Resistance to critically important antibiotics in hospital wastewater from the largest Croatian city

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ABSTRACT

The emergence of extended-spectrum β-lactamase (ESBL)- and especially carbapenemases in *Enterobacterales* has led to limited therapeutic options. Therefore, it is critical to fully understand all potential routes of transmission, especially in high-risk sources such as hospital wastewater. Wastewater samples were collected from two major hospitals in Zagreb during winter and summer 2020. Conventional culturing was performed to quantify coliform bacteria, and quantitative PCR was performed to monitor two ESBL and five carbapenemase (CP) genes, and four enteric opportunistic pathogens (EOPs) in the collected samples. The average concentrations of total, presumptive ESBL- and carbapenem-resistant coliforms for all samples combined were $3.4x10^4$, $4.7x10^3$ and $1.8x10^4$ CFU/mL, respectively. The most abundant resistance gene was bla_{KPC} (up to 10^{-1} gene copies/16S copies). *E. coli* was the most prevalent among EOPs (10^5 cell equivalents/mL). Sixty-nine ESBL- and 90 carbapenemase-producing *Enterobacterales* (CPE) isolates were isolated from hospital wastewater. All were multidrug-resistant and were mostly identified as *Escherichia coli*, *Citrobacter*, *Enterobacter*, and *Klebsiella*. Among ESBL isolates, $bla_{CTX\text{-}M\text{-}15}$ was the most prevalent ESBL gene, whereas in CPE isolates, $bla_{KPC\text{-}2}$ and $bla_{NDM\text{-}1}$ were the most frequently detected CP genes, followed by $bla_{OXA\text{-}48}$. Molecular epidemiology using PFGE, MLST and whole-genome sequencing (WGS) revealed that clinically relevant variants such as *E. coli* ST131 ($bla_{CTX\text{-}M\text{-}15}$/$bla_{TEM\text{-}116}$) and ST541 ($bla_{KPC\text{-}2}$), *K. pneumoniae* ST101 ($bla_{OXA\text{-}48}$/$bla_{NDM\text{-}1}$), and *Enterobacter cloacae* complex ST277 ($bla_{KPC\text{-}2}$/$bla_{NDM\text{-}1}$) were among the most frequently detected bacterial strains. WGS also revealed that these isolates contained resistance genes to multiple antibiotic classes and a diverse plasmidome. The $bla_{CTX\text{-}M}$, $bla_{OXA\text{-}48}$, and $bla_{KPC\text{-}2}$ genes were found to be associated with mobile genetic elements, particularly transposons and insertion sequences, suggesting the potential for mobilization. Our findings suggest the need to ensure effective

treatment of hospital wastewater to reduce or prevent the spread of critical priority pathogens and resistance genes into water systems.

Keywords: antibiotic resistance; hospital wastewater, carbapenemase, ESBL, *Enterobacterales*, multidrug

1. Introduction

The threat of increasing antibiotic resistance (AR) of pathogenic bacteria is one of the greatest challenges to global health. Of particular concern is the increasing bacterial resistance to β-lactam antibiotics such as 3rd generation cephalosporins and carbapenems, which are classified as "critically important for human medicine" by the World Health Organization (WHO) (WHO, 2019). It is particularly important to protect the efficacy of these and other antibiotics used as a last-resort, as their loss due to AR would result in treatment failures and deaths.

Hospital wastewater is considered a high-risk point source for the spread of antibiotic-resistant bacteria (ARB), antibiotic-resistance genes (ARGs), and enteric opportunistic pathogens (EOPs) in the environment (Hassoun-Kheir et al., 2020). Hospitalized patients are more likely to be treated with antibiotics than the general population, and therefore, higher concentrations of ARB or ARGs are often found in hospital wastewater than in municipal wastewater (Hassoun-Kheir et al., 2020; Paulus et al., 2019). These ARB/ARGs can spread to rivers and lakes through municipal wastewater treatment plants (WWTPs) because they are not always completely removed in WWTPs (Kehl et al., 2022; Puljko et al., 2022). Human-derived bacteria that do not persist in the aquatic environment can transfer ARGs to resident aquatic microorganisms, including pathogens, through horizontal gene transfer (HGT) (Gonzalez-Plaza et al, 2019; Larsson and Flach, 2022). This could lead to further potential transfer of ARGs from the environment to humans. Therefore, it is important to understand the AR gene pool of hospital wastewater, especially for bacteria of the order *Enterobacterales*, some of which are important nosocomial pathogens that can thrive in both the environment and the human gut, in order to track their spread from the hospital point source to the environment.

In contrast to many developed countries, hospital wastewater in Croatia is discharged directly into municipal WWTPs without any treatment, which may be a cause for concern, even

though hospital wastewater represents only a small proportion (less than 2%) of the total volume of wastewater treated in WWTP. Therefore, ARB/ARGs, which are typically found in low levels in wastewater, such as CP-producing *Enterobacterales* (CPE) and their mobile ARGs (e.g. bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{VIM} and bla_{IMP}), can spread rapidly and widely, posing a greater risk than other, more common environmental bacteria with intrinsic resistance mechanisms (Manaia et al., 2018). CPE and ESBL (extended spectrum ß-lactamase)-producing *Enterobacterales* (ESBL-E) have been listed by the WHO as critical priority pathogens for which research and the development of new antibiotics is urgently needed due to the emergence of multidrug resistance among these pathogens (WHO, 2017). In Europe, these species have been increasingly detected in clinical samples over the past decade (Kazmierczak et al., 2021). Croatia is one of the countries with a high prevalence of these strains, especially *Klebsiella pneumoniae* isolates (62% resistant to 3rd generation cephalosporins in 2021; ECDC, 2021). In addition, carbapenem resistance rates among clinical *K. pneumoniae* isolates increased from 2% in 2018 to 32.9% in 2021 in Croatia (ECDC, 2021). Previous studies have identified hospital wastewater as a high-risk point source for the spread of CPE and ESBL-E (Jelić et al., 2019; Kehl et al., 2022). However, more information on their phylogeny and genomic characteristics is needed to better assess the risk of spreading these clinically important ARB and their ARGs via hospital wastewater.

The most common resistance mechanism to β-lactams in *Enterobacterales* is the production of β-lactamases, and the most important enzymes in this family are the ESBLs, plasmid-mediated AmpC β-lactamases (pAmpC), and carbapenemases (CPs). ESBLs confer resistance to most β-lactam antibiotics, including penicillins, cephalosporins, and monobactam aztreonam, and the most common variants are TEM, SHV, CTX-M, and OXA (Bradford, 2001). pAmpC enzymes are generally less prevalent than ESBLs in *Enterobacterales*, but are still important because they contribute to ß-lactam resistance, which can also extend to

carbapenems when pAmpC are overproduced in combination with an impermeability defect (Barišić et al., 2014). Carbapenems are considered to be a last-resort treatment for Gram-negative infections, as they retain activity against chromosomal cephalosporinases and ESBLs. The production of CPs can confer resistance to virtually all β-lactams and is the most common mechanism of resistance to carbapenems among Gram-negative bacteria. Acquired CPs of clinical importance include KPC, VIM, NDM, IMP and OXA-48, and their geographic distribution is remarkably diverse (Nasri et al., 2017; Kazmierczak et al., 2021; Neidhöfer et al., 2021).

The aim of this study was to investigate the prevalence of *Esherichia coli* and other coliforms presumed to be ESBL- or carbapenemase-positive, as well as selected ESBL and CP genes and EOPs in wastewater from two hospitals in Zagreb using culture-based and molecular methods (real-time PCR). A total of 159 enterobacterial isolates (69 ESBL- and 90 CP-producing) was successfully isolated and identified. These isolates were characterized by phenotypic and genotypic assays to determine their AR profiles, molecular epidemiology, and ARGs present in them. In addition, the mechanisms of AR and their potential mobility in the selected isolates of *E. coli*, *Klebsiella* spp., and *Enterobacter cloaceae* complex were characterised using whole genome sequencing.

