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EDITED BY
Aurora Piazza,
University of Pavia, Italy

REVIEWED BY
Salome N. Seiffert,
Zentrum für Labormedizin (ZLM), Switzerland
Zhuofeng Yu,
University of Copenhagen, Denmark

*CORRESPONDENCE
Héctor Alex Saka
✉ alex.saka@unc.edu.ar

†These authors have contributed equally to this work and share first authorship

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Urban wastewater overflows as hotspots for dissemination of bacteria producing extended-spectrum β-lactamases and carbapenemases in the Suquía River, Argentina

Susana Eugenia Ruiz[1,2†], Fabrizzio Nicolas Morandini[3,4†], María Emilia Panzetta[5], Flavio Gabriel Lipari[6], María Gabriela Irrazábal[7], Ricardo Toselli[8], Martín Der Ohannesian[8], Cristian Amieva[2], María Eugenia Valdes[3,4], Federico Javier Giraudo[1], María del Rosario Rollán[1], Valeria Amé[3,4], Claudia Sola[3,4] and Héctor Alex Saka[3,4]*

[1]Facultad de Ciencias de la Salud, Universidad Católica de Córdoba, Córdoba, Argentina, [2]LACE Laboratorios, Córdoba, Argentina, [3]Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Córdoba, Argentina, [4]Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina, [5]Department of Integrative Immunobiology, Duke University, Durham, NC, United States, [6]Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina, [7]Facultad de Ciencias Agropecuarias, Universidad Católica de Córdoba, Córdoba, Argentina, [8]Facultad de Ciencias Químicas, Centro de Química Aplicada (CEQUIMAP), Universidad Nacional de Córdoba, Córdoba, Argentina

Antimicrobial resistance (AMR) is a critical global challenge, yet the role of environmental dissemination of antibiotic-resistant bacteria remains underexplored, particularly in developing regions. This study investigated urban wastewater overflows from public streets as vectors for extended-spectrum-β-lactamase (ESBL)- and carbapenemase-producing *Enterobacterales* and *Aeromonas* in the Suquía River (Córdoba, Argentina). Sixty-two water samples were analyzed for coliform counts, antimicrobial susceptibility, and resistance genes. Horizontal gene transfer was assessed by conjugation. Sixty-five ESBL- and/or carbapenemase-producing isolates were recovered, including six carbapenemase producers subjected to whole-genome sequencing (WGS). Urban wastewater exhibited coliform levels >10^8 MPN/100 mL, while river counts increased 2–5 logs at urban and downstream sites compared to upstream, where no resistant strains were detected. ESBL- and/or carbapenemase-producers occurred in ~70% of wastewater and river samples, mainly *Escherichia coli* harboring bla_{CTX-M}. Carbapenemase producers carried bla_{KPC-2} or bla_{NDM-1} in *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Aeromonas caviae*. WGS revealed extensive resistomes, virulence genes, and plasmid replicons, including IncU and IncA/C2 linked to carbapenemases. Conjugation confirmed plasmid-mediated transfer of β-lactamase genes, and genetic context analysis identified clinically recognized transposons. Notably, *Enterobacter kobei* and *Aeromonas caviae* from the river carried bla_{KPC-2} on plasmidic contigs combining clinical and environmental elements, consistent with genetic exchange within aquatic ecosystems and transfer of clinically

significant resistance determinants to species adapted for riverine survival. These findings identify urban wastewater overflows as AMR hotspots that facilitate the dissemination of multidrug-resistant bacteria and mobile resistance elements into urban and peri-urban aquatic environments, underscoring the need for integrated environmental AMR surveillance.

KEYWORDS

antimicrobial resistance, *Enterobacterales*, *Aeromonas*, extended-spectrum β-lactamases, carbapenemases, urban wastewater, environmental spread of resistance, antimicrobial resistance in rivers

1 Introduction

Antimicrobial resistance (AMR) currently represents a critical global health challenge, severely limiting anti-infective treatment options, increasing the burden of healthcare costs and exacerbating mortality rates. Recent estimations indicate that at least 1.27 million deaths/year are attributable to infections caused by antibiotic-resistant bacteria worldwide (Antimicrobial Resistance, 2022). Projections predict over 10 million deaths/year due to AMR and a decrease of 2.0–3.5% in global GDP by 2050 (O'Neill, 2016). Compounding this challenge, the discovery of new antimicrobial agents has been limited over the past decades, highlighting the urgent need for effective interventions to counteract the harmful impacts of AMR (Silver, 2011; Roca et al., 2015).

Enterobacterales are Gram-negative bacteria that inhabit the intestinal tract of humans and animals and are significant causes of infection. Over recent decades, antibiotic resistance within *Enterobacterales* has increased substantially, positioning them as major contributors to global mortality linked to antibiotic resistance (Morosini, 2017; Antimicrobial Resistance, 2022). In this context, extended-spectrum-β-lactamase (ESBL)-producing and carbapenemase-producing *Enterobacterales* are classified by the World Health Organization (WHO) as critical priority pathogens for which new active antibiotics are urgently needed (WHO, 2024). Importantly, ESBLs and carbapenemases are frequently encoded by mobile genetic elements, such as plasmids, transposons and integrons, whose ability for horizontal gene transfer expands their potential for dissemination across bacterial species (Bush, 2018).

The persistent increase in AMR indicates that traditional approaches, which heavily emphasize human health, are insufficient to manage this complex problem. In this context, there is a growing consensus that AMR should be viewed as a multifaceted issue arising from intricate interactions among humans, animals, and the environment, a perspective known as the "One Health" approach (Laxminarayan et al., 2013; White and Hughes, 2019).

Much remains to be uncovered about the environmental dimension of AMR, especially in developing countries (Larsson et al., 2018). Studies indicate that many of the antibiotic resistance genes found in clinically important bacteria are linked to environmental origins, emphasizing the environment's critical role in AMR (D'Costa et al., 2011; Forsberg et al., 2012). Anthropogenic activities seem to play an important role in this process. Intensive use and release of antimicrobials across sectors such as industry, agriculture, medicine, and veterinary medicine create conditions for selective pressure that promotes the emergence and spread of resistant microorganisms (Singer et al., 2016). A significant portion of the antibiotics administered to humans and animals are excreted in active form, mainly via urine and feces, accumulating in environmental matrices like surface water, groundwater, soil, and sediments. These sub-inhibitory concentrations in natural ecosystems may foster the selection of resistant strains within native microbial communities (Zhang et al., 2018; Mutuku et al., 2022). Furthermore, anthropogenic activities may aggravate the problem by releasing antibiotic-resistant bacteria and their genes to the environment (Czatzkowska et al., 2022; Larsson and Flach, 2022).

Pollution from wastewater spills containing antibiotic-resistant bacteria in public areas, though less studied, may be a significant factor driving environmental AMR (Manaia et al., 2016). Frequent wastewater overflows occur in public streets throughout the city of Córdoba, Argentina. Although official data are lacking, unofficial estimates suggest approximately 30 overflow events per day, totaling over ten thousand annually across multiple city locations (LaVoz, 2017). Water from these overflows is channeled through the city's stormwater drainage system, which discharges runoff mainly into La Cañada stream (a tributary of the Suquía River) or directly into the urban stretch of the Suquía River. Within this context, our study aimed to investigate the potential role of urban wastewater overflows from public streets as vectors for critically important antibiotic-resistant bacteria and their impact on the Suquía River basin. In addition, we carried out detailed genomic analyses to elucidate the resistome, virulome, plasmid content and genetic structure of carbapenemase-harboring strains recovered from these sources, and performed conjugation experiments to directly assess their horizontal transfer potential.

2 Materials and methods

2.1 Study area and sample collection

A total of 62 water samples were collected between 2016 and 2023, comprising 34 urban wastewater samples from Córdoba city, 22 surface water samples from the Suquía River and 6 surface water samples from Ansenuza National Park. Wastewater samples exhibiting organoleptic properties of sewage overflows were collected from public streets in Córdoba, the second most populous city in Argentina (1.6 million inhabitants). The Suquía River flows west to east/northeast, traversing the city of Córdoba and continuing for approximately 200 km before discharging into the Mar de Ansenuza. Sampling sites along the Suquía River were strategically selected to include both pre-urban locations (upstream of the city) and urban sites, covering the river's course through the

city as well as the area influenced by the municipal wastewater treatment plant (WWTP). The Mar de Ansenuza, within Ansenuza National Park, is a highly saline endorheic lagoon covering up to 8,000 km^2. It is the largest saline endorheic lagoon in South America and the fifth largest worldwide. Sampling points are detailed in Supplementary Figure S1. Information on individual samples is provided in Supplementary Table S1. Samples were collected in triplicate at ~5-min intervals. Each replicate (50–200 mL) was placed in sterile containers, refrigerated immediately, and processed within 24 h.

2.2 Coliform bacteria count

Total coliforms, fecal coliforms and *Escherichia coli* in water samples were enumerated according to the Standard Methods for the Examination of Water and Wastewater (APHA) using methods 9221A, 9221B, and 9221C for total coliforms; method 9221E for fecal coliforms; and method 9221F for *E. coli*. Results were expressed as the Most Probable Number per 100 mL (MPN/100 mL) (Lipps and Baxter, 2023).

