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Molecular epidemiology and mechanisms of carbapenem and colistin resistance in *Klebsiella* and other Enterobacterales from treated wastewater in Croatia

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ABSTRACT

Among the most problematic bacteria with clinical relevance are the carbapenem-resistant Enterobacterales (CRE), as there are very limited options for their treatment. Treated wastewater can be a route for the release of these bacteria into the environment and the population. The aim of this study was to isolate CRE from treated wastewater from the Zagreb wastewater treatment plant and to determine their phenotypic and genomic characteristics. A total of 200 suspected CRE were isolated, 148 of which were confirmed as Enterobacterales by MALDI-TOF MS. The predominant species was *Klebsiella* spp. (n = 47), followed by *Citrobacter* spp. (n = 40) and *Enterobacter cloacae* complex (cplx.) (n = 35). All 148 isolates were carbapenemase producers with a multidrug-resistant phenotype. Using multi-locus sequence typing and whole-genome sequencing (WGS), 18 different sequence types were identified among these isolates, 14 of which were associated with human-associated clones. The virulence gene analysis of the sequenced *Klebsiella* isolates (n = 7) revealed their potential pathogenicity. PCR and WGS showed that the most frequent carbapenemase genes in *K. pneumoniae* were $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$, which frequently occurred together, while $bla_{\text{KPC-2}}$ together with $bla_{\text{NDM-1}}$ was mainly detected in *K. oxytoca*, *E. cloacae* cplx. and *Citrobacter* spp. Colistin resistance was observed in 40% of *Klebsiella* and 57% of *Enterobacter* isolates. Underlying mechanisms identified by WGS include known and potentially novel intrinsic mechanisms (point mutations in the *pmrA/B*, *phoP/Q*, *mgrB* and *crrB* genes) and acquired mechanisms (*mcr-4.3* gene). The *mcr-4.3* gene was identified for the first time in *K. pneumoniae* and is probably located on the conjugative IncHI1B plasmid. In addition, WGS analysis of 13 isolates revealed various virulence genes and resistance genes to other clinically relevant antibiotics as well as different plasmids possibly associated with carbapenemase genes. Our study demonstrates the important role that treated municipal wastewater plays in harboring and spreading enterobacterial pathogens that are resistant to last-resort antibiotics.

1. Introduction

Antimicrobial resistance (AMR) is a multifaceted problem in which the relationships between humans, animals and the environment are closely intertwined. It is therefore essential to fully understand all potential transmission routes of AMR. In addition to surveillance of AMR in clinical settings, wastewater-based surveillance is considered a complementary approach to assess AMR in non-clinical isolates and to assess environmental exposure to potentially hazardous antibiotic-resistant bacteria (ARB) and their resistance genes (Tiwari et al., 2022).

According to the World Health Organization (WHO), Enterobacterales resistant to last-resort antibiotics such as carbapenems, are among the most challenging bacteria to treat in clinics (WHO, 2017). Resistance to carbapenems is often associated with enzymatic

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production of carbapenemases that can hydrolyze most carbapenems and other β-lactam antibiotics (Poirel et al., 2007). This resistance often occurs in combination with resistance to other classes of antibiotics, which limits treatment options and increases mortality (Budhram et al., 2020). In the last decade, the clinically relevant carbapenemases KPC, OXA-48, NDM, VIM and IMP have spread worldwide (Bonomo et al., 2018). In addition, genes encoding carbapenemases are often located on plasmids that can be spread between different species (Ramsamy et al., 2022). In addition to carbapenemases, carbapenem resistance in Enterobacterales is also associated with a reduction in outer membrane permeability in the presence/absence of extended-spectrum ß-lactamase (ESBL) and/or AmpC enzyme production (Barišić et al., 2014; Martínez-Martínez, 2008). The antibiotic colistin is one of the few therapeutic options for severe infections caused by carbapenem-resistant Enterobacterales (CRE), making colistin resistance a serious public health problem (Binsker et al., 2022). Resistance to colistin in Enterobacterales is attributed to mutations in certain chromosomal genes (*pmrA/B*, *phoP/Q*, *mgrB* and/or *crrB*) or to the acquisition of the plasmid-mediated *mcr* genes (Gogry et al., 2021). The detection of isolates carrying carbapenem and colistin resistance genes is therefore of particular importance, as there are only a few therapeutic options left.

In Europe, an increase in the prevalence of CRE, particularly carbapenem-resistant *Klebsiella pneumoniae*, has been observed, with prevalence increasing by almost 50% in the last four years. During the same period, the prevalence of carbapenem resistance in *K. pneumoniae* in Croatia, which causes bloodstream infections, increased by 110% (ECDC, 2023a). Remarkably, Croatia is among the six EU countries with the highest prevalence of carbapenem-resistant *K. pneumoniae* in clinical settings from 2020 to 2022 (ECDC, 2023b). This is in line with the high carbapenem use in the hospital and community sector in Croatia compared to other EU countries in the same three-year period (ECDC, 2023c). Therefore, understanding the factors that favor the spread of CRE, especially *Klebsiella* spp. outside of hospitals, is crucial to prevent their further spread.

Outside of hospitals, wastewater treatment plants (WWTPs) are reported to contribute to the emergence and spread of AMR (Karkman et al., 2018; Marano et al., 2020). Studies have shown the presence of CRE and associated antibiotic-resistance genes (ARGs) in wastewater after treatment in WWTPs (Marutescu et al., 2023). Our recent study in the same WWTP that we analyzed here showed an enrichment of some carbapenemase genes (bla_{VIM} and bla_{IMP}) in the treated wastewater compared to the raw wastewater (Puljko et al., 2022). In addition, the survival of carbapenemase-producing *K. pneumoniae* in the river into which the wastewater is discharged has also been found in Croatia (Jelić et al., 2019) and other countries (Kehl et al., 2022; Lepuschitz et al., 2019). Consequently, treated wastewater could serve as important monitoring sites for environmental exposure to hazardous ARB such as CRE and their mobile carbapenemase genes, with the risk of these being reintroduced into the community (Larsson et al., 2023). However, there is little information on the occurrence and detailed characteristics of CRE in treated municipal wastewater, especially on their epidemiology and genomic characteristics (Gomi et al., 2018; Hoffmann et al., 2023; Zurfluh et al., 2017). In accordance, this work aimed to isolate and identify CRE from treated wastewater and characterize them in terms of their epidemiology, phenotypic AMR and genetic mechanisms underlying resistance to carbapenems. A subset of isolates (n = 13), including isolates with concomitant carbapenem and colistin resistance phenotype (n = 8), was selected for whole-genome sequencing (WGS) to investigate their molecular epidemiology and the presence of ARGs, virulence genes and plasmid replicon types.

2. Materials and methods

2.1. Collection of treated wastewater, isolation and identification of suspected CRE

Treated wastewater samples from the Zagreb WWTP were collected as described in Puljko et al. (2022). Samples were then serially diluted (1:10 to 1:100,000) with 0.85% sterile NaCl, and each diluted sample was filtered through a filter membrane (47 mm diameter, 0.22 µm pore size, Whatman, GE Healthcare, Life Science, USA). For isolation of CRE, filters were aseptically transferred to CHROMAgar mSuperCARBA (CHROMAgar, France) plates and incubated for 24 h at 37 °C. This agar was selected because it can detect OXA-48 producers in addition to other carbapenemase producers (KPC, VIM, NDM and IMP). The suspected CRE colonies (n = 200), which appeared red for *Escherichia coli* and metallic blue for *Klebsiella, Citrobacter* and *Enterobacter*, were subcultured on the same medium to purity and stored at −80 °C in 20% glycerol for further analysis (Puljko et al., 2023).

The isolates were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) at the Ruđer Bošković Institute, as described in Puljko et al. (2023). Three isolates for which MALDI-TOF MS analysis was not successful (two *Enterobacter* and one *Citrobacter*; Table S1) were sent to Macrogen (Amsterdam, Netherlands) for sequencing of the 16S rRNA gene (Puljko et al., 2023).

2.2. Confirmation of carbapenemase production and AMR phenotypes

Isolates identified as Enterobacterales (n = 148) were tested for carbapenemase production using the CarbaNP test (Nordmann et al., 2012). All Enterobacterales isolates were screened for susceptibility to 12 different antibiotics using a Kirby-Bauer disk diffusion test according to the EUCAST (2020) guidelines. Antibiotics screened included amoxicillin (AML) amoxicillin/clavulanic acid (AMC), cephalexin (CL), cefuroxime (CXM), ceftazidime (CAZ), cefepime (FEP), ertapenem (ETP), imipenem (IPM), meropenem (MEM), gentamicin (GM), trimethoprim/sulfamethoxazole (SXT), and ciprofloxacin (CIP). Isolates identified as carbapenem-resistant by the disk diffusion method were additionally tested for their resistance to carbapenems (IPM, ETP and MEM) using the broth microdilution method according to EUCAST (2020) guidelines. In addition, the minimum inhibitory concentration (MIC) of colistin (COL) was determined for all isolates using the same broth microdilution protocol and EUCAST MIC interpretation.