2. Materials and methods

2.1. Sample collection

Untreated wastewater samples were collected from two large hospitals (abbreviated as H1 and H2) in Zagreb, Croatia. Both hospitals provide primary health care and emergency services and differ in the number of hospital beds (H1 – 1510 beds, and H2 – 570 beds). Samples were taken at three time points in winter (January) and summer (July) of 2020. Grab wastewater samples (2000 mL) were collected from the sewer system in sterile 2.5 L glass bottles before being discharged into the municipal sewer system. Hospital wastewater is not treated at the hospital before it enters the municipal sewers, as is common for all hospitals in Croatia. The collected samples were transported on ice in cool boxes to the laboratory and processed within 2 hours.

2.2. DNA extraction and real-time PCR (qPCR) assays

For DNA extraction from wastewater, triplicate subsamples (50 - 90 mL) were filtered through mixed cellulose ester membranes (47 mm diameter, 0.22 μm pore size, GE Healthcare, Life Science, USA). Total community DNA was extracted from the filters using the DNeasy Powersoil kit (Qiagen, USA) according to the manufacturer's recommendations. Before the extraction procedure, the filters were cut into small pieces with sterile scissors. DNA quality (260/280 ratio) was determined using a Nanodrop spectrophotometer (BioSpec Nano, Shimadzu, Japan), and DNA quantity was determined using a Qubit Fluorometer 3.0 (Thermo Fisher Scientific, USA). All extracts were stored at -20 °C until use.

qPCR was used to quantify two ESBL genes (*bla*$_{TEM}$ and *bla*$_{CTX\text{-}M\text{-}32}$), five CP genes (*bla*$_{KPC\text{-}3}$, *bla*$_{NDM}$, *bla*$_{OXA\text{-}48}$-like, bla$_{IMP,}$ and *bla*$_{VIM}$), colistin resistance gene (*mcr-1*), and the 16S rRNA gene (*rrn*) as a marker for total bacteria. In addition, marker genes for EOPs were also quantified: *ycc*T (*E. coli*), *glt*A (*K. pneumoniae*), *sec*E (*Acinetobacter baumanii*) and 23S rDNA (enterococci). Primers, qPCR conditions and generation of standard curves are as

described in Puljko et al. (2022). All qPCR assays were performed on the ABI 7300 real-time PCR thermocycler (Applied Biosystems, USA) with Power SYBR® Green PCR Master Mix (10 µL, Applied Biosystems, USA), 1 µM of each primer (Puljko et al., 2022, Table S1, Table S2), and 2 ng of DNA template in a total volume of 20 µL. Gene abundances were calculated per 1 mL sample (absolute abundance) and per number of *rrn* copies (relative abundance). The abundances of the *ycc*T gene of *E. coli*, the *glt*A gene of *K. pneumoniae, sec*E gene of *A. baumannii*, the 23S rRNA gene of enterococci, and the *rrn* gene of total bacteria were expressed as cell equivalents (CE)/mL. In the case of *E. coli*, *K. pneumoniae*, and *A. baumannii*, only one copy of the target gene is present in a cell (Clifford et al., 2012; Gadsby et al 2015); thus, one copy number is equivalent to one cell. However, in enterococci and total bacteria, average copy number of 23S rRNA and 16S rRNA genes is five and three, respectively (Stoddard et al 2015); therefore, 23S rRNA and 16S rDNA copies determined by qPCR were divided by 5 and 3, respectively, to convert them to CE.

2.3. Coliform counts and isolation of ARB

To enumerate *E. coli* and non-*E. coli* coliforms, a series of dilutions of wastewater samples were prepared in 0.85% NaCl (tenfold dilutions up to 1:10,000), and then filtered in triplicate through sterile mixed cellulose ester membrane filters (47 mm diameter, 0.22 µm pore size, Whatman, GE Healthcare, Life Science, SAD). Filters were then placed on 1) Rapid'*E.coli* 2 (Bio-Rad, France) for enumeration of total *E. coli* and non-*E. coli* coliforms; 2) Rapid'*E.coli* 2 agar plates supplemented with 4 mg/L cefotaxime (CTX) representing 3rd generation cephalosporins for enumeration of CTX-resistant (CTX-R) *E. coli* and non-*E. coli* coliforms and 3) CHROMagar mSuperCARBA (CHROMagar, France) agar plates for enumeration of carbapenem-resistant (CR) *E. coli* and non-*E. coli* coliforms. Plates were incubated at 37°C for 24 h, and colonies of total, CTX-R and CR *E. coli* and non-*E. coli* coliforms were enumerated,

and their concentrations were calculated as colony-forming units (CFU) per mililiter of wastewater (CFU/mL).

For isolation of ARB, a total of 200 colonies of presumptive *E. coli* and other coliforms were picked from Rapid′*E. coli* 2 with CTX and CHROMagar mSuperCARBA plates and re-streaked on the same medium to purity. The purified colonies were stored in a 20% glycerol stock at −80 °C.

2.4. Identification of isolates

Bacterial isolates were sent to the Laboratory for Mass Spectrometry and Functional Proteomics at the Ruđer Bošković Institute for identification using Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) analysis. Isolates were streaked on Mueller-Hinton plates (Oxoid, UK) and incubated overnight for 18 - 24 hours at 37°C. Colony material of pure cultures was transferred by direct smearing onto spots of the MALDI-TOF MS target with tooth-picks. Bacterial identification was reported to the species level if the score value was above 2.00 or to the genus level if the score was between 1.70 and 1.99. A minority of isolates that could not be successfully identified by MALDI-TOF MS were identified by sequencing of the 16S rRNA gene. For this purpose, a 1465p fragment of the 16S rRNA gene was amplified by PCR using primers 27F and 1492R (Weisburg et al., 1991). Thermocycling conditions were as follows: 5 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 1 s at 55 °C and 1:30 min at 72 °C, and a final extension step at 72 °C for 10 min. Amplicons were sent to Macrogen (Amsterdam, Netherlands) for Sanger sequencing in the forward direction. The resulting sequences were characterised using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/). All sequences were identified to species level (≥ 99% sequence identity).

2.5. Antibiotic susceptibility testing

All isolates were subjected to antibiotic susceptibility testing using the disk diffusion method (EUCAST, 2020). The antibiotics used were amoxicillin (AML, 25 µg), amoxicillin/clavulanic acid (AMC, 30 µg), cephalexin (CL, 30 µg), cefuroxime (CXM, 30 µg), ceftazidime (CAZ, 10 µg), cefepime (FEP, 30 µg), ertapenem (ETP, 10 µg), imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), gentamicin (GM, 10 µg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg) and ciprofloxacin (CIP, 5 µg). The AML, CL and CAZ disks were purchased from Oxoid, and the others from BD (BBL, USA). Isolates that were resistant to any of the carbapenems underwent minimum inhibitory concentration (MIC) determination by serial broth microdilution according to EUCAST (2020) guidelines. In addition, colistin (COL) resistance of all isolates was determined by MIC. Briefly, in a sterile 96-well plate, a starting concentration of 64 mg/L (COL, IPM, and MEM) or 16 mg/L (ETP) was used and serially diluted twofold to a final concentration of 1 mg/L (COL, IPM, and MEM) or 0.25 mg/L (ETP). The remaining two columns were used as a positive control for bacterial growth (no antibiotic) and a negative control (no bacteria added). The wells contained 90 µl of Mueller-Hinton broth (Merck, Germany) or cation adjusted Mueller-Hinton broth 2 (Sigma-Aldrich, Germany; in the case of COL) and serially diluted target antibiotics. Overnight bacterial cultures were diluted to a concentration of 5 x 10^5 CFU/mL, and each well was inoculated with 10 µl of the culture. The plates were incubated overnight at 37° C, and the lowest concentration at which no visible growth was observed was determined as the MIC of the sample. Strains *Escherichia coli* ATCC 25922 and *Escherichia coli* NCTC 13846 were used as quality controls. The isolates were classified as multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR) according to the definitions of Magiorakos et al. (2012). Finally, isolates were clustered into groups according to the similarity of their resistance patterns, and representatives of each group were used for further targeted PCRs.