2.3 Isolation of antibiotic-resistant bacteria

A volume of 0.2 mL of undiluted and serially diluted (10^{-1} to 10^{-4}) samples from wastewater, Suquía River or Ansenuza National Park surface water was inoculated onto ChromID ESBL (bioMérieux) plates for presumptive identification of ESBL- and carbapenemase-producing *Enterobacterales* and *Aeromonas*. Plates were incubated at 35–37 °C for 24–48 h. For samples collected from the Suquía River and Mar de Ansenuza, an enrichment step was additionally performed by inoculating 1 mL of sample into 10 mL of nutrient broth supplemented with cefotaxime (1 mg/L), followed by incubation at 35–37 °C for 24 h. Subsequently, 0.2 mL of undiluted or serially diluted enrichment broth was plated onto ChromID ESBL agar and incubated at 35–37 °C for 24–48 h. Colonies presumptively identified as *Enterobacterales* or *Aeromonas* were subcultured onto CLDE or MacConkey agar and identified to species-level using VITEK 2 Compact (bioMérieux, GN-ID card Ref. 21341). Isolates that could not be conclusively identified with VITEK 2 Compact, were further characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker, bioMérieux), using the MALDI BioTyper 3.1 software (Bruker Daltonics), MBT IVD reference library (version 2023). A total of 65 ESBL- and/or carbapenemase-producing isolates were identified (Supplementary Table S2) and cryopreserved at −80 °C in Mueller-Hinton broth supplemented with 20% glycerol.

2.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility of *Enterobacterales* to a variety of clinically relevant antibiotics was determined by disk diffusion following the Clinical&Laboratory Standards Institute (CLSI) M100 ED34 guidelines (CLSI, 2024). Antibiotics tested included: ampicillin (10 µg), ampicillin/sulbactam (10/10 µg), amoxicillin/clavulanic acid (20/10 µg), cefazolin (30 µg), cefixime (5 µg), ceftazidime (30 µg), cefotaxime (30 µg), piperacillin/tazobactam (100/10 µg), imipenem (10 µg), meropenem (10 µg), nitrofurantoin (300 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), ciprofloxacin (5 µg), gentamicin (10 µg) and amikacin (30 µg). For *Aeromonas*, CLSI M45-ED3 guidelines for disk diffusion were followed (CLSI, 2016) for ceftazidime (30 µg), cefotaxime (30 µg), piperacillin/tazobactam (100/10 µg), imipenem (10 µg), meropenem (10 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), ciprofloxacin (5 µg), gentamicin (10 µg) and amikacin (30 µg), while for nitrofurantoin (300 µg) CLSI recommendations for *Enterobacterales* were applied (CLSI, 2024). Ceftazidime/avibactam (10/4 µg) susceptibility was tested by disk diffusion in both *Enterobacterales* and *Aeromonas* according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for *Enterobacterales* (EUCAST, 2024). Phenotypic confirmation of ESBL production was carried out by the double-disk synergy test (Drieux et al., 2008). Carbapenemase production was confirmed by the modified carbapenem-inactivation method (mCIM) and the EDTA-modified mCIM method (eCIM) following CLSI recommendations (CLSI, 2024). For carbapenemase-producing strains, the minimum inhibitory concentration (MIC) of meropenem was determined by E-test (AB Biodisk) following manufacturer's instructions.

2.5 Mating assays

Mating experiments were used to assess horizontal transfer of β-lactamases for selected carbapenemase- and ESBL-producing strains, using *E. coli* ER1793 (rifampin resistant) as recipient strain. Conjugation mixtures were prepared at a donor-to-recipient ratio of 1:4 in physiological saline, with inocula adjusted to approximately a McFarland standard of 2. A 100 µL aliquot of the mixture was spot-inoculated onto pre-dried, cation-adjusted Mueller-Hinton agar plates and incubated at 37 °C for 24 h. Subsequently, bacterial growth was resuspended in 1 mL of physiological saline. Then, 100 µL of the suspension (undiluted and serially diluted 10^{-1} to 10^{-4}) was streaked onto Mueller-Hinton plates supplemented with rifampin (50–200 µg/mL) and either cefotaxime (4 µg/mL) or meropenem (0.5–2 µg/mL) for selection. Ten putative transconjugant colonies were re-isolated from each mating assay, identified as *E. coli* and subjected to disk diffusion susceptibility testing, double-disk and mCIM tests (as described above). PCR assays targeting the relevant resistance genes were conducted on transconjugants as detailed below.

2.6 PCR detection of selected ESBL- and carbapenemase-genes

Genetic determinants for selected ESBL and carbapenemases were investigated by PCR using specific primers (detailed in Supplementary Table S3). The 16S rRNA gene was amplified to verify the presence of amplifiable DNA. Total bacterial DNA was extracted using the boiling method as previously described (Queipo-Ortuno et al., 2008). PCR reactions were performed with an initial denaturation step of 5 min at 95 °C, followed by 30 amplification cycles consisting of 1 min at 95 °C, 1 min at the primer-specific annealing temperature (Supplementary Table S3), and 1 min at 72 °C,

with a final extension step (5 min, 72 °C). The PCR master mix was prepared to a final volume of 25 µL per reaction, containing 0.04 U of Taq DNA polymerase (Pegasus EA01M, PB-L), 1x reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.4 µM of each primer in molecular biology-grade water. Each reaction tube received 24 µL of master mix and 1.0 µL of DNA template. PCR products were resolved by electrophoresis on 1.0–1.5% agarose gels stained with SybrSafe (Invitrogen). Electrophoresis was conducted in 1x TAE buffer using a Bio-Rad electrophoresis chamber (100 V, 30–45 min). DNA ladder (MA02 100 bp, PB-L) was used as a molecular size marker. Amplicons were visualized using a UVP EC3 Imaging System. PCR amplifications were carried out on a Bio-Rad C1000 thermal cycler. *E. coli* ATCC 25922 was used as negative control for detection of antibiotic resistance genes. PCR reactions designed in this study were validated with reference strains *Klebsiella pneumoniae* ATCC BAA-1705 for bla_{KPC}, *E. coli* M1857 (Servicio Antimicrobianos INEI-ANLIS Malbrán reference strains collection) for bla_{PER}, *E. coli* M1890 (Servicio Antimicrobianos INEI-ANLIS Malbrán reference strains collection) for $bla_{CTX\text{-}M}$.

2.7 Whole genome sequencing, bioinformatic analysis and molecular typing

Genomic DNA from carbapenemase-producing strains was extracted using a column-based purification kit (PURO-Bacteria SA0701, PB-L) following manufacturer's instructions. DNA concentration was determined with a NanoDrop One spectrophotometer and DNA integrity was verified by electrophoresis on a 1% agarose gel in 1x TAE buffer. Purified DNA samples were subjected to WGS on the Illumina MiSeq platform (paired-end library, 150 pb insert size) at Novogene (United States). Raw sequence quality was assessed by FastQC (version 0.12.0; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low-quality reads were trimmed using Trimmomatic (default parameters, version 0.49) (Bolger et al., 2014). *De novo* genome assembly was performed with SPAdes (version 3.15.5) (Prjibelski et al., 2020). Genome assembly quality was evaluated for coverage and contamination using QUAST (version 5.22) (Mikheenko et al., 2018) and CheckM (version 1.2.3) (Parks et al., 2015) (Supplementary Table S4). Functional annotation was carried out with Bakta (version 1.8.1) (Schwengers et al., 2021). Species identity was confirmed by ribosomal multilocus sequence typing (rMLST) as described (Jolley et al., 2012) utilizing the online tool https://pubmlst.org/species-id. Clonal lineages were determined by multilocus sequence typing (MLST) (Jolley et al., 2018). Antimicrobial resistance genes were identified using AMRFinderPlus (Feldgarden et al., 2021). Virulence genes were detected with VFanalyzer (Liu et al., 2022) and protein BLAST (Camacho et al., 2009) against the Virulence Factor Database (Dong et al., 2024) with a cutoff value of ≥80% coverage and ≥60% identity and restricting reported genes to *Enterobacterales* and *Aeromonadaceae*. Mobile genetic elements were identified using ISfinder (Siguier et al., 2006). Plasmid analysis was performed with PlasmidFinder (version 2.0.1) (Carattoli et al., 2014), MOB-suite (Robertson and Nash, 2018) and Deeplasmid (Andreopoulos et al., 2022). To investigate the genetic context of carbapenemases, contigs containing carbapenemase-coding genes were extracted and used as query sequences for nucleotide BLAST searches against NCBI nr database (Sayers et al., 2025). Top hits where then retrieved to perform genome alignments, which were rendered using PyGenomeViz software (Shimoyama, 2024). Assembled genomes were deposited in NCBI database under accession numbers: JBMHEC000000000 (10Cfr), JBMHED000000000 (10Kmi), JBMHEE000000000 (31Ero), JBMHEF000000000 (34Eho), JBMHEG000000000 (1.4Eko), JBMHEH000000000 (4.5Aer).