Based on the observed AMR phenotypes and the criteria of Magiorakos et al. (2012), all Enterobacterales isolates were classified as multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR).

2.3. Molecular characterization of carbapenemase genes

Enterobacterales isolates were grouped according to their species and phenotypic AMR profiles. Representatives from each group were selected and tested for ARGs by PCR, with the exception of *Klebsiella* isolates, which were all tested by PCR. Briefly, isolates were revived from pure frozen stocks and grown in Luria-Bertani broth (LB) containing IPM (4 mg/L) at 37 °C with agitation (130 rpm). Genomic DNA was extracted from overnight culture using the Quick-DNA™ Miniprep Plus Kit (Zymo, USA). All isolates were screened for carbapenemase genes (bla_{KPC}, bla_{OXA-48}, bla_{NDM}, bla_{VIM} and bla_{IMP}) using the primers and conditions in Table S2. COL-resistant isolates were screened for plasmid-mediated COL resistance genes (*mrc-1-mcr-5*) by multiplex PCR (Table S2). PCR products were Sanger sequenced in forward direction (Macrogen, The Netherlands) and compared with sequences in the NCBI database using BLASTX search after editing.

2.4. Pulsed-field gel electrophoresis (PFGE) analysis and Multi-locus sequence typing (MLST)

E. coli, *Klebsiella* spp. and *Enterobacter cloacae* complex (cplx.) underwent standardized *Xba*I PFGE analysis, as described by Jelic et al. (2016). The dendrogram was constructed using BioNumerics (Applied Maths, Belgium) using the UPGMA and DICE similarity coefficient, and cut-off value of $\geq$ 85%. MLST analysis was performed by IDgenomics service (Seattle, USA) for representative isolates from clusters whose sequence type (ST) was not predicted by WGS (see below).

2.5. WGS, assembly and sequence data analysis

Thirteen isolates (1 *E. coli*, 7 *Klebsiella* spp., 3 *E. cloacae* cplx., and 2 *Citrobacter* spp.) were selected for WGS analysis. The isolates were selected based on their affiliation to the dominant PFGE clusters or the presence or absence of carbapenemase genes. Selected isolates were revived from glycerol stock onto LB plates with IPM (4 mg/L), and a single overnight grown colony was used to isolate genomic DNA using the Quick-DNA^{TM} Miniprep Plus Kit (Zymo, USA) according to the manufacturers recommendations. Sequencing was performed using the Ion Torrent PGM instrument as described in Puljko et al. (2023). SPAdes v.3.15.0 was used to assemble quality-filtered reads with the default parameters, and genomes were annotated with RAST (v.2.0).

2.6. Bioinformatic and phylogenetic analysis

ARGs were found in the assembled genomes and verified using raw reads with ResFinder 4.4.2. (Bortolaia et al., 2020). Plasmid replicon types were identified in the assembled genomes using PlasmidFinder 2.1 (Carattoli et al., 2014). For the *Klebsiella* spp. genomes, prediction of the capsular (K) and lipopolisacharide (LPS) O antigen locus and hypervirulence genes were done by Kleborate (v.2.2.0.) and Kaptive 2.0 tools implemented in Pathogenwatch (Argimón et al., 2021; Lam et al., 2021; 2022). For genomes that were differentially identified by MALDI-TOF MS and WGS, the average nucleotide identity (ANI) values (Goris et al., 2007) were calculated on the basis of a comparison between the genomes tested and the reference genomes using the online service (https://enve-omics.ce.gatech.edu/ani/). The $\geq$ 95% identity criteria were used to identify the species, and four reference genomes were used for comparison: *K. pneumoniae* HS11286 (GenBank accession number: NC_016845.1, *K. quasipneumoniae* subsp. *similipneumoniae* ATCC 700603 (GenBank accession number: NZ_CP014696.2), *K. michiganensis* THO-011 (GenBank accession number: NZ_AP022547.1) and *C. portucalensis* (GenBank accession number: CP044098.1). SerotypeFinder 2.0 and ClermonTyping were used to identify the serotype and phylogroup of *E. coli*, respectively (Beghain et al., 2018; Joensen et al., 2015). The STs of the genomes were identified using the MLST tool 2.0 (Larsen et al., 2012), and the virulence genes were identified using the Virulence Factor Database (VFDB, Chen et al., 2016). The VFanalyzer within the VFDB was used to search for virulence genes in *E. coli* and *Klebsiella* spp. isolates by comparing their nucleotide sequences with the reference sequences (*E. coli* str. K-12 substr. MG1655 and *K. pneumoniae* subsp. *pneumonia* MGH 78578). The virulence genes of *E. cloacae* and *Citrobacter* isolates were predicted based on the alignment of their nucleotide sequences with the nucleotide sequences of VFDB (set B). Nearly identical matches were filtered out based on bitscore values above 90, E-values below $10e^{-5}$, and sequence identity above 90%.

The relationship of the *mcr* gene of *K. pneumoniae* in this study to other *mcr* variants (*mcr-1-mcr-10*) from the NCBI database was analysed by constructing a phylogenetic tree. The tree was inferred using the maximum likelihood method with the LG model and gamma distribution. Bootstrap analysis was performed with 1,000 replicates. The tree was generated using the software MEGA7 (Kumar et al., 2016) and visualised using the online tool iTol (Letunic and Bork, 2016).

The contig containing the *mcr* gene was annotated with RAST and BLASTX. In addition, the entire contig was aligned with the NCBI nucleotide database to find closely related sequences. Visualization was done with Easyfig 2.2.5 (Sullivan et al., 2011).

Mutations in genes conferring COL resistance in sequenced *Klebsiella* and *E. cloacae* cplx. isolates (*pmrA, pmrB, phoP, phoQ, mgrB,* and/or *crrB*) were predicted by alignment of their nucleotide and amino acid sequences with reference sequences in the NCBI database. Reference genomes included wild-type *K. pneumoniae* HS11286 (GenBank accession number: NC_016845.1), *K. oxytoca* KONIH1 (GenBank accession number: CP008788.1), *K. quasipneumoniae* ATCC 700603 (GenBank accession number: NZ_CP014696.2) and *E. cloacae* ATCC13047 (GenBank accession number: CP001918). The effects of the observed mutations (neutral or deleterious) on the functionality of the proteins encoded by COL ARGs were assessed using the PROVEAN tool.

2.7. Data availability

The sequences of the ARGs have been submitted to GenBank under the following accession numbers: bla_{KPC-2} (OR903110-OR903146), bla_{VIM-1} (OR948827-OR948440), bla_{IMP-13} (OR948841), bla_{NDM-1} (PP024889-PP024923), bla_{OXA-48} (PP024935-PP024951) and *mcr-4.3* (OR948842-OR94884). The WGS data have been deposited under the BioProject PRJNA913323 with the BioSample accession numbers: SAMN38849958 (SE_SC_COL_47 *K. pneumoniae*), SAMN34152686 (SE_SC_COL_72 *K. pneumoniae*), SAMN34152684 (SE_SC_COL_96 *K. quasipneumoniae*), SAMN34152685 (SE_SC_COL_102 *K. quasipneumoniae*), SAMN34152690 (SE_SC_COL_103 *K. quasipneumoniae*), SAMN34152683 (SE_SC_COL_148 *K. quasipneumoniae*), SAMN34152689 (SE_SC_COL_140 *K. michiganensis*), SAMN34152691 (SE_SC_COL_159 *E. asburiae*), SAMN34152692 (SE_SC_COL_198 *E. asburiae*), SAMN34152693 (SE_SC_COL_80 *E. cloacae*), SAMN34152682 (SE_SC_COL_21 *E. coli*), SAMN34152695 (SE_SC_COL_83 *C. farmeri*), and SAMN34152694 (SE_SC_COL_124 *C. portucalensis*).

3. Results

3.1. Identification of Enterobacterales isolates

A total of 200 suspected CRE were isolated from treated wastewater samples of the Zagreb WWTP, of which 148 isolates were identified as Enterobacterales species (Fig. 1). The remaining 52 isolates were identified mainly as *Aeromonas* spp. (n = 45) and *Enterococcus* spp. (n = 7) (Fig. 1). Of the 8 Enterobacterales genera identified, the most frequently found genus was *Klebsiella* spp. (n = 47; 32%), consisting of 36 *K. pneumoniae* and 11 *K. oxytoca* isolates. The second and third most common genera were *Citrobacter* spp. (n = 40; 27%) and *Enterobacter cloacae* cplx. (n = 35; 24%). Species such as *Raoultella* spp. (n = 12), *E. coli* (n = 7), *Kluyvera cryocrescens* (n = 5), *Serratia marcescens* (n = 1) and *Leclercia adecarboxylata* (n = 1) together accounted for 18% of the identified CRE species.

