsiella spp.* (49%), and *Citrobacter spp*. (19%). In 17.2% of *Klebsiella spp.* Isolates, both $bla_{\text{CTX-M}}$ and bla_{SHV} genes occurred simultaneously. Furthermore, 11.5% of the total isolates harbored the three groups of β-lactamase genes ($bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV}). The broad-spectrum β-lactamase producers were more widespread in hospital and the nursing home effluents (68.9%) than in wastewater from other sources (WWTP, 24.1%, and municipal wastewater, 6.9%). None of the carbapenemase genes bla_{VIM}, bla_{IMP}, bla_{KPC}, $bla_{\text{OXA-48}}$, and bla_{NDM} was detected in the plasmid DNA of the carbapenem resistant isolates (n = 2, *E. coli,* and n = 7, *Klebsiella* species). However, carbepenem resistant *K. oxytoca* isolates were shown to carry the bla_{VIM} gene in the genome by a robust colony PCR test (Figure S1).

Table 4. The number and percentage distribution and co-occurrence of bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes among the ESBL positive isolates.

ESBL Gene Family	*E. coli*	*K. pneumoniae*	*K. oxytoca*	*Citrobacter*	Total (% of ESBL Positive, n = 87)
$bla_{\text{CTX-M}}$	50 (86)	22 (85)	11 (65)	4 (25)	87 (100)
bla_{TEM}	39 (67)	16 (62)	5 (29)	3 (19)	63 (72)
bla_{SHV}	0 (0)	14 (54)	1 (6)	0 (0)	15 (17)

bla_{CTX-M} + bla_{TEM}	36 (62)	16 (62)	5 (29)	3 (19)	60 (69)
bla_{CTX-M} + bla_{SHV}	0 (0)	14 (54)	1(6)	0(0)	15 (17)
bla_{CTX-M} + bla_{TEM} + bla_{SHV}	0 (0)	10 (38)	0 (0)	0 (0)	10 (11)
Total number (ESBL positive and ESBL negative)	58	26	17	16	126

Next Generation Sequencing of Plasmids

Selected isolates belonging to *E. coli* (n = 10), *Klebsiella spp.* (n = 10), and *Citrobacter spp.* (n = 1) were subjected to next generation sequencing and de novo assembly of plasmid sequences. Number, total length, and N50 of assembled contigs ranged between 17 and 64, 182 477–547 810 bp, and 11 403–62 343 bp, respectively (Table S1). According to maximal unique and exact match (MUM) indices the isolates were clustered into six groups, designated as G1–G6 (Figure 2). The two main groups contained the majority of *E. coli* (G1) and *K. pneumoniae* (G2) isolates and the four minor groups (G3–G6) enclosed single, unrelated isolates. Cluster G1 could be subdivided into 3 subgroups (G1-a, G1-b, and G1-c).

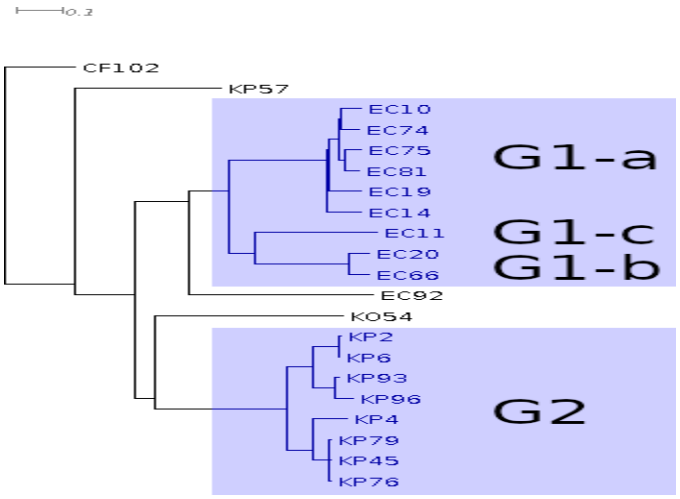

Figure 3. Representation of clusters based on MUMi distance of plasmid sequences. The major clusters are highlighted with blue