2.8 Statistical analysis

Statistical analyses were performed using MedCalc 10.2.0.0 and GraphPad Prism 9.2.0. For comparisons of proportions, a two-tailed Chi-square test was applied. For medians, a two-tailed Mann–Whitney test was used. The specific method employed is indicated in the corresponding table or figure legend. Statistical significance was considered for $p < 0.05$.

3 Results

3.1 Coliform bacteria in urban wastewater overflows, Suquía River and Ansenuza National Park

Frequent overflows of sewage wastewater occur in the city of Córdoba, with most discharges entering the Suquía River. As it flows through the city, the Suquía River serves as both a collector and a potential long-distance vector for wastewaters and associated microorganisms. In this context, wastewater samples were gathered from multiple points throughout the city, and surface water samples were taken from several locations along the Suquía River, covering pre-urban and urban zones before and after the impact of the city's WWTP. To examine the potential remote effects, additional samples were collected from Ansenuza National Park, including the Suquía River estuary (Laguna del Plata) and other points within the Mar de Ansenuza lagoon (Supplementary Figure S1). To evaluate the presence of fecal bacteria, total coliforms, fecal coliforms, and *E. coli* were enumerated (Figure 1; Supplementary Table S1). All wastewater samples exhibited very high counts (>10^8 MPN/100 mL). In contrast, samples taken from the Suquía River upstream of Córdoba city displayed fecal coliform counts ranging 10–10^2 MPN/100 mL, several orders of magnitude lower than those found in the urban stretch of the river (10^2–10^6 MPN/100 mL). Further increases were detected close to the WWTP, with values staying high for at least 10 km downstream (Figure 1A; Supplementary Table S1). At the mouth of the Suquía River, the highest fecal coliform values (10^2–10^3 MPN/100 mL) were observed in the estuary of Laguna del Plata. These values decreased to 1–10 MPN/100 mL at the interface with the saline waters of the Mar de Ansenuza, while counts became undetectable further offshore (Figure 1B). To further investigate the impact of the city of Córdoba in the Suquía River, we performed a quantitative analysis grouping samples by location: "pre-Urban" (upstream of the city), "Urban" (within the city and up to just before the WWTP), and "Urban + WWTP" (from the WWTP to 10 km downstream). Coliform counts exhibited statistically significant increases when comparing "Urban" or "Urban + WWTP" samples to "pre-Urban"

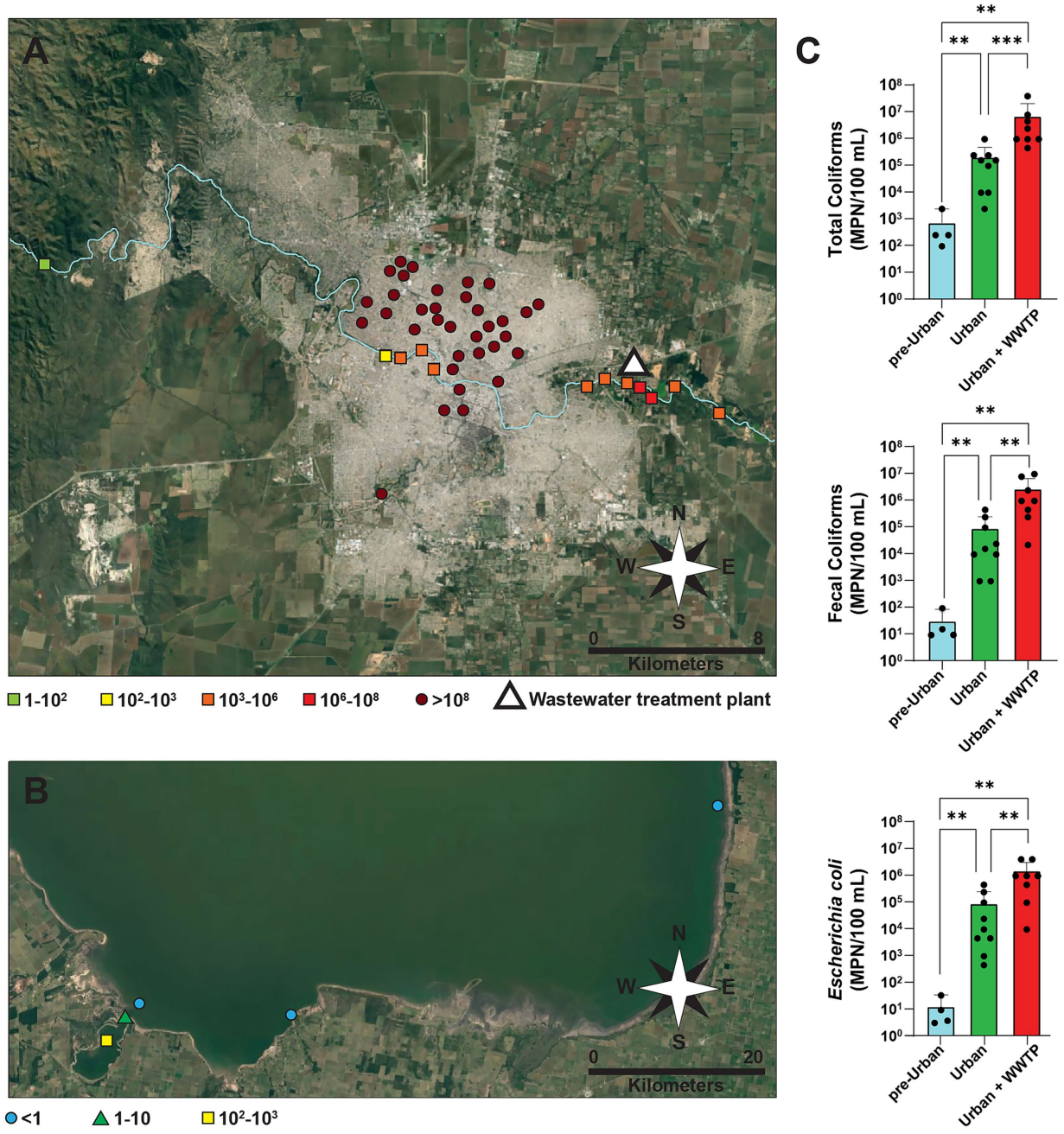

FIGURE 1
Enumeration of coliform bacteria in wastewater, surface water from the Suquía River and Ansenuza National Park sampling points. **(A)** Satellite image of Córdoba city showing wastewater overflows samples collected from public streets (circles) and surface water samples from the Suquía River (squares). The river flows from west to east. The Bajo Grande wastewater treatment plant (WWTP) is represented by a white triangle. Fecal coliform counts expressed as the most probable number per 100 mL (MPN/100 mL) are color-coded, as specified. **(B)** Satellite image of Ansenuza National Park showing surface water samples collected from the estuary of the Suquía River (Laguna del Plata) near its mouth (square), the intersection between Laguna del Plata and Mar de Ansenuza (triangle) and different locations in Ansenuza National Park (circles). Fecal coliform counts expressed as MPN/100 mL) are color-coded, as specified. **(C)** Total coliforms, fecal coliforms, and *E. coli* in surface water from the Suquía River of samples grouped into the following categories: Pre-Urban (the westernmost point, taken from the river before entering Córdoba city), Urban (seven points along the river within and exiting Córdoba city up to just before the WWTP), and Urban + WWTP (the four easternmost points, from WWTP to 10 km downstream). Columns represent the median (MPN/100 mL) and error bars correspond to the interquartile range. Black dots represent individual samples. Statistical significance was assessed using a two-tailed Mann–Whitney test, $^{**}p < 0.01$, $^{***}p < 0.001$.

samples (Figure 1C). Significant increases were also observed between "Urban + WWTP" to "Urban" samples.

These results indicate that the city of Córdoba contributes substantial quantities of coliform bacteria to the Suquía River, with increases of 2, 3, and 4–5 orders of magnitude for total coliforms, fecal coliforms, and *E. coli*, respectively.

3.2 ESBL- and carbapenemase-producing bacteria are present in high proportions in urban wastewater overflows and the urban stretch of the Suquía River

ESBL- and carbapenemase-producing *Enterobacterales* are classified as critical priority bacterial pathogens by the WHO (WHO, 2024), thus, their presence in water samples was investigated. Additionally, ESBL- and carbapenemase-producing *Aeromonas* spp. were studied, as these bacteria not only exhibit pathogenic potential but also, as part of aquatic microbial communities, may serve as environmental reservoirs for antibiotic resistance genes (Jones et al., 2023). A detailed list of all 65 ESBL- and/or carbapenemase-producing bacteria isolated in this study is provided in Supplementary Table S2. A high proportion of wastewater overflows collected from public streets (71%) and surface water from the Suquía River (68%) contained bacteria producing ESBL and/or carbapenemases (Figure 2A). In wastewater overflows, 62% of samples contained ESBL-producing bacteria, 6% had both ESBL- and carbapenemase-producing bacteria, and 3% contained carbapenemase producers. In surface water from the Suquía River, ESBL-producing bacteria were detected in 59% of samples, while ESBL- and carbapenemase-coproducers were identified in 9% of samples. In clear contrast, these bacteria were not detected from surface waters of the Mar de Ansenuza or from the Suquía River upstream of the city. The main genetic determinant of ESBLs was $bla_{CTX\text{-}M}$ (Figure 2A). Among carbapenemase-producing strains, five carried bla_{KPC} and one harbored bla_{NDM}. Regarding the distribution of species expressing these resistant phenotypes, ESBL-producing *E. coli* predominated (66% in wastewater and 62% in the Suquía River, respectively) (Figure 2B).