3.2. Carbapenemase production and AMR profiles of isolates

The CarbaNP assay confirmed that all 148 CRE isolates produced carbapenemase enzymes. The phenotypic resistance of these isolates to nine clinically relevant antibiotic classes was determined by Kirby-Bauer and broth microdilution methods (Fig. 2, Table S1). The resistance profile to all β-lactam antibiotics tested was similar in each of the 8 identified genera. For instance, all isolates were found to be resistant to AML (penicillins), AMC (penicillin + β-lactamase inhibitor), CL, CXM, and CAZ (1st, 2nd, and 3rd generation cephalosporins, respectively), and ETP and IPM (carbapenems) (Fig. 2). Intermediate resistance profiles to FEP (4th generation cephalosporin) were observed in *Klebsiella*, *Citrobacter, E. cloacae* cplx, *E. coli,* and *Roultella* isolates. In addition, resistance to MEM (carbapenem) was observed in all isolates except a

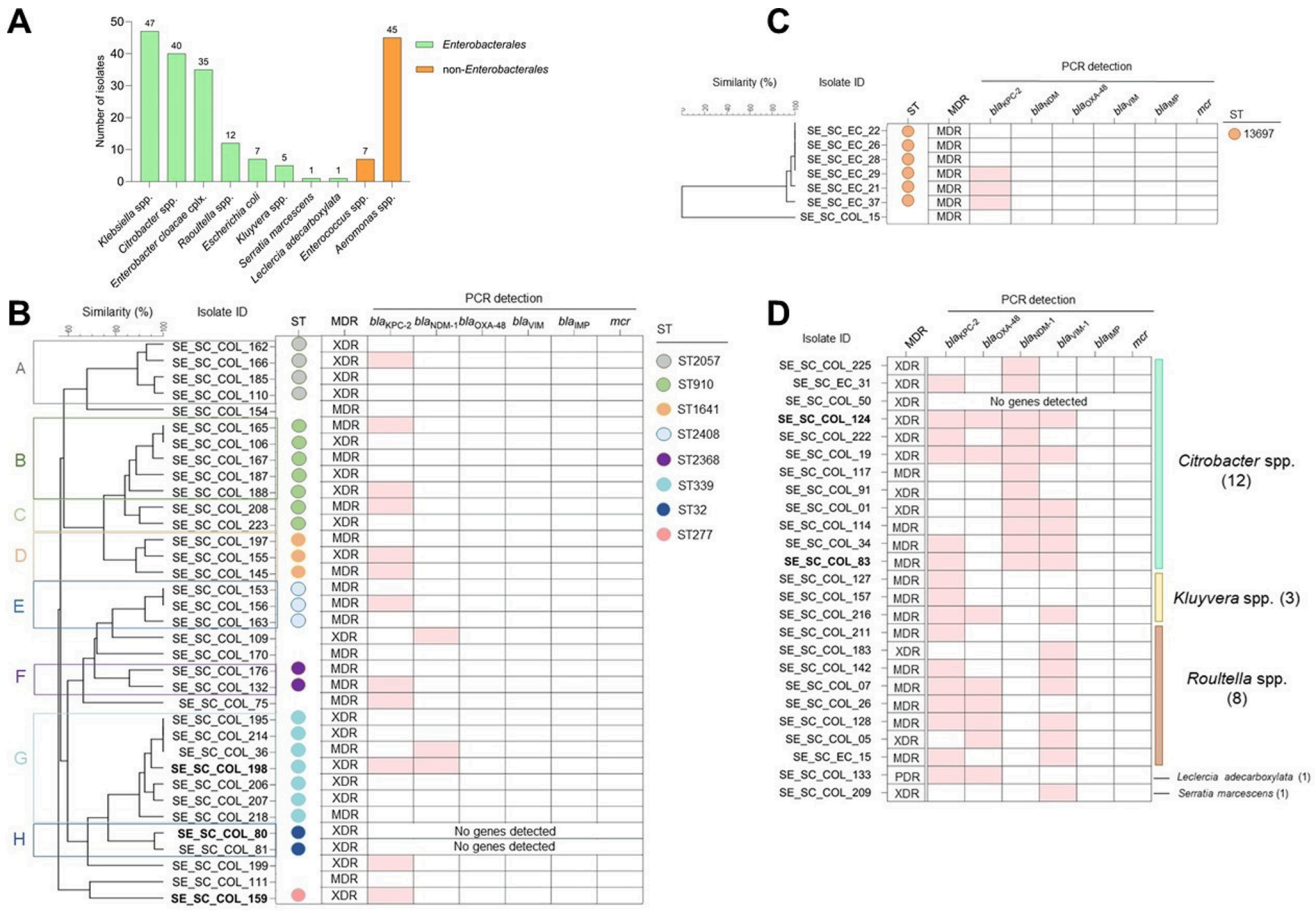

Fig. 1. Identification of 200 suspected CRE species from the treated wastewater samples (A) and cluster analysis based on *Xba*I-PFGE of carbapenem-resistant (B) *E. cloacae* cplx. isolates and (C) *E. coli*. The letters A to H in the dendrogram indicate the identified primary clusters. The coloured circles refer to different sequence types (ST). The light red coloured row in the table indicates the presence of the carbapenemase or colistin genes. (D) Table showing the presence (light red) or absence (white) of genes encoding carbapenemases or colistin resistance in a subset of CRE isolates (*Citrobacter, Kluyvera*, *Roultella, Leclercia,* and *Serratia*). MDR – multidrug-resistant, XDR – extensively drug-resistant, PDR – pandrug-resistant. Isolates subjected to whole-genome sequencing are shown in bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

small percentage of *Citrobacter* and *E. cloacae* cplx. (2% and 6%, respectively). Regarding resistance to non-β-lactam antibiotics, most isolates (>65% of each genus) were resistant to CIP (fluoroquinolones). The highest resistance to GM (aminoglycosides) and SXT (trimethoprim/sulfamethoxazole) was observed in *Klebsiella* (51% and 23%, respectively) and *Citrobacter* isolates (53% and 50%, respectively). In addition, the percentage of isolates resistant to COL (i.e. MIC > 2 μg/mL) was generally low, except for *Klebsiella* spp. (40%; MIC = 4–32 μg/mL) and *E. cloacae* cplx. (52%, the majority of which had an MIC > 64 μg/mL) (Fig. 2; Table S1). All 148 CRE were identified as MDR, with more than 50% classified as XDR, and 4 isolates (3 *K. pneumoniae* and one *L. adecarboxylacara*) were PDR (Table S1).

3.3. Genome and phylogenetic analyses of *E. cloacae* cplx. isolates

Phylogenetic typing revealed that 28/35 *E. cloacae* cplx. isolates could be assigned to one of 8 clusters (A-H) belonging to 8 known STs (Fig. 1B). MLST and WGS revealed that ST339 (cluster G) was the most prevalent, followed by ST910, ST2057, ST1641, ST2408, ST2368, and ST32. One nonclustered isolate was identified as ST277 (Fig. 1B). Most isolates associated with these STs carried the carbapenemase gene bla_{KPC-2}, except ST32, in which no tested carbapenemase genes were found, and ST339, which carried bla_{KPC-2} and bla_{NDM-1} simultaneously.

Three *E. cloacae* cplx. isolates (ST32, ST339 and ST277) were subjected to WGS analysis (Fig. 4). The presence of bla_{KPC-2} was confirmed by WGS in ST339 and ST277, as was the absence of known carbapenemase genes in ST32. In addition, other clinically important ARGs were found in sequenced isolates, including ESBLs such as bla_{OXA-1} (in ST277), $bla_{OXA-256}$ (in ST339), and bla_{OXA-10} (in ST32), as well as ARGs against aminoglycosides (*aac(6′)-lb-cr*, *aac(3)-I, aph(6)-Id,* and *aph (3″)-lb*), rifampicin (*aar-3*), chloramphenicol (*catB3*), and antiseptics (*qacE/qacH*) (Fig. 4). Although all three sequenced isolates were phenotypically resistant to COL, no plasmid-mediated *mcr* genes were found in their genomes. However, multiple point mutations were found in the genes corresponding to the PmrA/B, PhoP/Q and/or MgrB proteins, including deleterious (P147A, E218G, and N174S) and neutral mutations (Fig. 4, Table S5). All sequenced *E. cloacae* cplx. isolates contained between 11 and 22 different virulence genes (*fim*, *iutA, fep*, among other genes) (Table S6). PlasmidFinder identified 1 (IncP6), 3 (Col4401, IncFII, and IncR), and 7 plasmid replicons (Col(pHAD28), IncC, IncFIB, IncFII IncHI2, IncP6, and IncX5 types) in *E. cloacae* ST339, *E. asburiae* ST277, and *E. cloacae* ST32, respectively (Fig. 4).