2.6. Phenotypic identification of ESBLs, pAmpC and carbapenemases

For the detection of ESBL production, CTX-R isolates underwent the double disc synergy test according to EUCAST guidelines (http://www.amcli.it/wp-content/uploads/2015/10/EUCAST_detection_resistance_mechanisms_V1.pdf). Briefly, overnight cultures of isolates were diluted in saline to 0.5 McFarland concentration and plated on Mueller-Hinton agar plates with a sterile cotton swab. Paired CAZ (30 µg) and CTX (30 µg) discs were used, which were 20 mm and 30 mm (centre to centre) from the amoxicillin-clavulanate disc (AMC, 20+10 µg), respectively. Plates were incubated overnight at 37°C. An increase in the zone of inhibition (synergy with clavulanate) for one of the extended-spectrum caphalosporins was considered a positive result for ESBL production.

To screen for pAmpC production, CTX-R isolates were subjected to a combined disk test using phenylboronic acid (Gupta et al., 2014). Briefly, the cefoxitin disk (30 µg) alone and in combination with phenylboronic acid (300 µg) were placed on the inoculated Mueller-Hinton agar plates. After overnight incubation at 37° C, an increase in the zone of inhibition of ≥5 mm indicated pAmpC production.

To detect carbapenemase production, CR isolates were subjected to the in-house Carba NP test (Nordmann et al., 2012). Briefly, bacterial suspensions in Tris-HCL lysis buffer were mixed with 100 µL phenol red solution containing $ZnSO_4$ x $7H_2O$ (0.1 mM) and imipenem-cilastatin (12 mg/mL). After incubation at 37°C for a maximum of 2 hours, the bacterial strains that changed the color of the suspension from red to orange or yellow were considered to be carbapenemase producers.

2.7. Targeted PCRs

Targeted PCRs were performed on a subset of isolates with different AR profiles. Total bacterial DNA was extracted from bacterial overnight cultures using the Quick-DNA™ Miniprep Plus Kit (Zymo, USA) according to the manufacturer´s instructions. Isolates with confirmed ESBL production were tested for the presence of ESBL genes by multiplex PCR

(*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{GES}$ and *bla*$_{SME}$) and singleplex PCR (*bla*$_{CTX-M}$ groups 1, 2, and 9). In addition, these isolates underwent multiplex PCR for pAmpC genes (*bla*$_{MOX}$, *bla*$_{CIT}$, *bla*$_{DHA}$, *bla*$_{ACC}$, *bla*$_{EBC}$, and *bla*$_{FOX}$). All isolates with confirmed CP production underwent PCR for the following CP genes: *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, and *bla*$_{VIM}$. Bacterial isolates that were identified as colistin-resistant in broth microdilution assays underwent multiplex PCR for the following genes: *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5. Primer sequences and thermocycling conditions are listed in Table S3. All positive PCR products were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) and underwent Sanger sequencing in the forward direction (Macrogen). Resulting sequences were edited and compared with reference sequences in the NCBI database using the online BLASTX search.

2.8. Genotyping by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

Isolates of *E. coli, Klebsiella* spp., and *Enterobacter* spp. were subjected to genetic relatedness determination by PFGE of *Xba*I-digested genomic DNA using the CHEF-DR III system (Bio-Rad Laboratories, USA), as previously described (Jelic et al., 2016). Restriction patterns were analysed with BioNumerics software (Applied Maths, Belgium) using the DICE coefficient (tolerance 1.5%), and the dendrogram was generated with UPGMA. Isolates that had a similarity cut-off of ≥85 % of their banding patterns were assigned to the same cluster.

One representative of each cluster was analysed for the presence of a sequence type (ST) using the commercial service IDgenomics (Seattle, USA) or by whole genome sequencing (WGS, see below). In the case of the commercial service, the sequences of 7 housekeeping genes of 3 *E. coli* and 1 *K. pneumoniae* were typed using the database https://pubmlst.org/.

2.9. Whole genome sequencing (WGS) and sequence analysis

Based on the results of antibiotic susceptibility testing and their clinical significance, selected isolates of *E. coli*, *K. pneumoniae*, and *E. cloacae* complex (cplx) were subjected to WGS, resulting in the sequencing of 4 *E. coli*, 2 *K. pneumoniae*, 2 *Enterobacter asburiae*, 3 *Enterobacter cloacae*, 1 *Enterobacter ludwigii* and 1 *Enterobacter kobeii*. DNA was extracted from frozen isolates revived by two consecutive smears on LB agar plates with CTX or IPM (4 mg/L) and subculture overnight in LB broth with the appropriate antibiotic (4 mg/L CTX or IPM). Sequencing was performed on the Ion Torrent PGM platform (Life Technologies, USA) according to the manufacturer's instructions. The Ion Xpress Plus Fragment Library Kit was used to enzymatically shear 100 ng of genomic DNA. The target fragment size was 400 bp. Subsequently, the fragmented DNA was processed using the Ion DNA Barcoding Kit (Life Technologies, USA) and its size was selected using the E-Gel SizeSelect 2 % Agarose Kit (Life Technologies, USA). The size and distribution of DNA fragments were analysed using the High Sensitivity Kit (Agilent, USA). Further sample preparation was performed using the Ion OneTouch Kit (Life Technologies, USA). Finally, the amplified DNA was sequenced using the 318 Chip (Life Technologies, USA). The raw data were assembled *de novo* using the Assembler SPAdes software, ver. 3.1.0., which is part of the Assembler plugin on the Ion Torrent server. Genomes were annotated using the Rapid Annotation using Subsystem Technology (RAST) database (Aziz et al., 2008; Overbeek et al., 2014). ARGs were found using ResFinder (Bortolaia et al., 2020). STs and plasmid replicon types were identified using tools from the Center for Genomic Epidemology website (Larsen et al., 2012; Carratoli et al., 2014).

Screening for chromosomal mutations in genes associated with colistin resistance was performed using the reference genome of *E. cloacae* ATCC 13047 (NCBI GenBank Accession No. CP001918). *E. cloacae* ATCC 13047 was screened for reference amino acid sequences of PmrA, PmrB, PhoP, PhoQ, and MgrB. Whole genome sequences of 6 *Enterobacter* spp. isolates were used to search for chromosomal mutations causing resistance to colistin in *Enterobacter*

spp. by sequence BLASTing (http://blast.ncbi.nlm.nih.gov). The effect of mutation as neutral/detrimental was determined using the freely available PROVEAN (Protein Variation Effect Analyzer) v1.1.3 software (http://provean.jcvi.org/seq_submit.php).

2.10. Data analysis

Bacterial and gene concentration data were first log10-transformed before further analysis. The unpaired Welch's t-test was used to compare the average concentration of target organisms (total coliforms, *E. coli*, other coliforms) in hospital wastewater and the absolute concentration of EOPs between seasons. In addition, the relative abundance of ESBL and CP genes between seasons in each hospital wastewater was assessed using a multiple unpaired t-test. All statistical analyses and data visualisations were performed using GraphPad Prism ver. 9.4.0 for Windows (GraphPad Software, San Diego, California, USA).

2.11. Data accessibility

The submission of Sanger sequence data to GeneBank is underway. Whole genome sequencing data have been submitted under BioProject: PRJNA913323 with BioSample accession numbers SAMN32292703 - SAMN32292715.