To characterize accompanying resistance not conferred by β-lactamase production, susceptibility to a variety of non-β-lactam antibiotics was determined. The two main accompanying resistances observed were to ciprofloxacin and trimethoprim-sulfamethoxazole (Figure 2C). Analysis of resistance combinations (Figure 2D) revealed 12 different profiles. Among wastewater isolates, out of 11 profiles, the top four -accounting for 54%- were: ciprofloxacin (18%), ciprofloxacin plus trimethoprim-sulfamethoxazole (18%), ciprofloxacin plus gentamicin (10%), ciprofloxacin plus trimethoprim-sulfamethoxazole plus gentamicin (8%). For Suquía River isolates, out of seven profiles, the top four -representing 75%- were: trimethoprim-sulfamethoxazole (26%), ciprofloxacin plus trimethoprim-sulfamethoxazole plus gentamicin (19%), ciprofloxacin (15%), and ciprofloxacin plus trimethoprim-sulfamethoxazole (15%). As shown in Figure 2E, 76% of wastewater and 89% of Suquía River isolates exhibited accompanying resistance. The most frequent profile was co-resistance to two or more non-β-lactam antibiotics, underscoring the multi-resistant phenotype of these strains.

KPC- and NDM-type carbapenemases confer resistance to last-resort antibiotics (Bush, 2013; Perez and Bonomo, 2019), making the isolation of environmental strains harboring these carbapenemases particularly relevant. We summarize the origin, species, MLST type, carbapenemase genes and resistance profile of the carbapenemase-producing strains isolated in the context of this study in Table 1. None of the carbapenemase-producing strains corresponded to *K. pneumoniae* -the main driver of carbapenemase dissemination in clinical settings (David et al., 2019)-, but to other *Enterobacterales* and, interestingly, *Aeromonas caviae* from the Suquía River. Notably, two of the three wastewater overflows yielding carbapenemase-producing isolates (samples AR30 and AR31, Supplementary Table S2) were collected near healthcare centers. The third one (sample AR34, Supplementary Table S2) was obtained from a large-scale sewage overflow near the urban course of the Suquía River (LaVoz, 2021). The two Suquía River surface water samples with carbapenemase-producing bacteria (RS1.4 and RS4.5, Supplementary Table S2) were collected 6 km and 10 km downstream from the WWTP. For MLST types (Table 1), the KPC-producer isolate *C. freundii* (10Cfr) was identified as the high-risk clone ST22 (Biez et al., 2023), while the rest of carbapenemase-producing *Enterobacterales* strains harbored sporadic MLST types. Interestingly, the KPC-producer *A. caviae* strain (4.5Aer) was assigned to ST2216, identified in this study. Regarding the susceptibility profiles, each strain presented a distinct resistance pattern, but all exhibited accompanying resistance to at least one non-β-lactam antibiotic. Remarkably, the NDM-producer was resistant to all 5 non-β-lactam antibiotics tested (Table 1). All KPC-producers were susceptible to ceftazidime-avibactam, as expected. Meropenem MICs ranged from 1 to >32 mg/L; the highest was observed in the NDM-producer *E. hormaechei* (34Eho), whereas KPC producers MICs ranged 1–4 mg/L.

These findings demonstrate that urban wastewater overflows from Córdoba, as well as surface water from the urban stretch of the Suquía River up to 10 km downstream of the city's WWTP, carry viable ESBL- and/or carbapenemase-producing *Enterobacterales*, underscoring the environmental dissemination of critical-priority resistant bacteria.

3.3 β-lactamase genes are horizontally transferred by ESBL- and carbapenemase-producing environmental strains in mating assays

We performed conjugation assays using all carbapenemase-producing and one representative ESBL-producing strain to evaluate their ability to horizontally transfer β-lactamase genes (Table 2). Successful carbapenemase transfer was achieved for three strains: KPC-producers *C. freundii* (10Cfr) and *K. michiganensis* (10Kmi), and NDM-producer *E. hormaechei* (34Eho), the latter also co-transferring gentamicin and amikacin resistance. For strains co-producing KPC and ESBLs, *E. roggenkampii* (31Ero) and *E. kobei* (1.4Eko), only the ESBL determinants (bla_{GES} and bla_{VEB}, respectively) were transferred. No transfer was detected for the KPC-producing *A. caviae* strain despite repeated attempts. Additionally, the $bla_{CTX\text{-}M}$ determinant from the representative ESBL-producing strain *K. pneumoniae* (18Kpn) was successfully transferred, with co-transfer of gentamicin and trimethoprim-sulfamethoxazole resistance.

These results indicate that ESBL and carbapenemase genes in *Enterobacterales* from wastewater overflows and the Suquía River are mainly encoded on transferable plasmids, highlighting their potential for horizontal dissemination.

3.4 Carbapenemase-producing isolates from urban wastewater and the Suquía River harbor extensive resistomes and virulomes

WGS analysis of all carbapenemase-producing strains ($n = 6$) revealed a broad repertoire of antibiotic resistance genes. All isolates

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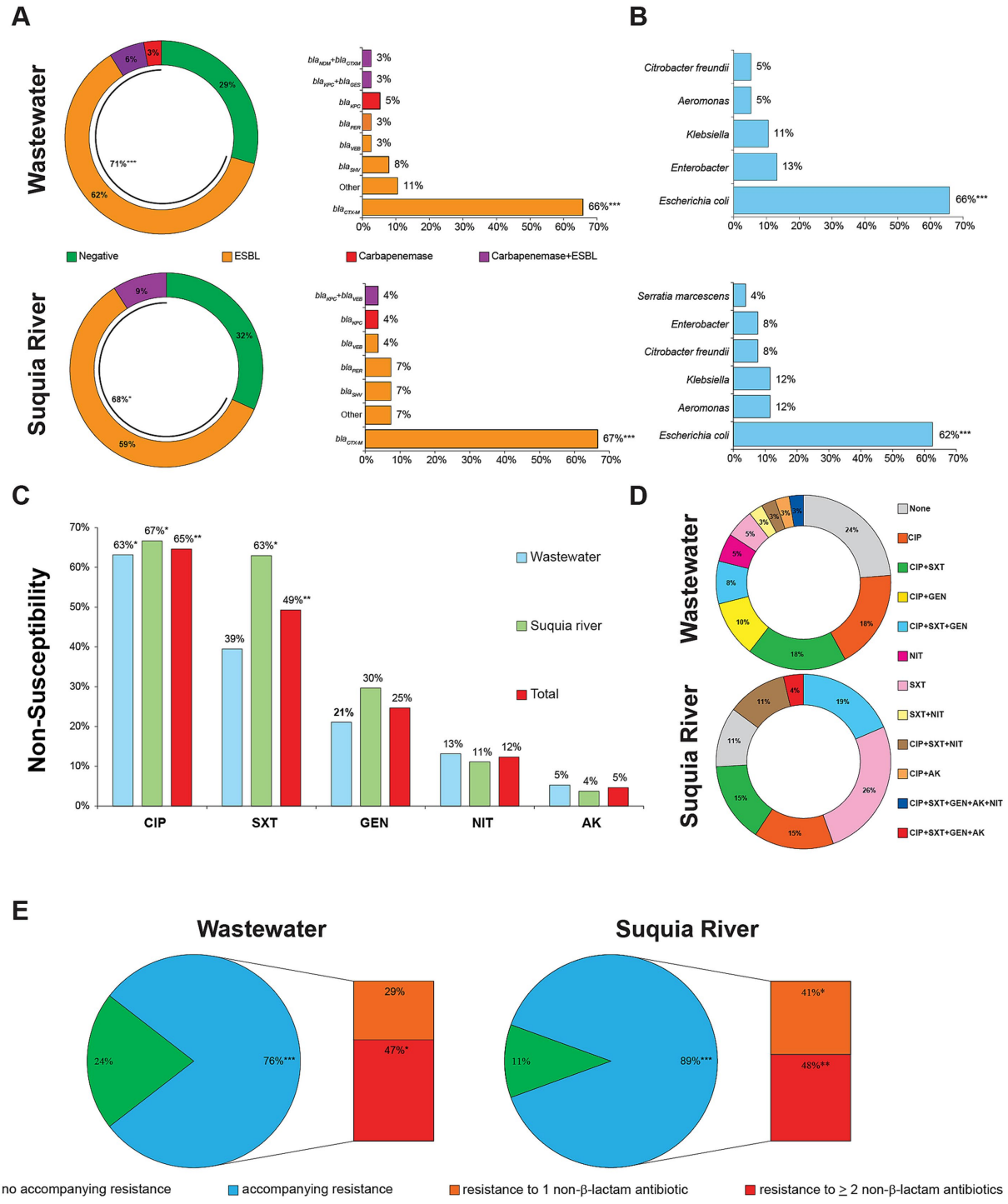

FIGURE 2
Antibiotic resistance profiles, genetic determinants and species distribution of bacteria harboring ESBLs and/or carbapenemases in wastewater overflows and surface water of the Suquía River. **(A)** Percent of wastewater overflows (n = 34) and Suquía River surface water (n = 22) samples containing ESBL and/or carbapenemase-producing bacteria and their ESBL- or carbapenemase-encoding genes. **(B)** Distribution of species harboring ESBL and/or carbapenemases in wastewater overflows (n = 38) and Suquía River surface water (n = 27). **(C)** Accompanying resistance to non-β-lactam antibiotics. CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; GEN, gentamicin; NIT, nitrofurantoin; AK, amikacin. **(D,E)** Combination and percentages of accompanying resistances to non-β-lactam antibiotics, respectively. Asterisks indicate statistical significance by Chi square test (*p < 0.05, **p < 0.01, ***p < 0.001).

carried genes conferring resistance to multiple antibiotics commonly used in clinical practice, consistent with their multidrug-resistant profiles (Figure 3; Supplementary Table S5). Notably, all strains harbored resistance determinants for at least four antibiotic classes, with resistance genes to β-lactams and aminoglycosides being the most numerous and diverse. Collectively, 53 genetic determinants conferring

TABLE 1 Carbapenemase-producing strains isolated from wastewater overflows (WW) and Suquía River (SR).