All isolates (n = 9) of G1 were identified as *E. coli* and were shown to harbor $bla_{CTX-M-27}$ type ESBL gene, aminogylcoside (*aadA5, aph(3")-Ib*, *aph(6)-Id*), folate inhibitor (*dfA17*, *sul1*, *sul2*), tetracycline (*tet(A)*), macrolide (*mph(A)*), and quaternary ammonium compound (*qacE*Δ) resistance genes (Table S1). The *dfrA17*, *aadA5*, *qacE*Δ, and *sul1* genes were part of a class I integron. The integron and the *mph(A)* gene were co-located on identical contigs in each isolate (Table S2). Besides, *aph(6)-Id, aph(3")-Ib*, and *sul2* were also found to be co-localized in all isolates, but the contigs carrying them differed according to the subgroups (Figure S2 and Table S2). In subgroup G1-a (Figure S2a–S2b), a *Tn*2 transposon carrying bla_{TEM-1} was inserted between *aph(6)-Id* and *aph(3")-Ib* and either *floR* or *tet(A)* was located upstream from this genetic structure. In subgroup G1b-G1c (Figure S2c and Table S2) *tet(A)* was located upstream from *aph(6)-Id, aph(3")-Ib*, and *sul2*. The presence of *floR* and *tet(A)* was shown for subgroup G-1a and all subgroups of G1, respectively. Different plasmid incompatibility groups where characteristic for the three subgroups: in subgroup G1-a IncB/O/K/Z, IncFIA, IncFIB, and IncFII, in subgroup G1-b IncFIA, IncFII, and IncI, and in subgroup G1-c IncFIA, IncFIB, IncFII, and IncI was

detected (Table S1).

Cluster G2 enclosed eight, closely related *K. pneumoniae* isolates. The presence of multiple β-lactamase genes (ESBL: *bla*$_{\text{CTX-M-27}}$, non-ESBL: *bla*$_{\text{TEM-1}}$, *bla*$_{\text{OXA-1}}$), aminoglycoside (*aac(6')-Ib-cr, aph(3")-Ib,* and *aph(6)-Id*), chloramphenicol (*catB3*), folate inhibitor (*dfrA14, sul2*), and quinolone (*qnrB1, aac(6')-Ib-cr*) resistance genes was characteristic for all isolates of G2. The *dfrA14* was carried by a class I integron which lacked the 3′ conserved sequence. The contigs carrying the aforementioned resistance genes where highly identical in all isolates (Table S2). Additionally, three isolates also harbored *aac(3)-IIa* on identical contigs. Two plasmid incompatibility groups (IncFIB and IncFII) were identified in this cluster (Table S1). The four unrelated isolates, namely *C. freundii* CF102, *K. pneumoniae* KP57, *K. oxytoca* KO54, and *E. coli* EC92, were shown to harbor either *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{CTX-M-30}}$, or *bla*$_{\text{CTX-M-1}}$ ESBL genes, respectively. Besides, a diversity in plasmid incompatibility groups and multiple antibiotic resistance genes (Table S1) were identified in these isolates, except for EC92 in which only the ESBL gene was detected. Genes *aac(6')-Ib*, *aadA1*, *bla*$_{\text{OXA-9}}$, and *bla*$_{\text{TEM-1}}$ were located on a transposon (*Tn*1331) in KP57 and KO54 (Figure S3). In addition to *Tn*1331, KP57 also harbored *aph(3")-Ib* and *aph(6)-Id* aminoglycoside resistance genes. In CF102 *aac(3)-IIa*, *bla*$_{\text{OXA-1}}$, *catB3*, and *aac(3)-IIa* were identified on contigs that were highly similar to those found in *K. pneumoniae* isolates of G2 (Table S2). Moreover, CF102 also carried *aph(3")-Ib*, *cmlB1*, and *tet(A)* genes as well.