3. Results

3.1. Concentrations of total, CTX-R and CR E. coli and other coliforms

Cultivation on non-selective and two different selective plates revealed the presence of total and presumptive CTX-R *E. coli* and non-*E. coli* coliforms (Biorad Rapid′*E. coli* 2 agar plates with CTX) and presumptive CR *E. coli* and non-*E. coli* coliforms (CHROMagar mSuperCARBA plates) in all hospital wastewater samples (Fig. 1). The average concentration

of total *E. coli* and non-*E. coli* coliforms in wastewater from the two hospitals was 9 x 10^3 and 2.5 x 10^4 CFU/mL, respectively. In comparison, significantly lower concentrations of presumptive CTX-R *E. coli* (4.8 x 10^2 CFU/mL) and non-*E. coli* (6.8 x 10^3 CFU/mL) were measured. However, the concentrations of presumptive CR *E. coli* and non-*E. coli* coliforms in the analysed wastewater samples were slightly higher than the corresponding CTX-R concentrations and were consistent with the total concentrations of the corresponding species (Fig. 1, Table S4). No significant seasonal changes were detected in presumptive CTX-R or CR *E. coli* and non-*E.coli* coliforms.

3.2. Abundance of ESBL and CP genes in hospital wastewater

Two ESBL (bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$) and five CP genes ($bla_{KPC\text{-}3}$, $bla_{OXA\text{-}48\text{-}like}$, bla_{NDM}, bla_{IMP}, bla_{VIM}) were detected in all hospital wastewater samples using qPCR (Fig. 2). The concentrations of bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$ were mostly between approx. 10^{-3} and 10^{-4} gene copies/*rrn* copies (Fig. 2A). Regarding seasonal variations, hospital H1 had significantly higher levels of bla_{TEM} in summer samples, whereas hospital H2 had significantly higher levels of $bla_{CTX\text{-}M\text{-}32}$ in the same season (unpaired t-test, $p < 0.01$). Among the CP genes, $bla_{KPC\text{-}3}$ was the most abundant in wastewater of both hospitals, with significantly higher levels in summer (approx. 10^{-1} gene copies/*rrn* copies) compared to winter samples (approx. 10^{-3} gene copies/*rrn* copies) (unpaired t-test, $p < 0.01$; $p < 0.001$) (Fig. 2B). The relative abundance of the other CP genes examined mostly ranged from approx. 10^{-3} to 10^{-4} gene copies/*rrn* copies with no significant seasonal differences observed, except for bla_{VIM} in H1 and $bla_{OXA\text{-}48}$ in H2 with significantly higher levels in summer samples. The colistin resistance gene *mcr-1* was not found in any of the hospital wastewater.

3.3. Concentrations of total bacteria and EOPs in hospital wastewater

The qPCR-based analyses of the bacterial *rrn* gene showed that the mean concentration of total bacteria in the winter wastewater samples was approx. 10^8 CE/mL and was significantly lower in the summer samples (10^7 CE/mL) (Welch's t-test, $p < 0.001$) (Fig. 3). In addition, quantification of specific taxonomic markers for EOPs such as *E. coli* (*ycc*T), *K. pneumoniae* (*glt*A), *A. baumannii* (*sec*E), and *Enterococcu*s spp. (23S rRNA) showed that there were no significant differences in gene abundances between seasons, although median levels for *A. baumanii* were considerably higher in summer than in winter samples. In general, *E. coli* was the most abundant species in the hospital wastewater samples (approx. 10^5 CE/mL), whereas the concentrations of the other EOPs were approx. 10^4 CE/mL, with the exception of *A. baumanii* in summer.

3.4. Identification of isolates

A total of 200 presumptive enterobacteria were successfully isolated on selective media supplemented with antibiotics (CTX or carbapenems) from wastewater samples from both hospitals. Of these, we identified 159 members of the order *Enterobacterales* and 41 strains not belonging to this order by MALDI-TOF or 16S rRNA gene sequencing (Fig. S1A). *Enterobacterales* isolates included 69 CTX-R (27 from H1 and 42 from H2) and 90 CR isolates (43 from H1 and 47 from H2) (Fig. S1B). The identified *Enterobacterales* from both hospitals belonged to eight different genera, namely *Escherichia* (n=58), *Citrobacter* (n=39), *Enterobacter* (n=29), *Klebsiella* (n=23), *Raoultella* (n=5), *Kluyvera* (n=3), *Morganella* (n=1), and *Serratia* (n=1) (Fig. S1C).

3.5. Phenotypic tests for detection of ß-lactamases

All presumptive CTX-R *Enterobacterales* isolates (n=69) were found to be positive for ESBL production in the double disk synergy test. Of these, 35 % of isolates (n=24) were also

409 phenotypically positive for pAmpC production in the phenylboronic acid disk test, and 32 %
410 (n=20) were positive for CP production in the Carba NP test. In addition, all presumptive CR
411 *Enterobacterales* isolates (n= 90) were confirmed as CPE by the Carba NP test.
412
413 ***3.6. Antibiotic susceptibility patterns***
414 All 159 *Enterobacterales* isolates from hospital wastewater (both ESBL-E and CPE) were
415 tested for antibiotic susceptibility by the agar disk diffusion and broth microdilution methods
416 (Fig. 4). Of the ß-lactam antibiotics tested, all isolates were resistant to penicillins (AML,
417 AMC), 1st (CL) and 2nd generation cephalosporins (CXM). More than 85% of ESBL-E and >
418 95% of CPE isolates were resistant to 3rd (CAZ) and 4th generation cephalosporins (FEP),
419 respectively. Almost all CPE isolates showed resistance to all three carbapenems tested,
420 whereas half of the ESBL-E isolates were found to be carbapenem-resistant (51%, ETP).
421 Regarding resistance to other classes of antibiotics, high rates of resistance to fluoroquinolones
422 (CIP; ≥87%) were observed in both groups of isolates. Resistance to aminoglycosides (GM)
423 occurred more frequently in ESBL-E (75%) than in CPE isolates (57%). Trimethoprim-
424 sulfonamide resistance (SXT) was confirmed in 36% of isolates in both groups. Colistin
425 resistance was found at relatively low levels, but in a higher percentage (27%) in CPE than in
426 ESBL-E isolates (9%).
427 Intrinsic resistance was considered in the evaluation of multidrug-resistant (MDR) and
428 extensively drug-resistant (XDR) profiles of *Enterobacterales* isolates (Magiorakos et al.,
429 2012). MDR was found in all isolates (ESBL-E and CPE), and approximately 50 % of ESBL-
430 E and 80 % of CPE isolates were XDR (Fig. 4). In addition, one CPE isolate (*E. kobei*) was
431 classified as pan-drug resistant (PDR), because it was resistant to all antibiotics tested.
432
433 ***3.7. Molecular detection of ARGs***

The subset of 42 ESBL-E isolates underwent targeted PCR to detect ESBL and pAmpC genes. Of these isolates, those identified as colistin- or carbapenem-resistant underwent a PCR-based analysis targeting plasmid-mediated colistin resistance genes or CP genes, respectively. Sanger sequencing of the amplicons was used to determine the gene variant (Table 1). The most frequently detected ESBL gene was *bla*$_{\text{CTX-M-1}}$ group genes, specifically *bla*$_{\text{CTX-M-15,}}$ which was present in 30 isolates, mainly *E. coli* (n=13) and *K. pneumoniae* (n=8), while *bla*$_{\text{CTX-M-3}}$ was detected in 4 isolates (Table 1). This was followed by *bla*$_{\text{TEM-116}}$, which was detected in 22 ESBL isolates. Other ESBL genes detected were *bla*$_{\text{SHV}}$ (*bla*$_{\text{SHV-12}}$ and *bla*$_{\text{SHV-28}}$) and *bla*$_{\text{GES-7,}}$ which were detected only in *Klebsiella* spp. Nineteen ESBL-E isolates possessed two ESBL genes, mainly *bla*$_{\text{CTX-M-15+TEM-116}}$ (Table 1). Additionally, 12/42 isolates possessed CP genes, mainly *bla*$_{\text{KPC-2}}$ (n=6) and *bla*$_{\text{OXA-48}}$ (n=5), whereas *bla*$_{\text{NDM-1}}$ was detected in only one isolate (*K. oxytoca*) (Table 1). The latter *K. oxytoca* was the only isolate in which 5 ß-lactamase genes were detected (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{GES-7}}$, *bla*$_{\text{EBC}}$ and *bla*$_{\text{NDM-1}}$) (Table 1). In addition, the pAmpC multiplex PCR revealed the presence of *bla*$_{\text{EBC}}$ (6/42 isolates, mainly in *E.* cloacae complex (cplx) and *Klebsiella* spp.), *bla*$_{\text{CIT}}$ (6/42, found only in *Citrobacter* spp.), and *bla*$_{\text{MOX}}$ genes (4/42, found only in *E. coli*).