Strain (origin)	Species (carbapenemase)	MLST	MIC meropenem	Susceptibility to selected antibiotics					
				NIT	SXT	GEN	AK	CIP	CZA
10Cfr (WW)	*Citrobacter freundii* (bla_{KPC})	ST22	4 mg/L	S	R	S	S	R	S
10Kmi (WW)	*Klebsiella michiganensis* (bla_{KPC})	ST143	24 mg/L	S	S	I	S	I	S
31Ero (WW)	*Enterobacter roggenkampii* (bla_{KPC})	ST40	2 mg/L	S	S	R	R	R	S
34Eho (WW)	*Enterobacter hormaechei* (bla_{NDM})	ST121	>32 mg/L	R	R	R	R	R	R
1.4Eko (SR)	*Enterobacter kobei* (bla_{KPC})	ST691	1 mg/L	S	R	S	S	S	S
4.5Aer (SR)	*Aeromonas caviae* (bla_{KPC})	ST2216*	2 mg/L	S	S	S	S	R	S

Multilocus sequence types (MLST), minimum inhibitory concentration (MIC) to meropenem and susceptibility phenotypes to selected antibiotics are indicated. S, susceptible; I, intermediate; R, resistant; NIT, nitrofurantoin; SXT, trimethoprim-sulfamethoxazole; GEN, gentamicin; AK, amikacin; CIP, ciprofloxacin; CZA, ceftazidime-avibactam. *Newly identified sequence type.

TABLE 2 Transferability of carbapenemase and ESBL determinants in mating assays.

Donor strain ID (origin)	Donor strain species (determinant/s of interest)	Donor strain accompanying resistance	Transconjugant strain ID (determinant/s of interest transferred)	Transconjugant strain accompanying resistance
10Cfr (WW)	*Citrobacter freundii* (bla_{KPC})	CIP, SXT	! C10Cfr (bla_{KPC})	None
10Kmi (WW)	*Klebsiella michiganensis* (bla_{KPC})	CIP, GEN	! C10Kmi (bla_{KPC})	None
31Ero (WW)	*Enterobacter roggenkampii* (bla_{KPC}, bla_{GES})	GEN, AK	! C31Ero (bla_{GES})	None
34Eho (WW)	*Enterobacter hormaechei* (bla_{NDM}, bla_{CTXM})	CIP, GEN, AK, NIT, TMS	! C34Eho (bla_{NDM})	AK, GEN
1.4Eko (SR)	*Enterobacter kobei* (bla_{KPC}, bla_{VEB})	None	! C1.4Eko (bla_{VEB})	None
4.5Aer (SR)	*Aeromonas caviae* (bla_{KPC})	CIP	None obtained	Not applicable
18Kpn (WW)	*Klebsiella pneumoniae* (bla_{CTX-M})	CIP, GEN, SXT	! C18Kpn (bla_{CTX-M})	GEN, SXT

Mating assays were performed using carbapenemase- and ESBL-producing strains isolated from wastewater overflows (WW) and the Suquía River (SR) as donors. *E. coli* ER1793 was used as the recipient strain. Resistance determinants and accompanying resistance to non-β-lactam antibiotics of donor and transconjugant strains are indicated. CIP, ciprofloxacin; GEN, gentamicin; AK, amikacin, NIT, nitrofurantoin; SXT, trimethoprim-sulfamethoxazole.

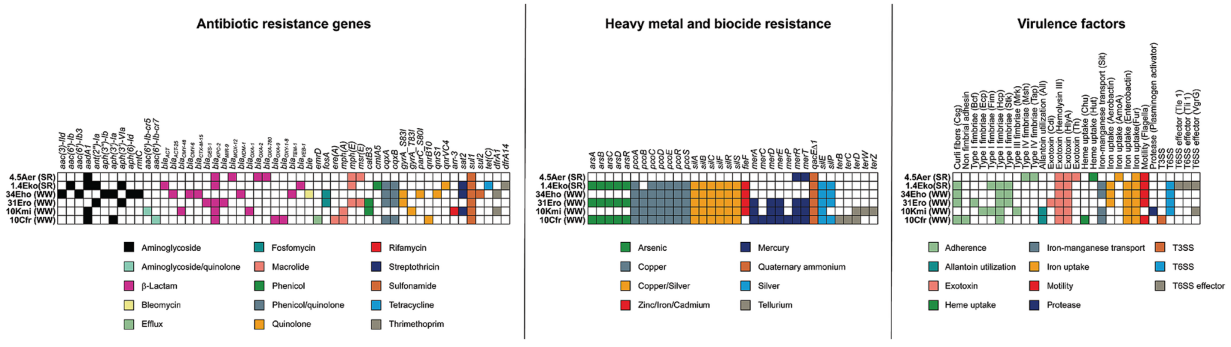

FIGURE 3
Antibiotic, heavy metals and biocide resistance genes and virulence determinants in carbapenemase-producing bacteria isolated from urban wastewater overflows (WW) and the Suquía River (SR). The presence of genes encoding antibiotic resistance (left panel), resistance to biocides and heavy metals (middle panel), and virulence factors (right panel) are indicated.

resistance to at least 12 different antibiotic classes were identified. Resistance gene counts varied among strains, ranging from 8 in KPC-producing *A. caviae* (4.5Aer) from the Suquía River to 22 in NDM-producing *E. hormaechei* (34Eho) from wastewater. Remarkably, all strains carried 3–5 β-lactamase genes. *K. michiganensis* (10Kmi) and *C. freundii* (10Cfr), recovered from a wastewater overflow near a healthcare center, carried bla_{KPC-2}. In addition to its intrinsic β-lactamase bla_{KOXY}, *K. michiganensis* (10Kmi) harbored the carbenicillinase bla_{OXA-9}. Meanwhile, *C. freundii* (10Cfr) possessed its intrinsic cephalosporinase bla_{CMY-48} and the carbenicillinase bla_{OXA-1}. *E. roggenkampii* (31Ero), isolated from wastewater near another healthcare center, also carried the bla_{KPC-2} carbapenemase. Alongside its natural cephalosporinase bla_{MIR-9}, this isolate presented bla_{GES-1}, a relatively rare ESBL. Regarding *E. hormaechei* (34Eho), recovered from wastewater overflow near the Suquía River, in addition to the bla_{NDM-1} carbapenemase, it harbored its intrinsic cephalosporinase bla_{ACT}, as well as three additional β-lactamases: bla_{CMY-6}, bla_{TEM-1} and the ESBL $bla_{CTX-M-15}$. Carbapenemase-producing strains recovered from the Suquía River, *E. kobei* (1.4Eko) and *A. caviae* (4.5Aer), both carried bla_{KPC-2} in addition to their species-specific AmpC cephalosporinases

(*bla*$_{ACT}$ and *bla*$_{MOX}$, respectively). *E. kobei* (1.4Eko) also carried *bla*$_{VEB-1}$ (an infrequent ESBL) and *A. caviae* (4.5Aer) harbored *bla*$_{OXA-2}$ and *bla*$_{OXA-780}$ (narrow-spectrum class D carbenicillinases). The detection of a *bla*$_{KPC-2}$-producing *A. caviae* isolate in the Suquía River was particularly interesting, as this species is a natural inhabitant of aquatic microbial communities (Fernandez-Bravo and Figueras, 2020) and this isolate represents, to our knowledge, the first report of a KPC-producing *A. caviae* strain from a river in Argentina. In summary, all KPC-producing strains carried *bla*$_{KPC-2}$, while the NDM-producing strain carried *bla*$_{NDM-1}$. Importantly, both carbapenemases are widely disseminated in clinical settings in Argentina and globally (Hammoudi Halat and Ayoub Moubareck, 2020). Also, all strains encoded narrow-spectrum β-lactamases (*bla*$_{TEM-1}$, *bla*$_{OXA-1}$, *bla*$_{OXA-2}$, *bla*$_{OXA-9}$, and/or *bla*$_{OXA-780}$), with three also carrying ESBLs (*bla*$_{GES-1}$, *bla*$_{VEB-1}$, or *bla*$_{CTX-M-15}$). Aminoglycoside resistance profiles varied: *A. caviae* (4.5Aer) contained only *aadA1*, while others had multiple aminoglycoside resistance genes (nucleotidyl/acetyl/phosphotransferases). Notably, the NDM-producing strain *E. hormaechei* (34Eho) contained six aminoglycoside resistance determinants, including the 16S methyltransferase *rmtC*.