3.4. Genetic characteristics of *E. coli* and other Enterobacterales isolates

Six of the seven MDR *E. coli* isolates formed a cluster belonging to ST13697 and phylogroup A with serotype O154:H4 (Fig. 1C and 4). The carbapenemase type KPC-2 was identified by PCR in isolates from this cluster (Fig. 1C). In addition, WGS identified the ß-lactamase type TEM-1 and the AmpC ß-lactamase type EC as well as the fluoroquinolone resistance gene *qnrS1* in sequenced *E. coli* ST13697. Two plasmid replicon types detected in this sequenced *E. coli* were Col156 and IncP6

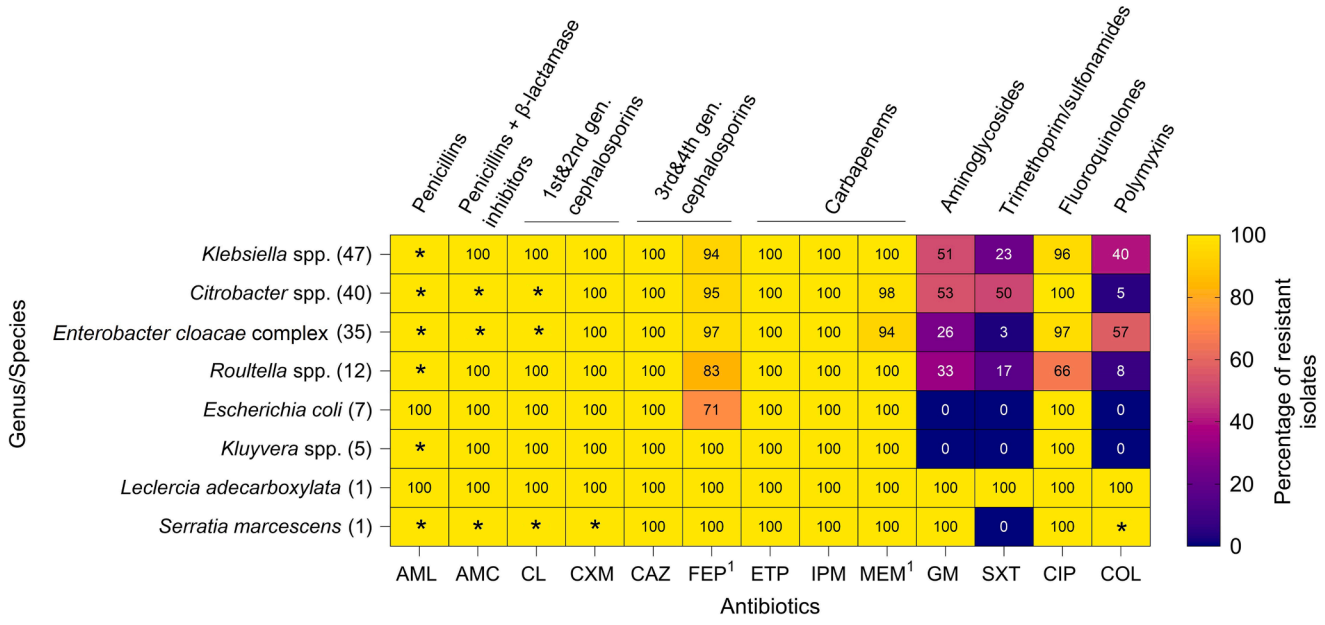

Fig. 2. Antimicrobial resistance patterns of CRE isolated from treated wastewater. Numbers in parentheses indicate the number of isolates from each genus or species. The numbers in the cells indicate the percentage of isolates that were resistant to each antibiotic tested. The asterisk represents intrinsic resistance. [1]The percentage of isolates with a resistant phenotype was not 100% because some isolates were intermediate susceptible. 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; COL: Colistin.

(Fig. 4). In addition, this isolate carried 35 different virulence genes related to colonization and biofilm formation, autotransporters, brain endothelial cell invasion etc. (Table S6).

In addition to *E. coli*, a subset of 25 other Enterobacterales isolates with different AMR profiles was selected for further targeted PCR for carbapenemase genes (Fig. 1D, Table S1). In *Citrobacter* isolates, bla_{NDM-1} was the most frequently detected gene (n = 11), followed by bla_{KPC-2} (n = 6), bla_{VIM-1} (n = 6), and bla_{OXA-48} (n = 2). Most *Citrobacter* isolates (n = 8) carried several carbapenemase genes simultaneously, with four carrying two genes (bla_{NDM-1} with bla_{KPC-2} or bla_{VIM-1}), two carrying three genes ($bla_{NDM-1,}$ bla_{KPC-2} bla_{VIM-1}) and two carrying four genes ($bla_{NDM-1,}$ $bla_{KPC-2,}$ $bla_{VIM-1,}$ bla_{OXA-48}). WGS analysis revealed that *C. freundii* was misidentified by MALDI-TOF MS and belonged to *C. portucalensis* (Fig. 4, Table S1). Furthermore, *in silico* MLST analysis associated *C. portucalensis* with ST641 and *C. farmer* with ST600. The bla_{KPC-2} and bla_{VIM-1} were identified by WGS in ST641 *C. portucalensis*, and bla_{KPC-2} was detected in ST600 *C. farmer* (Fig. 4). In addition to the carbapenemases, both sequenced isolates carried genes for TEM-1 ß-lactamase, and OXA-type (OXA-1 or OXA-10) or SHV-12 ESBLs. Genes for resistance to aminoglycosides (*aac(6′)-lb-cr, aph(3)-l* and *aph(6)-ld*), fluoroquinolones (*qnrB9* or *aac(6′)-lb-cr*), sulfonamides (*sul1*), chloramphenicol (*catA1),* trimethoprim (*dfrA1)* and antiseptics (*qacE*) were also found in sequenced isolates (Fig. 4). Both sequenced *Citrobacter* isolates had multiple virulence-associated genes (22 and 26, respectively), including *fepA*, *ompA, nmpC*, *acrB*, among other genes (Table S6). Analysis of the plasmid replicons revealed the presence of IncC, IncX5, IncX6 and IncY plasmids in the sequenced ST600 isolate, while the ST641 isolate had IncY and IncM1 plasmid replicons (Fig. 4).

Raoultella isolates mainly carried bla_{KPC-2} (6 isolates) or bla_{VIM} (6 isolates), followed by bla_{OXA-48} (4 isolates). Two carbapenemase genes in different combinations were found in 4 isolates. All three isolates of *K. cryocrescens* carried bla_{KPC-2}, one of them together with bla_{OXA-48} and bla_{VIM-1}. In *L. adecarboxylata* bla_{KPC-2} and bla_{OXA-48} were detected, and in *S. marscenscens* only bla_{VIM-1} (Fig. 1D).

3.5. Genome analyses of *Klebsiella spp.* isolates

3.5.1. Genetic diversity and carbapenemase gene variants

The PFGE analysis of *K. pneumoniae* showed that 29/36 tested isolates were clustered in 5 clusters (A-E) (Fig. 3A). MLST and WGS further revealed that isolates from the largest cluster A (n = 8 isolates) belonged to ST3590, while isolates from the next largest cluster B (n = 7 isolates) belonged to ST1697. There was also an unassigned ST (STND) that included 6 isolates that belonged to cluster E. Cluster D (n = 4 isolates) included ST1803, while isolates from cluster C (n = 4) belonged to ST629. Various carbapenemase genes were detected in these isolates based on PCR and sequencing analysis. All ST3590 isolates (XDR or MDR) carried only bla_{KPC-2}. In contrast, all XDR and MDR isolates of ST1697 contained bla_{NDM-1}, except one that contained bla_{NDM-1} together with bla_{OXA-48} and one that contained bla_{OXA-48} together with bla_{KPC-2}. All XDR and MDR isolates of STND were carriers of bla_{NDM-1} and bla_{OXA-48} simultaneously. The bla_{NDM-1} gene was common to most XDR ST1803 isolates, sometimes in combination with bla_{OXA-48}, except for one ST1803 isolate that carried only bla_{KPC-2}. The gene bla_{OXA-48} was detected in all isolates of the ST629 cluster, with most isolates (3/4) also carrying the *mcr-4.3* gene for resistance to COL (Fig. 3A).