Of the 43 CPE isolates selected for targeted PCR, CP genes were detected in almost all isolates (41/43). The *bla*$_{\text{KPC-2}}$ (20/43, 47%) and *bla*$_{\text{NDM-1}}$ (19/43, 44 %) genes were the two most frequently detected ones, especially in *Citrobacter spp.* (Table 2). *E. cloacae* cplx strains (n=5) and *E. coli* (n=4) were also frequent carriers of *bla*$_{\text{KPC-2,}}$ whereas *K. pneumoniae* was a frequent carrier of *bla*$_{\text{NDM-1}}$ (n=7).

Other CP genes detected were *bla*$_{\text{OXA-48}}$ (15/43 isolates, 35%, mostly in *K. pneumoniae*) and *bla*$_{\text{VIM-1}}$ (9/43, 21%, mostly in *E. cloacae* cplx and *Citrobacter* spp.) (Table 2), whereas the 17/43 CPE isolates had two or more CP genes. Among these 17 isolates, the most frequent combination of genes was *bla*$_{\text{OXA-48+NDM-1}}$ in *K. pneumoniae* (n=5) and *Citrobacter* spp. (n=1)

and *bla*$_{KPC-2+NDM-1}$ in *E. cloacae* cplx (n=2) and *Citrobacter* spp. (n=3) (Table 2). The most common combination of three CP genes were *bla*$_{KPC-2+NDM-1+VIM-1,}$ detected in *Citrobacter* spp (n=3).

Both ESBL-E and CPE isolates were negative for mobile colistin resistance genes (*mcr*1-*mcr*5).

3.8. Molecular epidemiology of E. coli, K. pneumoniae and E. cloacae cplx isolates

Clonal relatedness of all (ESBL-E and CPE) *E. coli* (n=58), *K. pneumoniae* (n=22) and *E. cloacae* cplx isolates (n=26) was determined by PFGE, and ST was determined for one representative isolate from each cluster.

For *E. coli*, a total of 6 PFGE clusters were found: A (n=2), B (n=4), C (n=7), D (n=8), E (n=15), F (n=2); 14 isolates could not be assigned to any cluster and 5 could not be typed. Five different STs were found among the clustered isolates: ST216, ST405, ST361, ST541, and ST131. Isolates from clusters D and F had the same ST131, while the ST of the largest cluster (E) could not be determined. The distribution of *E. coli* isolates among clusters showed partitioning between hospitals. The largest cluster (E) included 15 isolates (with *bla*$_{KPC-2}$ or *bla*$_{TEM-1/TEM-116}$+*bla*$_{CTX-M-15+}$*bla*$_{MOX+}$*bla*$_{KPC-2}$), all of which were from H2 hospital wastewater. Most of them showed the same AR profile and were sensitive only to SXT and COL. The *E. coli* strains of another dominant cluster (D) were ST type ST131 (*bla*$_{TEM-1}$ or *bla*$_{TEM116+}$*bla*$_{CTXM-15}$), and all but one were from H2. The isolates of the third dominant cluster (C) belonged to ST541 (*bla*$_{KPC-2}$), were all derived from H1. They were only sensitive to GM, SXT and COL. Isolates from the latter two clusters (C and D) had phenotypic resistance to a lower number of antibiotics tested than those from the largest cluster E.

Among the 22 *K. pneumoniae* isolates, 3 PFGE clusters were found among 13 isolates, whereas the remaining 9 isolates were singletons (Fig. 5B). Three different ST were identified: ST16, ST101, and ST307. The largest cluster B included 9 isolates from H2 wastewater

belonging to ST101, and most of them showed resistance to all antibiotics tested, except colistin, and carried *bla*$_{\text{OXA-48}}$ or *bla*$_{\text{OXA-48}}$+*bla*$_{\text{NDM-1}}$.

For the *E. cloacae* cplx, PFGE genotyping was assessed per species (Fig. 5C). The *E. cloacae* (n=16) were grouped into 3 clusters (n=11), and the remaining 5 isolates were singletons. Cluster C was the largest and included 6 isolates from H2 wastewater that were ST type ST277 and showed phenotypic resistance to carbapenems (*bla*$_{\text{KPC-2}}$) and colistin but were sensitive to GM and SXT (Fig. 5C). The ST type of the second largest cluster (B) could not be determined (*bla*$_{\text{VIM-1}}$), whereas 2 isolates of cluster A belonged to ST32. Of the *E. asburiae* isolates (n=7), only 4 were in a cluster (cluster A), originated from H2 and belonged to ST277 (*bla*$_{\text{KPC-2}}$+*bla*$_{\text{NDM-1}}$). The remaining 3 isolates were singletons (Fig. 5C). Three *E. ludwigii* isolates from H2 wastewater had a similarity percentage >94%, and belonged to ST277. These isolates had the same genotypic (*bla*$_{\text{KPC-2}}$) and phenotypic resistance profile as *E. cloacae* isolates from cluster C (Fig. 5C). Two *E. bugadensis* isolates were singletons, and one *E. kobei* was not included in this analysis.

3.9. Presence of ARGs, STs and plasmid replicon types in isolates by WGS analysis

A total of 13 isolates were subjected to WGS analysis - four *E. coli*, two *K. pneumoniae*, three *E. cloacae*, two *E. asburiae*, one *E. ludwigii*, and one *E. kobei* (Table S8). This selection mainly included isolates from dominant and distinct clusters with clinically-relevant features, such as resistance to carbapenems and/or colistin.

Of the four different *E. coli* isolates, the STs were identified for three of them, including ST361, ST131, and ST541 while one isolate could not be typed. All three ESBL-producing *E. coli* (ST361, ST131 and ND) contained *bla*$_{\text{CTX-M}}$ genes (*bla*$_{\text{CTX-M-15}}$ and/or *bla*$_{\text{CTX-M-194}}$) and the *bla*$_{\text{OXA-1}}$ gene, whereas one ST361 strain additionally possessed *bla*$_{\text{TEM-1B}}$. In addition, all *E. coli* isolates contained the pAmpC *bla*$_{\text{EBC}}$ gene. Phenotypic resistance to aminoglycosides was

supported by the detection of *acc(6')-Ib-cr* and/or *aac(3)-IIa* genes, whereas resistance to fluoroquinolones was supported by the presence of *qnr*B1 and/or *acc(6')-Ib-cr*, to chloramphenicol by the detection of *cat*B3 and to trimethoprim/sulfamethoxazole by *dfr*A12 gene. Other resistance genes found comprised the *lnu*F gene (lincomycin resistance), *tetA* (tetracyline resistance), and *sitABCD* gene (resistance to biocides - hydrogen peroxide). Two ESBL-producing *E. coli* isolates (ST361 and ND) were phenotypically identified as carbapenem resistant, but WGS analysis did not detect carbapenem resistance genes in them, whereas the presence of $bla_{\text{KPC-2}}$ was detected by PCR in one of them. This gene was also detected in CP-producing *E. coli* (ST541), and phenotypic resistance to fluoroquinolones was confirmed by the detection of the *qnr*S1 gene.

Two CP-producing *K. pneumoniae* isolates belonged to ST16 and ST101, respectively (Table S8). According to WGS, these two isolates carried the $bla_{\text{OXA-48}}$ gene; however, targeted PCR additionally detected the presence of $bla_{\text{NDM-1}}$. They also possessed ESBL genes such as $bla_{\text{CTXM-15}}$, $bla_{\text{SHV-205}}$, and $bla_{\text{OXA-1}}$. Additional genes responsible for resistance to trimethroprim/sulfamethoxazole (*dfrA14*), (fluoro)quinolones (*aac(6')-lb-cr, oqxA, oqxB, qnrB1*), fosfomycin (*fosA, fosA5*), or chloramfenicol (*catB3*) were also detected in these isolates.