DISCUSSION

Hungary ranks among the countries with the lowest antimicrobial drug consumption rate (defined daily dose per 1000 inhabitants per day) both in hospitals and in the community sector in the European Union/European Economic Area based on the annual European Surveillance of Antimicrobial Consumption Network (ESAC-Net) report (ECDC, 2020). Despite the low consumption, disposal of untreated hospital effluents containing antimicrobials or their metabolites may select for the development of antibiotic resistant bacteria based on our findings. We observed a more or less similar rate of resistance across the hospital and the nursing home effluents, measured by the multiple antibiotic resistance indices (MAR index), despite a huge variation in the bed capacity. H2 and H3, with the lowest bed capacities (106 beds and 127 beds, respectively), recorded high MAR indices (0.592 and 0.683). This may imply that the resistance rate largely depends on the regularly prescribed classes of antibiotics and the presence of different departments at each hospital as opposed to the number of patients accommodated in the facilities. All the isolates and all the sites reported multiple antibiotic resistance index values higher than 0.2. MAR index values greater than 0.2 indicate a high level of antibiotic contamination at the source (Osundiya, Oladele, & Oduyebo, 2013). The elevated MAR index values observed in *E. coli, Klebsiella* species, *E. cloacae* and *Citrobacter* species are consistent with the MAR index values reported in the same members of Enterobacterales isolated from urinary tract infections in a tertiary-care hospital in Hungary in a surveillance study conducted between 2008-2017, where *E. coli* and *Klebsiella* species reported higher MAR index values compared to CES (*Citrobacter*, *Enterobacter* and *Serratia*)(Gajdács, 2019). Notably, there was an enrichment of the ARB in the sewage sludge after thermophilic digestion (MAR index 0.560, from 0.5000 in the activated sludge). The detection of increased antibiotic resistant bacteria in wastewater treatment plants' effluent has been reported in other studies, (Galler et al., 2014; Hocquet et al., 2016; White et al., 2016). However, the increase in resistance development among susceptible bacteria facilitated by WWTP processes has not yet been established (Galvin et al., 2010). Available data suggest that β-lactam agents (especially penicillins and cephalosporins) are the most frequently used class of antibacterial agents across Europe in both hospital and community settings (Connor et al., 2017; Penalva et al., 2019). Although our study used ceftriaxone to screen for the β-lactam resistant bacteria, high resistance rate to other third-generation cephalosporins (cefpodoxime, cefotaxime, and ceftazidime) was attributed to cross-resistance. High levels of resistance to the same antimicrobial agents in Enterobacterales were found in effluents

from WWTPs in Navarra, Northern Spain (Ojer-Usoz, Gonzalez, Garcia-Jalon, & Vitas, 2014). The *bla*$_{CTX-M}$ type extended-spectrum β-lactamase (ESBL) observed in *E. coli*, *K. pneumoniae* and *C. freundii* in this study was largely responsible for resistance to extended-spectrum cephalosporins as reported in previous studies (Coque et al., 2008). When comparing the plasmid sequences from 21 selected isolates, a cluster of *bla*$_{CTX-M-27}$ harboring *E. coli* and another group of *bla*$_{CTX-M-15}$ carrying *K. pneumoniae* isolates were revealed. In Hungary, CTX-M-15 and CTX-M-27 are found to be the dominant ESBL types among clinical isolates of *K. pneumoniae* and *E. coli* respectively, which is in correspondence with our findings (Nagy et al., 2021; Tóth, Tóth, Kamotsay, Németh, & Szabó, 2022). Considering that the *bla*$_{CTX-M-27}$ harboring *E. coli* and *bla*$_{CTX-M-15}$ carrying *K. pneumoniae* isolates identified in the hospital and nursing home effluents can be of fecal origin from patients and nursing home residents, it can be presumed that their dominance in our samples resembles their prevalence among local inhabitants. The highly identical contigs shared within a cluster raises the possibility of clonal relatedness of the isolates. Unfortunately this question could not be addressed, because the DNA samples subjected to next generation sequencing was enriched for plasmids, and therefore the coverage of chromosomal fragments was too low to be suitable for MLST analysis. Besides the two major clusters, CTX-M-15 producing *C. freundii*, CTX-M-1 producing *E. coli*, SHV-12 producing *K. pneumoniae* and CTX-M-30 producing *K. oxytoca* were also detected in our study. The majority of the isolates carried multiple antibiotic resistance genes, and many of these genes occurred to be co-located on defined contigs. These findings might explain the high frequency of associated/co-resistance and elevated MAR indices revealed in this study. The ESBL producers were observed more frequently in hospital effluents and WWTP, which appears to mirror similar observations made in South Africa, Tunisia, and Spain, reporting high rates of ESBL prevalence from hospital effluent and urban wastewater treatment plants (King, Schmidt, & Essack, 2020; Sghaier et al., 2019). The presence of a high proportion of ESBL producers observed among isolates from hospital effluents may suggest increased prescription of certain extended-spectrum ß-lactam antimicrobials. Hsu et al. observed a significant increase in prescription of certain extended-spectrum β-lactam antibiotics, which were associated with high levels of ESBL producers in hospital effluents in Singapore (Hsu et al., 2010). The *E. cloacae* species were non-ESBL producers and showed resistance to cefoxitin (a cephamycin), which can be supported by the observation that ESBL- producing *E. cloacae* are less prevalent and hence rarely reported as most *Enterobacter* species carry AmpC cephalosporinases, which are not inhibited by clavulanic acid (Ojer-Usoz et al., 2014). Although carbapenemases were not reported in the plasmid DNA of our isolates, a *bla*$_{VIM}$ gene was detected among the *Klebsiella oxytoca* isolates by colony PCR. This is in support of certain reports regarding the emergence of carbapenemase-producing *Klebiella* spp. from environmental samples (Isozumi et al., 2012; Koh et al., 2012; Thomas et al., 2013). *Klebsiella* species harboring the *bla*$_{VIM}$ gene have been previously reported among isolates at the Clinical Centre University of Pécs (Melegh et al., 2014), which is located within this same catchment area, suggesting that hospital effluents may be reservoirs of carbapenemase producers that can be linked to clinical sources. The high rate of susceptibility to meropenem observed in this study is consistent with a similar observation regarding low carbapenem resistance in Enterobacterales reported from wastewater treatment plants (Ojer-Usoz et al., 2014). Clinical surveillance data in a tertiary care hospital in Hungary among Enterobacterales for the period 2004-2015 reported zero resistance to carbapenems; imipenem, meropenem, and ertapenem (Magyar et al., 2017).