The PFGE analysis of *K. oxytoca* (n = 11) grouped them to four clusters (A-D), except for two isolates that did not belong to any cluster (Fig. 3B). The MLST analysis assigned the clustered isolates to ST68 (n = 3), ST1697 (n = 2), ST43 (n = 2), and ST17 (n = 2). The presence of carbapenemase genes was variable among these isolates. The bla_{KPC-2} gene was detected in ST68, ST1697 and ST17 isolates, sometimes in combination with bla_{OXA-48} (in ST68) or bla_{NDM-1} and bla_{VIM-1} (in ST1697). One XDR ST43 isolate carried bla_{NDM-1} and bla_{IMP-13}, while the other ST43 isolate carried only bla_{NDM-1} (Fig. 3B).

3.5.2. Genetic determinants related to AMR, plasmid replicon types and virulence in sequenced isolates

To better understand the characteristics of carbapenem-resistant *Klebsiella* isolates from treated wastewater, five *K. pneumoniae* isolates (1 PDR, 3 XDR, and 1 MDR isolate) representing the above clusters and 2 XDR *K. oxytoca* isolates were selected and subjected to WGS. A summary

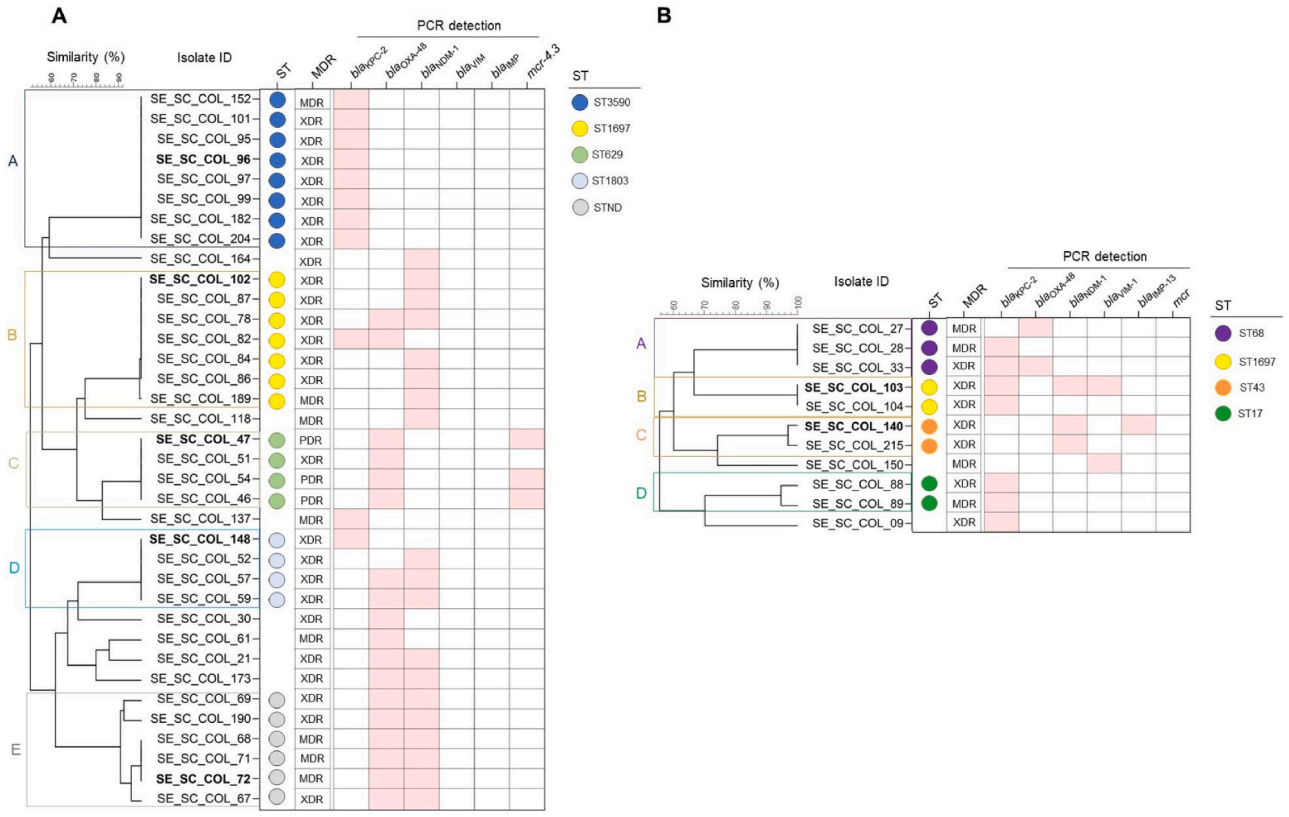

Fig. 3. Cluster analysis based on *Xba*I-PFGE of carbapenem-resistant (A) *K. pneumoniae* and (B) *K. oxytoca* isolates. The letters A through E in the dendrogram denote the primary clusters identified. The coloured circles refer to different sequence types (STs). MDR – multidrug-resistant, XDR – extensively drug-resistant, PDR – pandrug-resistant. The light red row in the table indicates the presence of the carbapenemase gene, and the white row indicates its absence. Isolates that underwent whole-genome sequencing are in bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the WGS data is shown in Fig. 4.

The analysis of 16S rRNA genes in *Klebsiella* genomes revealed that two *K. pneumoniae* isolates (SE_SC_COL_47 and SE_SC_COL_72) identified by MALDI-TOF MS belong to *K. pneumoniae*. In contrast, three *K. pneumoniae* isolates (ST3590, SE_SC_COL_96; ST1697, SE_SC_COL_102; ST1803, SE_SC_COL_148) and one *K. oxytoca* (ST1697, SE_SC_COL_103), previously identified by MALDI-TOF MS, were later identified by WGS as *K. quasipneumoniae* subsp. *similipneumoniae*, while the other *K. oxytoca* (ST43, SE_SC_COL_140) belonged to *K. michiganensis* (Fig. 4). The ANI calculator provided values of $\geq$ 97.7% for *K. quasipneumoniae* subsp. *similipneumoniae* or of 98.8% for *K. michiganensis*. Six sequenced *Klebsiella* isolates harbored various intrinsic ARGs such as $bla_{\text{SHV-1}}$, $bla_{\text{OKP-B}}$, $bla_{\text{OXY-1-7}}$, *fosA* and *oqxA/B*, where $bla_{\text{OKP-B}}$ and *fosA* were only detected in *K. quasipneumoniae* species. Of note, in the *Klebsiella* isolate SE_SC_COL_96 the $bla_{\text{OKP-B}}$ gene was only detected in the raw sequence data (not assembled). Several acquired carbapenemase genes ($bla_{\text{NDM-1}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{OXA-48}}$) and ESBL genes ($bla_{\text{SHV-12}}$, $bla_{\text{SHV-98}}$, $bla_{\text{OXA-1}}$, $bla_{\text{OXA-9}}$, $bla_{\text{C-15}}$ and $bla_{\text{C-3}}$) were identified in various combinations in sequenced *Klebsiella* spp. In addition, ARGs for other antibiotic classes were also found, including ARGs for fluoroquinolones (*qnrB*, *qnrS* and *aac(6′)-Ib-cr*), aminoglycosides (*aac(6′)-Ib-cr*, *aac(3)-I*, *aph(3)-I* and *aadA1*), sulfonamides (*sul1* and *sul2*), trimethoprim (*dfraA14*), macrolides (*mphA, mphE* and *msrE*), tetracyclines (*tetA*), chloramphenicol (*catB3*) and polymyxins (*mcr-4.3*). Two sequenced isolates carried the antiseptic-resistance gene *qacE*.

All sequenced *Klebsiella* isolates carried more than one plasmid

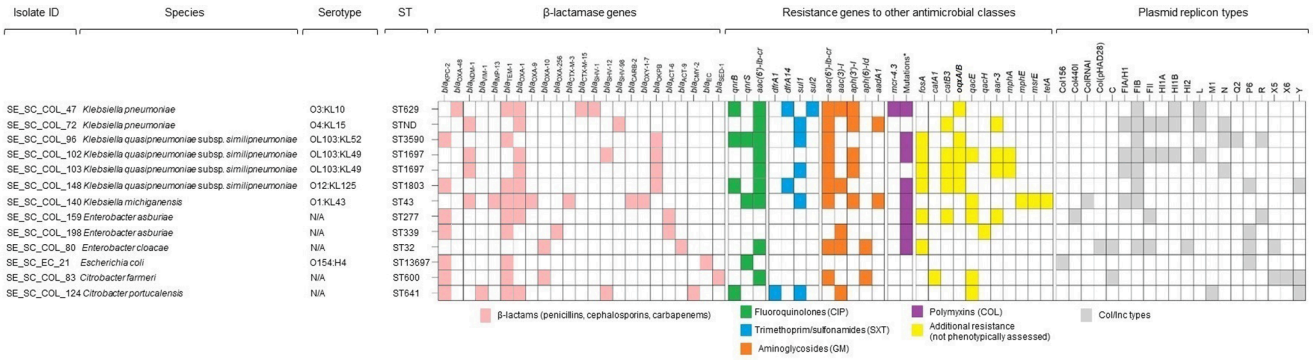

Fig. 4. Summary of whole-genome sequencing data for 13 selected CRE isolates. Colours indicate that the resistance gene or plasmid replicon was present. *The mutations are chromosomal point mutations in the *pmrA/B, phoP/Q, mgrB* and/or *crrB* genes that can confer colistin resistance (see Table S5 for a complete list of point mutations for colistin resistance). N/A – not applicable.

replicon, with IncFIB present in all (Fig. 4). The plasmid replicon type IncFIA/H1 was found in 4 isolates (STND, both ST1697, and ST43). IncHI1B, IncL, and IncN were found in three isolates each.