E. cloacae cplx included ST277 isolates (2 *E. asburiae*, *E. cloacae*, and *E. ludwigii*), ST32 (*E. cloacae*) and ST501 (*E. kobei*), whereas one isolate could not be assigned into any ST. Carbapenemase production activity was supported by the WGS-based detection of $bla_{\text{KPC-2}}$ in ST277 and ST501 isolates or $bla_{\text{VIM-1}}$ genes (unknown ST), and targeted PCR additionally detected $bla_{\text{NDM-1}}$ in two *Enterobacter* isolates (ST277, ST501). In addition to the bla_{TEM} gene, which was detected in all but one *Enterobacter* spp., other resistance genes for ß-lactams were observed, including $bla_{\text{CTX-M-3}}$ (in ST501), $bla_{\text{OXA-1}}$ (in ST277), $bla_{\text{OXA-10}}$ (in ST277, ST32), and $bla_{\text{OXA-14}}$ (in ST501). Each isolate contained one $bla_{\text{ACT}}/bla_{\text{MIR}}$ gene. Three isolates with

phenotypic gentamicin resistance contained *aac(6')-Ib-cr*, *aac(3)-I*, *aph(3'')-Ib*, and *aph(6')-Id* genes, and four gentamicin-susceptible isolates contained *aac(3')-1* and *aadA11* or *aac(6')-Ib-cr*. Of the 7 isolates displaying phenotypic ciprofloxacin resistance, two contained *qnrB1* or *qnrS1* genes and no ciprofloxacin resistance genes were detected in the five isolates. Two *Enterobacter* isolates with phenotypic trimethoprim/sulfamethoxazole resistance contained the *sul1*, *sul2* and *dfrA14* genes. Other genes responsible for resistance to fosfomycin (*fos*A), rifampicin (*arr*-3), chloramphenicol (*catB3*), and the biocides (*qacE*) were also found sporadically. No plasmid-mediated *mcr* colistin resistance genes were detected in ST277, ST32 and ST501 strains that were phenotypically resistant to colistin. These isolates had mutations in three or more genes (*pmrA*, *pmrB*, *phoP*, *phoQ* and *mgrB*) which are associated with colistin resistance. The total number of numerous mutations detected in all six *Enterobacter* spp. isolates was 343 and the analysis in PROVEAN revealed 91 unique mutations (6 deleterious - PmrA – P174A; PmrB – E218G, S308Q, G309R, L310S; PhoP – N174S; and 85 neutral mutations) (Table S9). Multiple amino acid substitutions were noted for all proteins except for MgrB that had one amino acid substitution (V10I) found in 4 *Enterobacter* spp. isolates. The L133I mutation in the *phoQ* gene was reported for the first time in this study, as indicated in Table S9.

The genetic context of the $bla_{\text{CTX-M}}$, $bla_{\text{OXA-48}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{VIM-1}}$ genes was analysed using RAST annotations (Fig. 6). The $bla_{\text{CTX-M}}$ genes ($bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-194}}$ and $bla_{\text{CTX-M-3}}$) were flanked by insertion sequences (*ISEc9* or IS6 family) and/or transposases (Tn*3* family) in all genomes (analysed by WGS) in which these genes were detected (6/13). The $bla_{\text{OXA-48}}$ gene was flanked by a *lys*R gene in *K. pneumoniae* ST101 and ST16 and by an IS4 family insertion sequence in *K. pneumoniae* ST101. The $bla_{\text{KPC-2}}$ gene was flanked in five *Enterobacter* spp. (ST277 and ST501) by genes belonging to the Tn*3*-based transposon family (*ISKpn6* and *ISKpn27*). Two ST277 *Enterobacter* isolates (*E. cloacae* and *E. ludwigii*) carried $bla_{\text{KPC-2}}$

560 together with the *bla*$_{TEM-1}$ gene as part of this Tn*3* transposon. Similarly, *bla*$_{KPC-2}$ was present in
561 ST541 *E. coli* associated with the Tn*3*-based transposon, Tn4401. The *bla*$_{VIM-1}$ was associated
562 in *E. cloacae* (unknown ST) with genes encoding resistance to aminoglycosides (*aac(6')-Ib-cr,*
563 *aadA1),* biocides *(qacEΔ1),* and sulfonamides (*sul1).*

564 Finally, plasmids were detected in all 13 sequenced isolates, with eleven isolates
565 containing more than one replicon (Table S8). In total, 19 different plasmid replicon types were
566 identified in *E. coli*, *K.pneumoniae*, and *Enterobacter* spp. The most frequently detected
567 plasmid replicon types were IncFIB (n=9) and IncHI2 (n=6).

568

569 ## 4. Discussion

570 This study focused on the resistance to 3rd generation cephalosporins and carbapenems,
571 especially among *Enterobacterales*, which are now among the most prevalent ARB threatening
572 human health. Monitoring of these bacteria and their ARGs from high-risk point sources such
573 as hospital wastewater is critical to obtain the information needed to track their spread in the
574 environment.

575 The culture-based enumeration of *E. coli* and other coliforms resistant to CTX (3rd
576 generation cephalosporin) or carbapenems in hospital wastewater analysed here showed that
577 concentrations ranged from 10^3 to 10^4 CFU/mL, which is up to two orders of magnitude higher
578 than in the influent of the receiving WWTP in Zagreb (Puljko et al, 2022). This high prevalence
579 of CTX-R and CR *E. coli* and non-*E.coli* coliforms in wastewater from the two large hospitals
580 in Zagreb is comparable or lower than in several previous studies, including hospital wastewater
581 from neighbouring Austria and Slovenia (Rozman et al, 2020). The lack of on-site treatment of
582 sewage in Zagreb hospitals exacerbates the potential for further spread and resulting health
583 impact of bacterial resistance to last-line antibiotics such as carbapenems.

The further quantification of five CP genes (*bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC-3}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{IMP}}$, and *bla*$_{\text{VIM}}$) by qPCR in wastewater from both hospitals showed that *bla*$_{\text{KPC-3}}$ was detected at the highest levels and reached relative levels of up to 10^{-1} gene copies/*rrn* copies. These levels were unusually high compared to previously published concentrations of *bla*$_{\text{KPC}}$ in hospital wastewater samples (around 10^{-5} gene copies/*rrn* copies) (Zhang et al, 2020). This high prevalence of *bla*$_{\text{KPC}}$ in the studied hospital wastewater is consistent with the frequent detection of KPC-producing isolates in Croatian hospitals, especially *K. pneumoniae* (Bedenić et al, 2015, 2021; Jelić et al, 2016). The potential risk of this gene would be exacerbated by the possibility of horizontal transmission between strains, as has already been demonstrated in clinical isolates in Croatia (Jelić et al, 2016). Finally, the fact that *bla*$_{\text{KPC}}$ is predominantly associated with hospital wastewater but rarely detected in the environment (Jelić et al., 2019; Hooban et al., 2020) may lead to the prioritisation of monitoring this gene to detect potential leakage from inadequately treated hospital wastewater. The concentrations of other CP genes, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{VIM}}$, and *bla*$_{\text{IMP}}$, as well as two ESBL genes, *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$, were within the range of previous studies (~10^{-3} to 10^{-4} gene copies/*rrn* copies) (Rodriguez-Mozaz et al., 2015; Flach et al., 2021). Moreover, the qPCR-based quantification of the WHO priority pathogens *E. coli, K. pneumoniae, A. baumanii,* and *Enterococcus* spp. showed that hospital wastewater contained all of these pathogens at concentrations of 10^{3} to 10^{5} CE/mL (or 10^{-3} to 10^{-5} CE/*rrn* copies), which were comparable to those measured in German hospital wastewater samples (Alexander et al 2022). All these results confirm that hospital waste is an important reservoir for high-priority pathogens and ARGs and a pathway for their dissemination in water systems.