Biocides, heavy metal resistance and virulence genes were analyzed (Figure 3; Supplementary Table S5), since they are important for bacterial survival in clinical and environmental settings. All carbapenemase-producing isolates carried *qacEΔ1* (encoding an efflux pump for quaternary ammonium disinfectants). Except for *A. caviae* (4.5Aer), all strains also carried the *pco* and *sil* systems (copper and silver resistance, respectively). Resistance determinants for arsenic, mercury, tellurium and zinc/iron/cadmium exhibited strain-specific variability. All isolates encoded virulence factors across at least five functional categories, including adhesins, exotoxins, and iron uptake systems. The Type VI secretion system (T6SS) was found in *Enterobacter* strains (1.4Eko, 34Eho, 31Ero) and *K. michiganensis* (10Kmi), which lacked flagellar genes. Surprisingly, the KPC-producing high-risk clone *C. freundii* ST22 (10Cfr) encoded a Type III secretion system with structural genes and the effector *ipaC/sipC*.

Overall, these findings demonstrate that carbapenemase-producing strains isolated from urban wastewater overflows and Suquía River surface water harbor extensive genetic repertoires for antibiotic resistance and virulence, underscoring their multidrug-resistant profiles and pathogenic potential.

3.5 Carbapenemase-producing isolates encode multiple plasmid replicons

Plasmid replicon analysis revealed that all carbapenemase-producing strains harbored multiple plasmid types, ranging from 2 in *A. caviae* (4.5Aer) to 7 in *E. kobei* (1.4Eko) (Supplementary Table S6). The Col440I replicon was present in all strains except *K. michiganensis* (10Kmi), indicating its widespread distribution. The IncL/M replicon, identified in *C. freundii* (10Cfr), *K. michiganensis* (10Kmi) and *E. roggenkampii* (31Ero), represented the second most broadly distributed plasmid type. Importantly, the IncU replicon identified in *E. kobei* (1.4Eko) was located on the contig encoding *bla*$_{KPC-2}$, while the IncA/C2 replicon in *E. hormaechei* (34Eho) was found within the contig carrying *bla*$_{NDM-1}$, indicating the plasmid-mediated dissemination of these resistance genes.

These results demonstrate that carbapenemase-producing strains isolated from urban wastewater overflows and the Suquía River possess a variety of plasmid types, including those encoding carbapenemases, underscoring their capacity for horizontal transfer of critically important antibiotic resistance.

3.6 Genetic context of carbapenemase genes in environmental strains include clinically recognized arrangements

Detailed analysis of the sequences surrounding *bla*$_{KPC-2}$ in strains recovered from wastewater overflows and the Suquía River (Figure 4A), showed that *K. michiganensis* (10Kmi), *E. roggenkampii* (31Ero) and *A. caviae* (4.5Aer) carry the same transposon. This transposon is characterized by flanking insertion sequences *ISKpn27* and *ΔISKpn6*, with *Δbla*$_{TEM-1}$ upstream of *bla*$_{KPC-2}$. This genetic arrangement, *ΔISKpn6-Δbla*$_{TEM-1}$*-bla*$_{KPC-2}$*-ISKpn27*, has been reported in clinical KPC-producing *Enterobacterales* in Argentina and other South American countries and is referred to as the "Non-Tn4401 variant 1a" (Gomez et al., 2011; Reyes et al., 2020). Interestingly, BLAST analysis shows that this genetic context has also been detected in an *Enterobacter asburiae* isolate from raw sewage in Argentina [strain WW-19C, CP080110.1 (Ghiglione et al., 2021)]. In contrast, *E. kobei* (1.4Eko) lacks *Δbla*$_{TEM-1}$ and *ΔISKpn27* upstream of *bla*$_{KPC-2}$, suggesting a native variant. For *C. freundii* (10Cfr), a complete characterization of the genetic context was not possible due to insufficient sequence coverage upstream of *bla*$_{KPC-2}$. The *bla*$_{NDM-1}$ gene in *E. hormaechei* (34Eho) resides within the ARI-A resistance island (Figure 4B), featuring a complex hybrid transposon with *ΔISAba125*, *ISKpn14*, *ISEcp1*, and a class I integron upstream, as previously described on IncC plasmids from clinical NDM-producing *Enterobacterales* in Argentina (Weber et al., 2019).

These findings indicate that carbapenemase genes in strains from wastewater overflows and the Suquía River are mobilized by transposon elements, including clinically recognized genetic arrangements.

3.7 Plasmid localization and genetic structure analysis of carbapenemase-harboring contigs

Since the *bla*$_{KPC}$ and *bla*$_{NDM}$ carbapenemase genes are predominantly plasmid-borne (Bush, 2018), we employed the deep learning-based bioinformatic tool Deeplasmid to predict their plasmid localization. This tool has been demonstrated to accurately distinguish plasmid sequences from bacterial chromosomal DNA (Andreopoulos et al., 2022). As shown in Supplementary Table S7, Deeplasmid analysis predicted plasmid localization with high-confidence scores (approaching 1.0) for carbapenemase-bearing contigs. *C. freundii* (10Cfr) was excluded from this analysis because its contig consisted almost entirely of the transposable element carrying *bla*$_{KPC-2}$, precluding reliable plasmid prediction. Nevertheless, conjugation assays confirm that *bla*$_{KPC-2}$ is plasmid-encoded in this strain.

To further characterize the genetic architecture of these resistance determinants, we performed BLASTn-based comparative analyses of the carbapenemase-containing contigs. Analysis of strains from wastewater

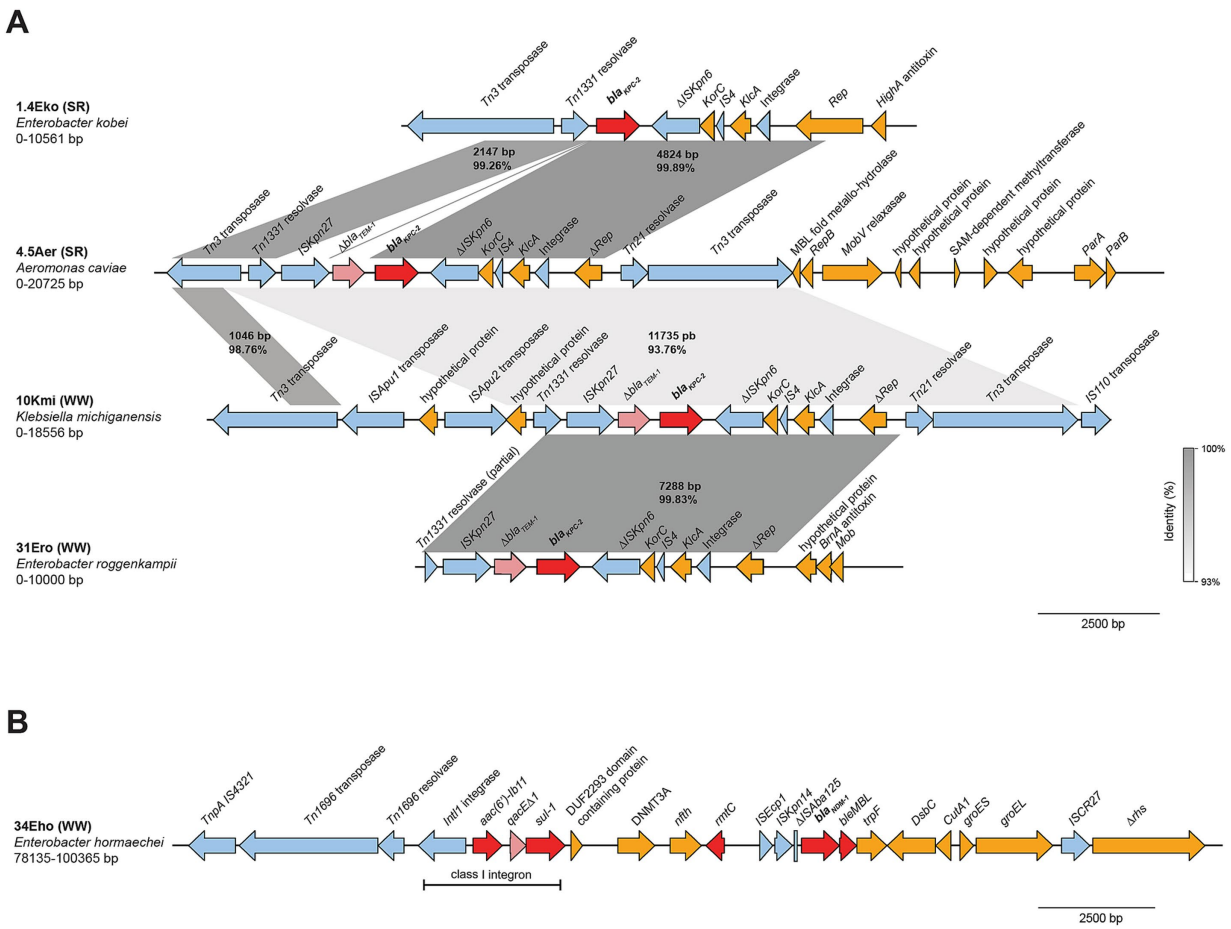

FIGURE 4
Genetic context of carbapenemases identified in strains isolated from wastewater overflows (WW) and Suquía River (SR). Linear arrangements of DNA sequences corresponding to the genetic context of bla_{KPC-2} **(A)** and bla_{NDM-1} **(B)** are shown. Gene annotations are depicted as colored arrows: antimicrobial resistance genes (red), truncated antimicrobial resistance genes (light red), insertion sequences, transposases, resolvases and integrases (light blue), all other genes (gold). Shaded boxes indicate the percent identities of aligned regions. Scale bars represent DNA size in base pairs (bp).