The VFanalyzer predicted between 16 and 70 different virulence genes in the *Klebsiella* genomes analyzed, with the highest number in ST43 *K. michiganensis* and ST1697 *K. quasipneumoniae* (Fig. 5). Virulence genes included genes encoding biofilm formation (*mrkA-mrkJ)*, adherence (*fimA-fimK)*, siderophores such as aerobactin (*iutA*), enterobactin *(entA-entS* and *fepA-feS)*, yersiniabactin (*fyuA* and *ybt*) and salmochelin (*iroE* and *iroN)*, and secretion systems (T6SS-I, T6SS-II and T6SS-III). Only ST43 *K. michiganesis* possessed yersiniabactin genes, and all isolates possessed genes *acrAB* for efflux pumps associated with AMR and virulence in *Klebsiella* spp. (Fig. 5). Kleborate analysis revealed no hypervirulence-associated genes in these isolates, with the exception of *K. michiganensis*, in which the presence of yersiniabactin-associated genes (*fyuA* and *ybt*) was confirmed. Finally, *K. pneumoniae* isolates had capsules of the KL10- and KL15-type (O3– and O4-type LPS, respectively), *K. quasipneumniae* of the KL52-, KL49- and KL125-type, while *K. michiganensis* had the capsular serotype KL43 (LPS serotype O1) (Fig. 4).

3.5.3. Colistin resistance mechanisms in sequenced Klebsiella spp.

Three ST629 *K. pneumoniae* isolates in this study that were phenotypically identified as COL-resistant contained the *mcr-4.3* gene (Fig. 3A). Phylogenetic analysis revealed that this gene from the sequenced *K. pneumoniae* isolate (SE_SC_COL_47) is closely related (100% nucleotide identity) to *mcr-4.3* from species of 6 genera (*Enterobacter, Acinetobacter*, *Shewanella*, *Salmonella*, *Leclercia*, and *Lelliottia*) (Fig. 6A, Table S3). These isolates were identified on 4 continents in 10 countries (Brazil, China, Czech, Republic, Germany, Italy, Netherlands, Norway, Singapore, Taiwan, and USA). Most of these strains were *E. cloacae* cplx. and *Acinetobacter* species (11/19), mainly isolated from humans. In addition, *mcr-4.3* was also found less frequently in strains from animals (n = 4) and wastewater (n = 1). Furthermore, the phylogenetic tree revealed that *mcr-4.3* and other *mcr-4* variants form a group that is more closely related to the large *mcr-3* gene family (mainly *E. coli* and *Aeromonas* spp.) than to the *mcr-1/2* family (mainly *E. coli* and *Moxarella* spp.) (Fig. 6A).

Sequencing also revealed that the *mcr-4.3* gene was present in the *K. pneumoniae* isolate (SE_SC_COL_47) on a 110,506 bp long contig, which also contains the IncHI1B plasmid replicon (Fig. 6B). In addition, a number of genes involved in conjugation (*tra/trh*) were found downstream of *mcr-4.3* on this contig. The BLASTn search for the entire contig resulted in a significant match (99.95% identity, 75% coverage) with a 230,982 bp plasmid (pC763_1, GenBank accession number: CP067474) from *K. pneumoniae*, which does not contain *mcr-4.3* (Fig. 6B). Nucleotide sequences found upstream of *mcr-4.3* include the *phd* gene of the type II toxin-antitoxin (TA) system and the Tn*3*-family transposon (Fig. 6B). The BLASTn search revealed that this region (Tn*3*-TA-*mcr-4.3*) has 100% identity to the similar region in the genome of *Shewanella frigidimarina* (GenBank accession number: CP000447.1), as well as high similarity (99.97%) to the plasmid of *A. baumannii* (GenBank accession number: NZ_MK360916.1) (Fig. 6B) and other *Acinetobacter* sp. (Table S4).

Finally, WGS showed that four sequenced *Klebsiella* spp. (ST1803, ST3590, ST1697, and ST43) that were phenotypically resistant to COL but lacked plasmid-mediated *mcr* genes had mutations in one or more genes associated with COL resistance: *pmrA/B, phoP/Q, mgrB*, and *crrB* (Fig. 4, Table S5). In ST1803 *K. quasipneumoniae*, 29 point mutations were found that resulted in amino acid changes, 9 of them for the first time (PmrA – S64A, L140Q, E119D; PmrB – T8N, N105S, R256S; PhoP – R165H, Q424L, and PhoQ – Q140K). Of these, R256S and Q424L were predicted to have deleterious effect on PmrB and PhoP function, respectively, based on PROVEAN analysis. In ST3590 *K. quasipneumoniae*, two novel neutral mutations were found in PmrB (M175V) and PhoP (K149E), whereas in ST1697 *K. quasipneumoniae*, a nucleotide deletion of 31 nucleotides (position 601–632) was detected in the *pmrB* gene. In ST43 *K. michiganensis* all investigated genes that can confer resistance to COL were found as wild type. Of note, ST629 *K. pneumoniae,* which contains the *mcr-4.3* gene, also had a neutral mutation in the CrrB protein (Q287K) (Table S5).

4. Discussion

WWTPs are increasingly recognized as a reservoir and pathway for the spread of priority MDR pathogens from the community into natural waters and pose a threat to public health (Lepuschitz et al., 2019). However, there is limited understanding regarding their role in the environmental spread of CRE. We report for the first time the phenotypic and genomic characteristics of CRE remaining in wastewater after treatment in the largest Croatian WWTP, which treats not only municipal wastewater but also wastewater from nine hospitals. A total of 148 carbapenemase-producing Enterobacterales were isolated and characterized, with *Klebsiella* spp. dominating, followed by *Citrobacter* spp. and *E. cloacae* cplx. Some of the isolates of *K. pneumoniae, K. oxytoca* and *C. freundii* were misclassified by MALDI-TOF MS and identified by WGS as *K. quasipneumoniae* subsp. *similipneumoniae, K. michiganensis* and *C. portucalensis*, respectively. This taxonomic reclassification was also supported by ANI analysis (values $\geq$ 97.7%), which has been shown to be a powerful tool for clarifying the distinction between species (Rodríguez-Medina et al., 2019). In addition, the bla_{OKP} gene, normally located on the chromosome of *K. quasipneumoniae*, has been reported to contribute to the reliable differentiation of *K. quasipneumoniae* from other *Klebsiella* spp. (Chew et al., 2021). The detection of $bla_{OKP\text{-}B}$ only in genomes associated with *K. quasipneumoniae* confirmed the correct identification of this species in this study. Of note, the raw data for the detection of $bla_{OKP\text{-}B}$ showed higher sensitivity than the assembled genomes, which could be due to errors in the repeat regions or contamination affecting genome assembly (Cooper et al., 2020). Misidentification of *Klebsiella* spp. and *Citrobacter* spp. by MALDI-TOF

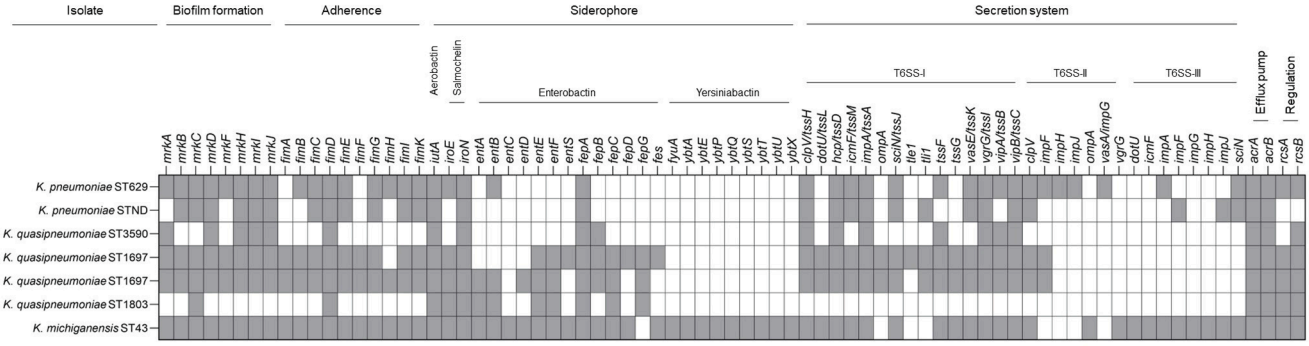

Fig. 5. The content of virulence-encoding genes in relation to their functions in sequenced *Klebsiella* isolates from treated wastewater.