To place the obtained ARG data in a medical context, culture-based methods paired with molecular methods such as WGS and PCR were used to investigate the phenotypic and molecular mechanisms of resistance in CR and CTX-R enterobacterial isolates. Sixty-nine CTX-R and 90 CR *Enterobacterales* were successfully isolated from our hospital wastewater

samples. The mechanisms underlying resistance to CTX and carbapenems in these isolates were the production of ESBL and carbapenemases, respectively. Among the CPE isolates, *Citrobacter* spp. (34 %), *Enterobacter* spp. (26 %), *E. coli* (18 %) and *Klebsiella* spp. (16 %), dominated, whereas *E. coli* (61 %), *Klebsiella* spp. (13 %), and *Citrobacter* spp. (12 %) were predominant among ESBL-E isolates. All isolates tested were found to be MDR, consistent with the ability of enterobacteria to acquire various ARGs via HGT, which is mostly mediated by plasmids (Cantón et al., 2012)

ESBL-E isolates had high rates of resistance to fluoroquinolones (87 %), aminoglycosides (75 %), and even carbapenems (ETP, 51%), in addition to 3rd and 4th generation cephalosporins. Further genetic characterization of these isolates revealed that the most common ESBL genotype was $bla_{\text{CTX-M-15}}$ (71 %) and $bla_{\text{TEM-116}}$ (52 %), whereas bla_{SHV} (12 %) ($bla_{\text{SHV-12}}$ and $bla_{\text{SHV-28}}$) was rare. Moreover, $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-116/TEM-1}}$ co-occurred in the majority of our *E. coli*, *Klebsiella* spp. and *Citrobacter* spp. isolates, whereas $bla_{\text{CTX-M-15}}$, bla_{TEM}, and bla_{SHV} co-occurred only in *K. pneumoniae* isolates (10 %). This is consistent with previous reports from Zagreb hospitals, including H1 studied here, describing a frequent association of these genes in clinical enterobacterial isolates (Bedenić et al., 2016; D'Onofrio et al., 2020). In addition, co-production of ESBL ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}), pAmpC (bla_{MOX}, bla_{EBC} or bla_{CIT}), and CP genes ($bla_{\text{KPC-2}}$, $bla_{\text{OXA-48}}$ or $bla_{\text{NDM-1}}$) was observed in some of the ESBL-E isolates analysed here, consistent with a worldwide survey of clinical enterobacterial isolates (Kazmierczak et al., 2021).

CPE isolates showed a high rate of resistance to all ß-lactam antibiotics tested, including carbapenems, and to fluoroquinolones (99 %), but also to a lesser extent to aminoglycosides (57 %). However, 27 % CPE isolates were resistant to colistin, which may lead to treatment failure if spread further, as carbapenems and colistin are considered as last choice antibiotics for the treatment of MDR bacteria. The $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ were the most

frequently detected CP genes in this study, particularly in *Citrobacter* spp. These species are becoming increasingly important in the hospital setting as emerging carriers of CPs, with KPC-2, OXA-48 or VIM predominating depending on the geographic location (Arana et al., 2017; Babiker et al., 2020; Yao et al., 2021).The presence of *bla*$_{\text{VIM-1}}$ and *bla*$_{\text{NDM-1}}$ has also been reported in *Citrobacter freundii* from hospital H1 studied here (Atalić et al., 2013; Bedenić et al., 2016). Our results also show the co-occurrence of two CPs (mostly *bla*$_{\text{KPC-2+NDM-1}}$) in *Citrobacter* spp. which is consistent with some previous studies (Yao et al, 2021), or even three different CPs such as *bla*$_{\text{KPC-2+NDM-1+VIM-1}}$, which to our knowledge has not been reported before. This indicates that these *Citrobacter* spp. could be a relevant reservoir for potentially transmissible carbapenem resistance in hospital wastewater. Moreover, in the present study, carbapenem-resistant *K. pneumoniae*, which are an emerging public health problem in Croatia and other EU countries, were found to contain predominantly *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$ genes, which frequently co-occur. This is consistent with previous findings showing that *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$ are the most frequently detected CP genes in clinical *K. pneumoniae* in Zagreb hospitals (Bedenić et al, 2016, 2022). Isolation of this WHO priority pathogen from hospital wastewater provides a secondary reservoir and possible transmission route for these bacteria to natural waters and the community.

Molecular epidemiology using PFGE coupled with WGS identification of ARGs and MLST was further performed on critical priority pathogens (WHO): ESBL- and CP-producing *E. coli*, *K. pneumoniae*, and *Enterobacter* spp. Among the *E. coli* isolates, the ST of the largest cluster could not be identified, possibly due to their environmental origin. In addition, WGS analysis of a representative of this cluster revealed multiple ARGs for ß-lactams, including ESBL (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-194}}$, *bla*$_{\text{TEM-116}}$) and CP genes (*bla*$_{\text{KPC-2}}$), and for other priority antibiotics, as well as the presence of several plasmids, including those already associated with the transmission of *bla*$_{\text{CTX-M-15}}$ (IncFIA and IncFIB) or *bla*$_{\text{KPC-2}}$ (IncR and Col440I) (Chen et al.,

2014; Nicolas-Chanoine et al., 2014; Rocha-Gracia et al., 2022). *E. coli* ST131 and ST541 were among the most common sequence types detected in this study. Interestingly, ST131 has also been found in human MDR isolates from hospitals in Croatia (Krilanović et al., 2020) and other countries (Price et al., 2013), and in the environment including municipal wastewater (Hocquet et al., 2016; Lopes et al., 2021). In this study, *E. coli* ST131 had ESBL (*bla*$_{\text{CTX-M-15 + TEM116}}$) and pAmpC genes (*bla*$_{\text{EBC}}$) and was found by WGS to have ARGs to other antibiotics such as gentamycin and chloramfenicol and biocides (peroxides). It also contained the plasmid replicon types IncFIA and IncFIB which have been associated with the spread of the *bla*$_{\text{CTX-M-15}}$ gene (Nicolas-Chanoine et al., 2014; Rocha-Gracia et al., 2022). In another ST, ST541, detected in CP-producing *E. coli* strains, *bla*$_{\text{KPC-2}}$ and ARGs to several other antibiotic classes were detected. This ST is rare and has been detected in livestock in Asia (Chan et al., 2014; Qiu et al., 2019).

Among the *K. pneumoniae* isolates examined in this study, ST101 was the most prevalent multidrug-resistant clone, with phenotypic resistance to all ß-lactams including carbapenems. This ST was predominantly associated here with the *bla*$_{\text{OXA-48}}$ gene which was flanked by the IS4 family transposase IS10A, previously found predominantly in pOXA-48 plasmids (Hendrickx et al., 2021). This suggests that ST101 *K. pneumoniae* has the potential to spread carbapenem resistance through horizontal transmission. In agreement with our results, the presence of *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$ in ST101 *K. pneumoniae* isolates has recently been reported in Italian and Slovenian hospitals (Nucleo et al., 2020; Benulič et al., 2020). In addition, this ST has also been detected in hospitals and treated hospital wastewater in Serbia and Romania, respectively (Novović et al., 2017; Popa et al., 2021) Other STs detected in *K. pneumoniae* isolates were ST16, associated with CP producers carrying *bla*$_{\text{OXA-48}}$ and/or *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{CTX-M-15}}$ ESBL, and ST307, associated with ESBL producers carrying *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{SHV-28}}$. Previous studies from Croatia have reported the occurrence of *bla*$_{\text{NDM-1}}$ or