overflows (Figure 5A) showed *K. michiganensis* (10Kmi) and *E. roggenkampii* (31Ero) bla_{KPC-2} contigs shared >99.9% identity with IncP6 plasmids pCRE-KPC (MH919378.1) (Dong et al., 2020) and pKPC2_045096 (CP028566.1), respectively, from clinical and environmental strains in China. While both 10Kmi and 31Ero strains, shared high sequence identity and coverage with IncP6 plasmids from *Aeromonas* (data not shown), this replicon was not detected in the mentioned strains. The bla_{NDM-1} contig in *E. hormaechei* (34Eho) included multiple resistance genes and matched the IncA/C2 conjugative plasmid pCFR17394 (MH995506.1) from a clinical *C. freundii* previously isolated in Argentina (Martino et al., 2019). For Suquía River strains (Figure 5B), the top BLAST hit for bla_{KPC-2} contig *of E. kobei* (1.4Eko) corresponded to a clinical *Serratia marcescens* plasmid pKPC-2-HENAN1602 from China (CP047392.1), with an upstream IncU replicon matching a small *A. caviae* plasmid pAeca3 (CP039626.1) lacking antibiotic resistance genes and identified from wastewater also in China. Lastly, analysis of the bla_{KPC-2} contig of *A. caviae* (4.5Aer) revealed a 12,771 bp fragment sharing over 99% identity to an IncL/M-type plasmid from a U. S. clinical *K. pneumoniae* ST307 isolate (CP137926.1). Similarly to 1.4Eko, immediately upstream of this region, the top hit corresponded to a small *A. hydrophila* plasmid (CP180566.1) lacking resistance genes. These genetic arrangements combining clinical and environmental elements, are consistent with integration of carbapenemase genes into native plasmids via genetic exchange in the environment.

Together, these analyses indicate that carbapenemase genes in these environmental isolates reside on plasmids, including genetic structures consistent with combined clinical and environmental sequences in strains from the Suquía River.

4 Discussion

This study provides new insights into the environmental dissemination of AMR by characterizing ESBL- and carbapenemase-producing *Enterobacterales* and *Aeromonas* in urban wastewater overflows and Suquía River surface waters in Córdoba, Argentina. Results show that wastewater overflows release high levels of coliforms and *E. coli*. Interestingly, this observation coincides with increased coliform counts along the river's urban stretch. Further rises were found near the city WWTP, a relatively common occurrence in low-resource settings due to overloads and/or infrastructure issues (Wang et al., 2022; Lee et al., 2023). Thus, our findings strongly suggest that wastewater overflows introduce coliform bacteria into the river, likely via the stormwater system, representing an entry point of

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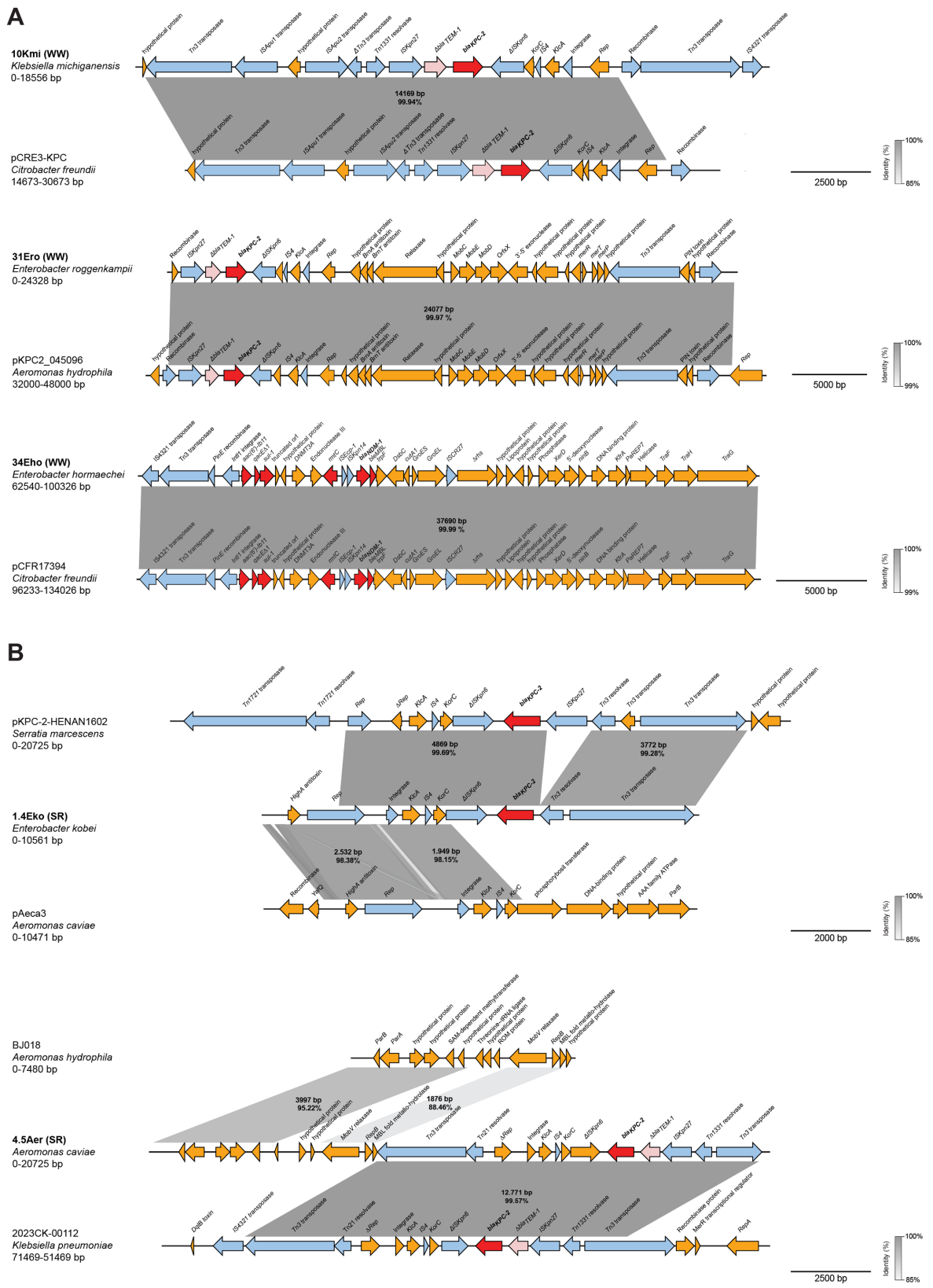

FIGURE 5
Genetic structure and comparative analysis of carbapenemase genes-harboring contigs. Linear arrangements of contigs harboring carbapenemase genes identified in strains isolated from **(A)** wastewater overflows (WW) and **(B)** Suquía River (SR) aligned with highly homologous sequences are shown. Gene annotations are depicted as colored arrows: antimicrobial resistance genes (red), truncated antimicrobial resistance genes (light red), insertion sequences, transposases, resolvases and integrases (light blue), all other genes (gold). Shaded boxes indicate the percent identities of aligned regions. Scale bars represent DNA size in base pairs (bp).

contamination with antibiotic-resistant bacteria. Persistence of high coliform levels up to 10 km downstream of the WWTP support the idea that these bacteria can remain viable during river transport, creating an extended contamination zone. Although counts declined near the river's mouth and approached detection limits in the hypersaline Mar de Ansenuza lagoon, the observed distribution underscores the potential for long-distance dissemination of AMR determinants through interconnected aquatic environments.