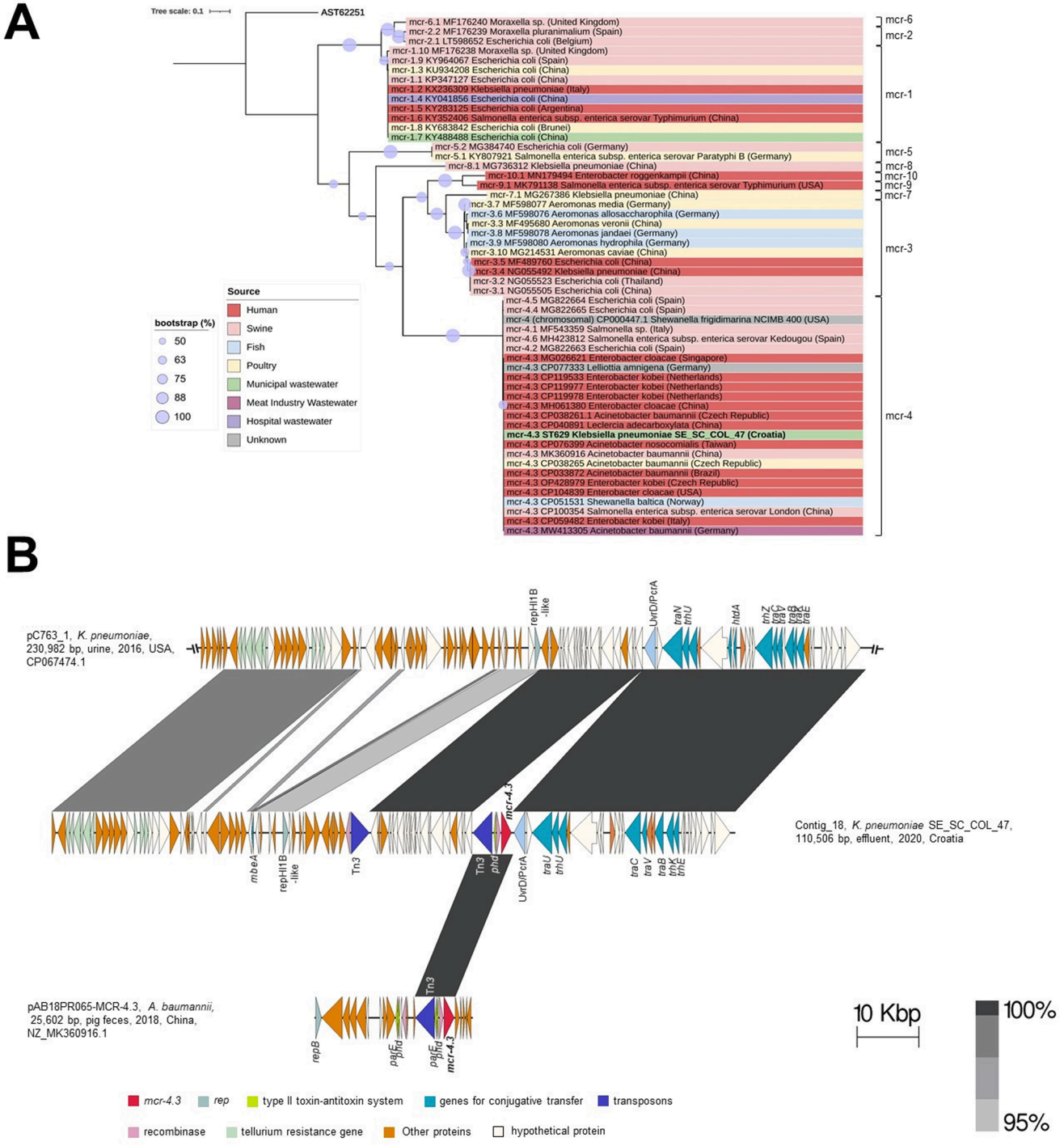

Fig. 6. (A) Phylogenetic tree of *mcr-4* gene sequences. The nucleotide sequence of *mcr-4.3* from *Klebsiella pneumoniae* SE_SC_COL_47 obtained in this study was compared with other known *mcr* variants derived from different isolates and different origins. The tree was rooted with *Escherichia coli* Z1140 with non-functional phosphoethanolamine transferase gene (GenBank accession number: AST66929). The *mcr-4.3* gene from Croatia is indicated in bold letters. The origin of the isolates is noted on the coloured strips according to the indicated key, and the country of isolation is indicated in parentheses. The nucleotide accession number of each member is indicated in the figure. (B) Genetic context of *mcr-4.3* on the contig of *K. pneumoniae* SE_SC_COL_47 together with the linearized partial map of the plasmids of *K. pneumoniae* (GenBank accession number: CP067474.1) and *Acinetobacter baumannii* (GenBank accession number: NZ_MK360916.1). The arrows indicate the direction of transcription of the genes. Grey shaded areas indicate homologous regions with ≥ 95% nucleotide sequence identity with the contig carrying the *mcr-4.3* gene. Different colors indicate the predicted functions of the different genes.

MS has also been observed in other studies (Nobrega et al., 2023; Rodrigues et al., 2018), and it has been hypothesized that *K. quasipneumoniae* is more common in wastewater than *K. pneumoniae* (Liu et al., 2023).

The majority of *Klebsiella*, *Citrobacter* and *E. cloacae* cplx. isolates (≥54% of each genus) were defined as XDR because they were resistant to all but one or two of the antibiotic classes tested. These XDR bacteria, especially *Klebsiella*, pose a significant public health problem as they often cause difficult-to-treat infections and, in some cases, deaths in affected individuals (Navon-Venezia et al., 2017). PCR and WGS

analyses further revealed genetic mechanisms underlying carbapenem resistance in these isolates. The bla_{NDM-1} and the bla_{OXA-48} were the most frequently detected carbapenemase genes, which frequently co-occurred in our *K. pneumoniae* isolates. This carbapenemase combination was also recently detected in *K. pneumoniae* isolates from patients in hospitals and hospital wastewater in Zagreb (Bedenić et al., 2023; Puljko et al., 2023) and in clinical *K. pneumoniae* isolates from 13 European countries, suggesting transboundary dissemination (Ludden et al., 2020). On the other hand, carbapenemase KPC-2 was predominantly detected in our *K. oxytoca* and *E. cloacae* cplx. isolates as well as in *Citrobacter* isolates, but in combination with carbapenemase NDM-1. Interestingly, we frequently observed the presence of two or three different carbapenemases in our Enterobacterales isolates from wastewater. A possible reason for this accumulation of carbapenemase genes in enterobacteria could be the high consumption of carbapenem antibiotics during the COVID-19 pandemic in Croatia (Bedenić et al., 2023). In fact, Croatia is among the three and five EU countries with the highest carbapenem consumption in the hospital and community sectors in 2020 and 2021, respectively (ECDC, 2022). Other carbapenemase genes detected in our isolates were bla_{VIM-1} and bla_{IMP-13}. The bla_{VIM-1} gene was frequently found in *K. oxytoca*, *Citrobacter*, *Kluyvera* and *Roultella* isolates together with two or three other carbapenemase genes. In contrast, bla_{IMP-13} was detected in an XDR isolate of *K. michiganensis* together with bla_{NDM-1}. The identification of the bla_{IMP-13} gene in *K. michiganensis* was an unusual finding, as this gene is normally present in *Pseudomonas aeruginosa* and sporadically in clinical enterobacteria, including *E. coli* and *E. cloacae* cplx, as well as in *Aeromonas media* previously isolated from the same wastewater analyzed here (Cañada-García et al., 2022; Drk et al., 2023).

MLST analysis identified some of the human-associated *K. pneumoniae* (ST3590, ST1803, ST629) and *K. oxytoca* (ST43 and ST17) clones in our wastewater isolates (Aires et al., 2017; Jolley et al.,; Šuto et al., 2022; Takei et al., 2022; Wan et al., 2023). Some of these clones were also found in non-human sources such as animals and soil (ST43 and ST1697) (Jiang et al., 2023; Liao et al., 2019). With the exception of ST629, all these clones were detected for the first time in Croatia. The presence of virulence determinants such as aerobactin, enterobactin and/or yersiniabactin-related genes in *Klebsiella* genomes (ST3590, ST1803, ST629, ST43, STND, and ST1697) has shown that they are likely to be associated with human infections (Shankar et al., 2018). However, due to the absence of other hypervirulence-associated markers, including hypervirulence-associated capsules, these isolates cannot be classified as hypervirulent.