Fluoroquinolones hold the fifth position in the European antimicrobial market, with a maximum of 3.04 DDD (defined daily dose/1000 inhabitants) (Penalva et al., 2019). Our findings showed a high rate of resistance to ciprofloxacin, consistent with previously reported resistance to fluoroquinolones among isolates from various environmental compartments (Da Costa, Vaz-Pires, & Bernardo, 2006; Moore, Guzman, & McGee, 2008). Increased resistance to fluoroquinolones among Enterobacterales from urinary

tract infections has been reported in clinical surveillance data (Gajdács, 2019; Magyar et al., 2017). Consistent with our finding, a recent study in South Africa also found an increased rate of co-resistance between third-generation cephalosporins and fluoroquinolones in *Klebsiella* spp. (King et al., 2020). Other studies have demonstrated remarkable co-resistance to fluoroquinolones and broad-spectrum cephalosporins among *E. coli* and *K. pneumoniae* isolated from wastewater (Conte et al., 2017). The presence of the quinolone resistance protein *qnrB1* and the aminoglycoside modifying enzyme *aac(6')-Ib-cr* variant associated with low-level fluoroquinolone resistance identified in *Klebsiella* isolates indicates that acquired genes contribute to fluoroquinolone resistance among *Klebsiella* spp. from the environment. Ciprofloxacin resistance among *E. coli* was, however, mainly attributed to accumulation of double serine mutations in the DNA gyrase and topoisomerase IV genes, as reported by Fuzi et al. (Fuzi, Szabo, & Csercsik, 2017), since they did not carry acquired resistance genes. Similarly, resistance to gentamicin in this study occurred frequently among *Klebsiella* strains, which is consistent with a previous observation in *Klebsiella* spp. from wastewater treatment plants and hospital effluents in KwaZulu-Natal, South Africa (King et al., 2020). $bla_{\text{CTX-M}}$ harboring plasmids are often known to carry other genes of resistance, particularly to aminoglycosides, tetracycline, sulfonamides, and trimethoprim, suggesting co-selection, co-expression,