684 *bla*$_{\text{OXA-48}}$ in clinical ST16 *K. pneumoniae,* but the co-occurrence of *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$ has
685 not yet been reported in this lineage in Croatia (Bedenić et al., 2016; Jelić et al., 2018; Kocsis
686 et al., 2016). In other countries, ST16 is frequently associated with co-occurrence of *bla*$_{\text{OXA-232}}$
687 and *bla*$_{\text{NDM-1}}$ (Abe et al., 2022; Avolio et al., 2017; Espinal et al., 2019). Furthermore, ST307
688 has also been described in CTX-M-15-producing *K. pneumoniae*, which caused a nosocomial
689 outbreak in Germany (Haller et al., 2019). In addition, WGS showed that both ST16 and ST101
690 contained ARGs for several antibiotic classes other than ß-lactams and several plasmid replicon
691 types, including IncFIA, IncFIB, and IncR that have previously been associated with the
692 carriage of *bla*$_{\text{CTX-M-15}}$ in *K. pneumoniae* (Silva et al., 2022; Wyres et al., 2019). Apart from the
693 likely plasmid association, *bla*$_{\text{CTX-M-15}}$ was flanked by insertion sequences and Tn3 type
694 transposon in our *K. pneumoniae* and *E. coli* isolates, highlighting the role of these platforms
695 in its further dissemination (Zhao and Hu, 2013; Grevskott et al., 2020).
696 The majority of *E. cloacae* cplx isolates analysed in this study were carbapenemase
697 producers belonging to ST277, which to our knowledge has not been previously detected in
698 humans or environmental samples. This ST was MDR with carbapenem and colistin resistance
699 being the most commonly detected resistance phenotypes. WGS showed that these isolates
700 harboured CP genes *bla*$_{\text{KPC-2}}$ or *bla*$_{\text{KPC-2}}$+*bla*$_{\text{NDM-1}}$ and several other ARGs for ß-lactam and
701 other antibiotic classes and biocides, but no mobile colistin resistance genes. However, point
702 mutation analysis of these *E. cloacae* cplx isolates identified mutations in the *pmrA*, *pmrB*,
703 *phoP*, *phoQ* or *mgrB* genes that most likely confer the observed colistin resistance, suggesting
704 chromosomally associated resistance mechanisms. In addition, WGS showed that these isolates
705 contained a diverse plasmidome, including plasmid replicon types associated with the carriage
706 of *bla*$_{\text{KPC-2}}$ (IncFII, IncN, IncP6, IncR, IncX5, and Col440I) (Chen et al., 2014; Souza et al.,
707 2019; Yao et al., 2017) or *bla*$_{\text{NDM-1}}$ (IncFIB, IncN, and IncR) (Wu et al., 2019). This suggests
708 that these CP genes may spread further via HGT. Furthermore, *E. cloacae* isolates whose ST

classification could not be successfully identified (cluster B) contained *bla*$_{VIM}$ and mobilizable plasmids such as IncHI2, IncHI2A, and IncC, which are commonly associated with the transmission of this gene (Arcari et al., 2020; Sadek et al., 2020). The *bla*$_{VIM}$ gene was genetically linked to several ARGs for other classes of antibiotics or antiseptics in these isolates, suggesting possible common transmission of these genes via HGT. Finally, a ST501 *E. kobei* isolate that had previously successfully colonized and persisted in hospital sinks and plumbing (Aranega-Bou et al., 2021), was phenotypically identified here as PDR. This was supported by the presence of a variety of ARGs, including the carbapenemases KPC-2 and NDM-1, which could be associated with the detected plasmids IncN and IncR, respectively (Chen et al., 2014; Gamal et al., 2016; Wang et al., 2018). Finally, the analysis of the genomic context of *bla*$_{KPC-2}$ showed that *Enterobacter* isolates carried this gene as part of a non-Tn4401 transposon, as also reported in clinical *Enterobacter* isolates from Colombia (De La Cadena et al., 2018) and environmental *Klebsiella* isolates from Brazil (Janssen et al., 2021), suggesting the potential for mobilization.

5. Conclusions

The results of this study, which is the first of its kind in Croatia, show that the wastewater from the two major hospitals in Zagreb contains relatively high levels of coliform bacteria resistant to 3rd generation cephalosporins and carbapenems, as well as clinically significant ESBL and CP genes. Of concern is the presence of the multidrug-resistant WHO priority pathogens with both intrinsic (point mutations involved in colistin resistance) and acquired resistance mechanisms (ESBL and carbapenemase) previously reported in patients from local hospitals as well. So there is a possibility that these pathogenic strains and antibiotic-resistant strains can be transmitted into the water systems and then back to humans and animals. Effective treatment of hospital wastewater with advanced treatment methods such as UV and

ozone treatments must therefore be ensured to reduce or stop the spread of ARB and ARGs of clinical concern in the natural environment.

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Figure Captions

Fig. 1. Boxplot comparison of concentrations (CFU/mL) of total, presumptive CTX-R and CR *E. coli* (A) or non-*E. coli* coliforms (B) in wastewater from both hospitals. Boxes indicate median and quartiles, and whiskers represent minimum and maximum values. Asterisks indicate significant difference between seasons (*$p < 0.05$, multiple Welch's t-test).

Fig. 2. Relative abundance of ESBL ($bla_{\text{CTX-M-32}}$ and bla_{TEM}) (A) and carbapenemase (CP) genes ($bla_{\text{OXA-48}}$, $bla_{\text{KPC-3}}$, bla_{NDM}, bla_{IMP}, and bla_{VIM}) (B) in wastewater from two hospitals during winter and summer sampling. A significant difference between gene abundance in samples from different seasons in each hospital was determined using an unpaired t-test and is indicated by asterisks (**$p < 0.01$; ***$p < 0.001$, multiple unpaired t-test).

Fig 3. Quantification of *E. coli* (*ycc*T), *K. pneumoniae* (*glt*A), *A. baumannii* (*sec*E), *Enterococcus* spp. (23S rRNA) and total bacteria (16S rRNA) (cell equivalents (CE)/mL) from winter and summer samples in two hospitals. The boxes indicate the median and quartiles, and the whiskers represent the minimum and maximum values. Asterisks indicate significant difference between seasons (*** $p < 0.001$, multiple Welch's t-test).

Fig. 4. Percentage of ESBL- and carbapenemase-producing *Enterobacterales* (ESBL-E and CPE) isolates from hospital wastewater samples identified with an antibiotic resistance phenotype. AML: Amoxicillin; AMC: Amoxicillin/Clavulanic acid; CL: Cephalexin; CXM: Cefuroxime; CAZ: Ceftazidime; FEP: Cefepime; ETP: Ertapenem; IPM: Imipenem; MEM: Meropenem; GM: Gentamicin; SXT: Trimethoprim/Sulfamethoxazole; CIP: Ciprofloxacin; MDR: Multidrug-resistant, XDR: Extensively drug-resistant.

Fig. 5. Dendrogram generated with Bionumerics software showing cluster analysis of *Xba*I-PFGE patterns of (A) *Escherichia coli*, (B) *Klebsiella pneumoniae*, and (C) *Enterobacter* spp. isolates along with their antibiotic resistance phenotypes and genotypes, and multilocus sequence types (MLST). Red squares represent resistance, yellow squares represent intermediate resistance, and green squares represent susceptibility to the indicated antibiotics (AML- ampicillin, AMC – ampicillin/clavulanic acid, FEP – cefepime, CAZ – ceftazidime, CXM – cefuroxime, CL – cefalexin, CIP- ciprofloxacin, ETP – ertapenem, GM – gentamicin, IPM- imipenem, MEM – meropenem, SXT – trimethoprim/sulfamethoxazole, COL –colistin). ID stands for the name of the isolate. ND indicates that a MLST could not be completely identified. Medium indicates the selective antibiotic (CTX – cefotaxime or CARB – carbapenem) contained in the culture medium. Isolates selected for whole genome sequencing are underlined.

Fig 6. Schematic presentation of the genetic environment of ESBL ($bla_{\text{CTX-M}}$) and carbapenemase genes ($bla_{\text{OXA-48}}$, $bla_{\text{KPC-2}}$ and $bla_{\text{VIM-1}}$). Numbers (1-3) denote predicted regions for cefepime resistance (1), aztreonam resistance (2), ciprofloxacin resistance (3), and mannose-1-phosphate guanylytransferase (4). ST*ND*- sequence type could not be completely identified. Analyses were performed using RAST annotation and the Gene Graphics web application (https//:genegraphics.net).