The ST types detected among *E. cloacae* cplx. isolates included mainly human-associated MDR- or XDR-type clones (ST910, ST1641, ST339, ST32, ST277) (Izdebski et al., 2015; Jolley et al., 2018; Zelendova et al., 2023), some of which were also previously detected in wastewater (ST910, ST277, ST32) and waterways (ST910) (Cherak et al., 2021; Cirkovic et al., 2023; Falgenhauer et al., 2019; Puljko et al., 2023). The remaining three (ST2057, ST2408 and ST2368) have already been detected in *Enterobacter* spp. from wastewater, animals and food (Jolley et al., 2018). In addition, STs detected in *Citrobacter* isolates, such as ST641 and ST600 and *E. coli* ST13697, were previously reported in clinical *Citrobacter* spp. (Jolley et al., 2018; Zhou et al., 2020). The profile of virulence genes in *E. cloacae* cplx., *Citrobacter* and *E. coli* isolates suggests that these genes may play an important role in survival in different environments (Amaretti et al., 2020; Mustafa et al., 2020; Yu et al., 2022), although the pathogenic potential of these isolates cannot be determined based on their virulence gene content.

We further determined the plasmid content and potentially associated carbapenem ARGs in draft genomes of *Klebsiella*, *E. cloacae* cplx. and *Citrobacter* isolates to assess their potential for the acquisition and spread of carbapenem resistance. *Klebsiella* isolates carrying the bla_{NDM-1} gene had multiple plasmid replicons of Col-, IncF-, IncH-, IncL-, and IncN-types in various combinations, with IncFIA and IncFIB detected in all four sequenced isolates (STND, both ST1697, and ST43). These two conjugative plasmids and other detected IncH- and IncN-type conjugative plasmids were previously associated with the spread of bla_{NDM-1} in Enterobacterales (Wu et al., 2019). In addition, the two *Klebsiella* isolates ST3590 and ST1803 also had an IncFIB-type plasmid replicon, which may be related to the spread of the bla_{KPC-2} gene, as previously reported for the sewage *K. pneumoniae* isolate in Croatia (Kvesić et al., 2022). Interestingly, the KPC-2-producing *K. quasipneumoniae* ST1803 contained an additional, emerging IncP6 plasmid replicon with a broad-host range. This IncP6 plasmid was reported as a conjugative plasmid (Yao et al., 2017) and was also found in KPC-2-producing *E. cloacae* cplx. ST339 and *E. coli* ST13697 isolates in this study, suggesting that the bla_{KPC-2} gene was likely acquired through IncP6. This is consistent with previous reports of transmission of bla_{KPC-2} by IncP6 in Enterobacterales isolates from wastewater from Croatia and other countries (Ghiglione et al., 2021; Kvesić et al., 2022; Yao et al., 2017). In *E. cloacae* cplx. ST277 and KPC-2-producing *Citrobacter* ST600 and ST641 isolates analyzed in this study, several other plasmid replicons were detected, including IncX5, IncX6, IncM1, IncFII, and IncR types previously reported to be conjugative and associated with transmission of bla_{KPC-2} (Jia et al., 2023; Li et al., 2018; Raro et al., 2020; de Souza et al., 2019; Xie et al., 2023). Seven different plasmid replicon types were found in *E. cloacae* ST32 without the detection of carbapenemase genes. The carbapenem resistance of this isolate may be due to the presence of the AmpC gene bla_{ACT-9} or the ESBL gene bla_{OXA-10} in combination with porin loss, as previously reported (Alonso-García et al., 2023; Lee et al., 2011). In addition, the *C. portucalensis* ST641 carrying bla_{VIM-1} had the plasmid replicon type IncY, which was reported to be conjugative and to harbor bla_{VIM-1} in *E. coli* (Roschanski et al., 2017). Finally, the carbapenemase-producing PDR *K. pneumoniae* ST629 isolate, which carried IncL, IncHI1B and IncFIB plasmid replicons, also had the bla_{OXA-48} gene. The conjugative IncL plasmid (Poirel et al., 2012) may be associated with bla_{OXA-48} transmission, as reported in clinical and environmental isolates in Croatia (Kvesić et al., 2022; Šuto et al., 2022) and other countries (Hamprecht et al., 2019). In addition to carbapenem resistance, this *K. pneumoniae* ST629 isolate exhibits resistance to COL, one of the last available antibiotics, which is mediated by the *mcr-4.3* gene. To the best of our knowledge, this is the first detection of the *mcr-4.3* gene in *K. pneumoniae* and in Croatia in general. Interestingly, the contig with the *mcr-4.3* gene also carried the IncHI1B plasmid replicon, suggesting that the acquisition of *mcr-4.3* in this ST629 isolate may be related to the IncHI1B plasmid. Previous studies have reported the association of the *mcr-2, mcr-3* and *mcr-8* genes with IncHI1B in *K. pneumoniae* (Salloum et al., 2020; Stosic et al., 2021), but an association of the IncHI1B plasmid with *mcr-4.3* has not yet been reported. In addition, the presence of *tra/trh* genes on the contig with the IncHI1B replicon type suggests that IncHI1B may be a conjugative plasmid. Further analysis of this contig revealed that the *mcr-4.3* gene and the upstream neighboring region (Tn*3* transposon and the type II TA system) are identical to the genomic sequence of *S. frigidimarina* and nearly identical to the plasmid sequence of *A. baumannii* and other *Acinetobacter* spp. This supports the conclusion of Zhang et al. (2019) that *mcr-4.3* originated from *S. frigidimarina*, from where it was mobilized onto plasmids in Enterobacterales, potentially including *K. pneumoniae* in this study, but also other bacteria such as *A. baumannii*, as previously reported (Ma et al., 2019). The localization of *mcr-4.3* on the conjugative plasmid may contribute to the rapid spread of COL resistance via wastewater and increase the risk of retransmission to humans.

In addition, novel resistance mechanisms to COL have been observed in some sequenced *Klebsiella* isolates. This is evidenced by novel deleterious mutations in PmrB (R256S) and PhoQ (Q424L) (in *Klebsiella* ST1803) or mutations in *pmrB* by nucleotide deletion (ST1697) or the absence of known resistance mechanisms (ST43 and ST3590). In contrast, known resistance mechanisms to COL were observed in *Enterobacter* isolates. These were based on known deleterious mutations in PmrA, PmrB, PhoP, and/or PhoQ proteins as previously reported for clinical and wastewater *E. cloacae* cplx. isolates (Liao et al., 2022; Puljko

et al., 2023; Uechi et al., 2019).

5. Conclusion

The results of this study show that treated wastewater is a source of hazardous CRE, in particular *Klebsiella* spp. as well as *Citrobacter* spp. and *E. cloacae* cplx. into the aquatic environment. These opportunistic pathogens are phylogenetically related to human-associated MDR-type clones, some of which were detected for the first time in Croatia, and often carry several clinically important carbapenemase genes simultaneously. Five *Klebsiella* spp. were misidentified with MALDI-TOF MS and correctly identified with ANI and by the presence of the species-specific bla_{OKP} gene. WGS analyses also revealed a diverse range of plasmids in the sequenced isolates that are possibly associated with the carbapenemase genes detected. In addition, a significant proportion of *Klebsiella* and *E. cloacae* cplx. isolates demonstrated phenotypic resistance to the last-line antibiotic colistin. The mechanisms underlying its resistance include known and potentially novel intrinsic mechanisms (point mutations in genes for COL resistance) and acquired mechanisms (*mcr-4.3* gene). The colistin resistance gene *mcr-4.3* was identified for the first time in *K. pneumoniae* and in Croatia and is located on the conjugative IncHI1B plasmid. This illustrates the potential of this gene to spread rapidly in the environment and possibly reintroduce into the community, resulting in very limited treatment options. Thus, our monitoring of treated municipal wastewater contributes to the assessment of the risk of spreading resistance to last-line antibiotics from WWTPs into the environment and underlines the need for more effective wastewater treatment.

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CRediT authorship contribution statement

Ana Puljko: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Ivan Barišić:** Writing – review & editing, Writing – original draft, Resources, Formal analysis, Conceptualization. **Svjetlana Dekić Rozman:** Writing – review & editing, Investigation. **Stela Križanović:** Investigation. **Ivana Babić:** Writing – review & editing, Investigation, Data curation. **Marko Jelić:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Ana Maravić:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Nikolina Udiković-Kolić:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.108554.

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