Bacteria resistant to critically important antibiotics were detected in ~70% of urban wastewater overflows and Suquía River samples. ESBL production (~60%) was most common, mainly linked to *E. coli* carrying $bla_{CTX\text{-}M}$, while carbapenemases (~9%) occurred in *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Aeromonas* via bla_{KPC} or bla_{NDM}. These findings are in line with international studies, although prevalence varies widely likely due to methodological and geographic differences: 24.8% ESBL-producing *Enterobacterales* in hospital and municipal wastewater in a meta-analysis (Zaatout et al., 2021); 95% in municipal wastewater (Gomez-Sanz et al., 2023) and 36.2% in rivers/lakes in Switzerland (Zurfluh et al., 2013); 98% in polluted rivers in Ghana (Banu et al., 2021). Similarly, carbapenemase-producers have been detected with varied prevalence: carbapenemase-producing *Enterobacterales* were found in all municipal WWTP influent and nearby waters in a U.S. study (Mollenkopf et al., 2025), in 33% hospital wastewater in Iran (Khavandi et al., 2024) and in 68.5% of Nairobi River in Kenya (Too et al., 2024). In addition, both ESBL- and carbapenemase-producers are widespread in China's Tuojiang and Yangtze rivers (Zhang et al., 2020). Regarding the predominance of $bla_{CTX\text{-}M}$ and the presence of bla_{KPC} and bla_{NDM} in our study, it aligns with their known global dissemination (Bevan et al., 2017; Wu et al., 2019; Zou et al., 2019; Bush and Bradford, 2020; Gomi et al., 2022; Teixeira et al., 2022; Wasko et al., 2022; Kotzamanidis et al., 2024; Monge-Olivares et al., 2025).

Although metagenomic approaches provide greater sensitivity (Knight et al., 2024), our culture-based methodology enabled phenotypic characterization, identification of resistance genes and detailed molecular analysis within specific strains. In this regard, ESBL- and carbapenemase-producing strains from wastewater overflows and the Suquía River exhibited multidrug-resistant profiles, frequently including co-resistance to ciprofloxacin and trimethoprim-sulfamethoxazole, resembling clinical isolates. Comparable findings have been reported elsewhere: in Colombia, 63.2% *E. coli* isolates were multidrug-resistant (Aristizabal-Hoyos et al., 2019), while in Ireland, up to 53.98% of fecal coliforms exhibited multidrug-resistance, with ciprofloxacin and trimethoprim-sulfamethoxazole among the most frequent co-resistances (Smyth et al., 2020). The identification of ESBL- and carbapenemase-producing *Aeromonas* species in our study is in agreement with their emerging role as environmental reservoirs of clinically relevant resistance genes (Sekizuka et al., 2019; Drk et al., 2023). Notably, these isolates were detected at river sites with high coliform loads and presence of multidrug-resistant bacteria. While a direct link is uncertain, wastewater-impacted riverine environments could facilitate genetic exchange among bacteria that rarely coexist naturally, including *Aeromonas* species and coliform bacteria.

A remarkable finding of our study is that multidrug-resistant bacteria were detected only in the urban stretch of the Suquía River, underscoring Córdoba's anthropogenic impact. Antibiotic-resistant species found in the river closely matched those from wastewater overflows, supporting their role as a source. Interestingly, our finding of ciprofloxacin as the most frequent co-resistance in ESBL producers, is consistent with the reported presence of subinhibitory levels of cephalosporins and fluoroquinolones in the Suquía River (Valdes et al., 2021). It has been shown that sustained low levels of antibiotics in the environment can facilitate selection of resistance (Chukwu et al., 2023). In this context, wastewater overflows may act as vectors of coliform bacteria, antibiotic-resistant bacteria of critical importance (ESBL- and carbapenemase-producing *Enterobacterales*), and likely also subinhibitory concentrations of antibiotics into the river via the stormwater system. This aligns with other studies identifying stormwater systems as pathways for anthropogenic contamination of rivers (Eramo et al., 2017; Almakki et al., 2019). The complex mixture transported by these waters likely creates microenvironments that facilitate microbial interactions, gene exchange and selection of antibiotic resistance.

We used WGS to characterize all carbapenemase-producing bacteria from wastewater and the Suquía River, revealing ample resistomes. All KPC-producing strains carried $bla_{KPC\text{-}2}$, while the NDM-producer carried $bla_{NDM\text{-}1}$, alleles widespread in clinical strains globally (David et al., 2019; Estabrook et al., 2023; Faccone et al., 2023). Interestingly, the KPC-producing *A. caviae* strain (4.5Aer) from the Suquía River carried only 8 resistance genes and exhibited a novel MLST profile, consistent with an environmental autochthonous origin. Even though in most strains the $bla_{KPC\text{-}2}$ gene was found within the clinically-recognized "Non-Tn4401 variant 1a" transposon (Gomez et al., 2011; Reyes et al., 2020), the unique arrangement found for this gene in *E. kobei* (1.4Eko) from the Suquía River supports the existence of distinct environmental variants. Overall, WGS analysis provides molecular confirmation that carbapenemase-producing bacteria carrying mobile genetic elements identical to those found in clinical strains, are present in wastewater overflows discharged into public areas of Córdoba and in the Suquía River influenced by this discharge.

Regarding the virulome, these isolates carry virulence genes like those in clinical strains (Morgado et al., 2024; Rahmat Ullah et al., 2024), indicating significant virulent potential. Notably, Type III secretion system genes were found in a KPC-producing *C. freundii* wastewater strain (10Cfr) of high-risk clone ST22, a lineage already associated with carbapenemase production, environmental reservoirs, and hospital outbreaks (Jolivet et al., 2021; Riccobono et al., 2023).

Bioinformatic analyses showed all carbapenemase-producing strains carry multiple plasmids, with carbapenemase genes predicted to be plasmid-borne. In two cases, the carbapenemase gene was on the same contig as a plasmid replicon: $bla_{KPC\text{-}2}$ with IncU in *E. kobei* (1.4Eko) from the Suquía River, and $bla_{NDM\text{-}1}$ with IncA/C2 in *E. hormaechei* (34Eho) from wastewater. IncU plasmids, which have broad host range and low copy number and are proposed to form a single incompatibility group with IncP6 plasmids (Haines et al., 2006; Rozwandowicz et al., 2018), are rarely reported in KPC-producing strains but have been found in both clinical and environmental isolates (Rozwandowicz et al., 2018; Wu et al., 2021). IncA/C plasmids, first identified in the 1970s in multidrug-resistant fish pathogens (Aoki et al., 1971), efficiently spread resistance genes among *Enterobacterales* (Fricke et al., 2009; Lindsey et al., 2011). Recently, they have gained prominence as major vectors in the plasmid-mediated dissemination of $bla_{NDM\text{-}1}$ (Kopotsa et al., 2019). Consistently, the $bla_{NDM\text{-}1}$–carrying *E. hormaechei* (34Eho) transferred the carbapenemase in conjugation assays, and genomic analysis predicts $bla_{NDM\text{-}1}$ on a conjugative IncA/C2 megaplasmid (Martino et al., 2019).

One of the most significant findings of our study is the genetic structure analysis of carbapenemase-containing contigs, particularly in strains from the Suquía River. In both *E. kobei* (1.4Eko) and *A. caviae* (4.5Aer), the bla_{KPC-2} gene was located on plasmidic contigs combining clinically associated resistance elements with adjacent regions nearly identical to *Aeromonas* plasmids lacking antibiotic resistance genes. This genetic architecture suggests that resistance elements typically associated with clinical settings have become incorporated into environmental plasmids, most likely as a result of gene exchange between antibiotic-resistant coliforms of anthropogenic origin and native river microbiota. Such events may enable the transfer and persistence of clinically significant resistance determinants in species well adapted to free-living survival in riverine habitats.

Among the limitations of our study, the sampling was restricted to various times and locations, which may not fully capture seasonal variations or the complete temporal dynamics of antimicrobial resistance in the studied environments. In addition, the use of culture-based methods likely underestimated the overall diversity of resistance genes compared to metagenomic approaches and excluded non-culturable bacteria. It is also important to note that our evidence for genetic exchange is indirect, inferred from genetic structures in which clinically recognized elements are adjacent to sequences highly similar to *Aeromonas* plasmids. Finally, as risk assessment was beyond the scope of this study, exposure risks for human populations to these environmental sources remain to be determined.

To our knowledge, this study provides the first report of pollution by ESBL- and carbapenemase-producing bacteria in urban wastewater overflows from public streets in a major Argentine city and constitutes one of the few such investigations in Latin America. The data show that sewage overflows in public areas of the city carry coliform bacteria resistant to critically important antibiotics, making them an AMR hotspot and a significant factor in the dissemination of multidrug-resistant bacteria across urban and peri-urban aquatic environments. This scenario is relevant to other cities, particularly in developing countries, where wastewater overflows are common. Thus, expanding research on environmental antimicrobial resistance is essential to inform effective control and prevention strategies. In an interconnected world, coordinated regional actions and a comprehensive, multisectoral One Health approach, along with increased awareness among healthcare professionals and the public, are key to preserving antibiotic efficacy amid the global antimicrobial resistance crisis.

Data availability statement

The datasets presented in this study can be found in online repositories. The repositories can be found at: https://www.ncbi.nlm.nih.gov/ (accession numbers: JBMHEC000000000, JBMHED000000000, JBMHEE000000000, JBMHEF000000000, JBMHEG000000000 and JBMHEH000000000).

Author contributions

SR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing. FM: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. MP: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. FL: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. MI: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. RT: Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing. MO: Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. CA: Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. MV: Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. FG: Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. MR: Conceptualization, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. VA: Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. CS: Conceptualization, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. HS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1669531/full#supplementary-material

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