and hence co-resistance (Pai, Kim, Seo, Choi, & Oh, 2006). This finding can be linked to plasmid encoded aminoglycoside modifying enzyme encoding genes, *aph(3'')-Ib* and *aph(6)-1d* (phosphotransferases), *aadA1* and *aadA5* (adenylyltransferases) and *aac(3)-IIa* (acetyltransferase), which were identified in *E. coli* and *Klebsiella* spp, some of which are associated with gentamicin and tobramycin resistance. An increased rate of resistance to sulfamethoxazole/trimethoprim among the isolates can be associated with sulfonamide resistance genes (*sul1* and *sul2*) and *dfrA* (*dfrA14* and *dfrA17*) expressing dihydropteroate synthase and dihydrofolate reductases responsible for target replacement, conferring resistance to sulfonamides and diaminopyrimidines. A recent clinical surveillance data on *E. coli* from urinary tract infections indicated a high resistance rate of sulfamethoxazole/trimethoprim (Gajdács, 2019), implying that the resistance observed in wastewater isolates may be clinical in origin. It is notable that even though the isolates originated from different spots of the wastewater system, they were found to carry more or less the same plasmids groups. The main plasmids were the IncF replicons and their subtypes (FIA, FIB, and FII) which were evident in all the sequenced isolates. Acquired antibiotic resistance genes in bacteria are frequently carried on plasmids, with F plasmids being the most common conjugal plasmids in Enterobacterales linked to antibiotic resistance ("R factors") (Moran, Anantham, Pinyon, & Hall, 2015). According to Stephens et al. antibiotic resistance genes have been found in plasmids with a narrow host range, including IncI complex replicons (Z, B/O, K, or I1), but the majority of antibiotic resistance genes were associated with F replicons, and in most cases, multiple subtypes of F replicons were found on the same plasmids (Stephens et al., 2020). F- and I-complex replicons are frequently found in association with conjugating plasmids (Stephens et al., 2020). ESBL genes, carbapenemase genes, genes coding aminoglycoside-modifying enzymes, and plasmid-mediated quinolone resistance (PMQR) genes are the most frequently described resistance genes on IncF plasmids (Rozwandowicz et al., 2018). Multiple antibiotic resistance phenotype occurred at high frequency in the hospitals and the nursing home where individuals are likely to be put on a treatment regimen on a regular basis. In a related study, the percentage of multiple drug resistance in *E. coli* was higher in a nursing home than in hospital effluents (Oberlé et al., 2012). Co-resistance between cephalosporin and ciprofloxacin in this study was more frequent among isolates from the four hospitals, while that of cephalosporin and trimethoprim/sulfamethoxazole occurred more frequently among those from the nursing home. These findings may be related to antimicrobial drug prescriptions and demonstrate that antimicrobial drug resistant bacteria are likely to be selected in the human gastro-intestinal tract due to antimicrobial usage (Tenaillon, Skurnik, Picard, & Denamur, 2010). The discharge of untreated hospital effluent into the urban

wastewater network for co-treatment with the rest of municipal wastewater at the WWTP before releasing it into the environment, which is a general practice across many countries in Europe (Morris, Harris, Morris, Commins, & Cormican, 2016) may be directly linked to the high resistance rate observed in the hospital effluents. Isolates of clinical origin may disseminate resistance to environmental microbes, although resistant isolates from hospital effluents have not been correlated with those of clinical origin (White et al., 2016).

CONCLUSION

Our findings demonstrate that wastewater from human sources may serve as an important reservoir of multiresistant Enterobacterales, including ESBL and carbapenemase producers, and it may be likely that some of these strains could be traced to clinical sources. Notably, multiresistant Enterobacterales harboring plasmid-mediated extended-spectrum β-lactamases primarily of CTX-M, TEM, and SHV types that degrade broad-spectrum cephalosporins are more common in hospital effluents and their presence can be attributed to the development of resistance in the source population, and/or its buildup in the aqueous environment through selection pressure as well as resistance dissemination of the phenotype via horizontal gene transfer. Besides the plasmid borne β-lactamases, metallo-β-lactamase VIM also contributes to the resistance phenotype. Additionally, the interpretative readings of the inhibition zones of cephamycin suggest the possible presence of endogenous AmpCs β-lactamases. The findings present a clear indication that acquired genes contribute to multiresistance in Enterobacterales from the wastewater environment, contrary to certain reports linking acquired resistance only to clinical isolates. Monitoring wastewater of anthropogenic origin is a promising strategy for generating valuable data that can be correlated to the prevalence of clinically important resistant bacteria from the source population and may provide a cheaper alternative in regions facing challenges that limit clinical surveillance.

SUPPLEMENTARY MATERIALS: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11060776/s1, Table S1: Antimicrobial resistance profiles, antibiotic resistance genes and plasmid groups of enteric bacteria from the wastewater. Figure S1: Original gel images of the detected β-lactamase encoding genes.

Author contributions: Conceptualization, C.M., Z.G., and S.M; methodology, Z.G., S.M., and P.U.; software, E.V., and S.M.; validation, Z.G; formal analysis, C.M., E.V., and S.M.; investigation, C.M., K.K., and P.U.; resources, Z.G.; data curation, E.V.; writing—original draft preparation, C.M.; writing—review and editing, S.M., and Z.G.; visualization, Z.G. and S.M.; supervision, Z.G.; project administration, Z.G.; funding acquisition, Z.G.

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Data Availability Statement: The relevant data are provided along with the manuscript and additional information can be accessed at https://data.mendeley.com/datasets/j3mkwhzh84/1 Virág, Eszter; Mutuku, Christopher (accessed 4 May 2022), "Enterobacterales Plasmids", Mendeley Data, V1, doi: 10.17632/j3mkwhzh84.1 "Plasmid sequences (1).rar" contains the identified plasmids, and "Omicsbox_annot_table.zip" contains the localization and annotation of the genes found in the given plasmids.

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