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*Article*

# High Occurrence of Diverse ESBL-, AmpC- and Carbapenemase-Producing *Escherichia coli* and *Klebsiella pneumoniae* in Surface Waters, Southern Italy, 2023-2024

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**Abstract**

Antimicrobial resistance (AMR) is recognised as a major global public health threat, with the environment increasingly acknowledged as a key reservoir and dissemination pathway for resistant bacteria and resistance genes. In this study, 148 surface water samples were collected between 2023 and 2024 from six rivers and three canals discharging wastewater into two lake waters in southern Italy to assess the occurrence and genomic features of extended-spectrum β-lactamase (ESBL)-, AmpC- and carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae*. Relevant isolates were obtained using selective culturing, and tested for antimicrobial susceptibility by broth microdilution. Major β-lactam resistance genes were detected by Real-Time PCR. Whole-genome sequencing (WGS) was performed on presumptive carbapenemase-producing isolates. ESBL- and/or carbapenemase-producing Enterobacterales were detected in 67.6% of samples, yielding 176 non-duplicate isolates. The most prevalent gene was *bla*$_{\text{CTX-M}}$, detected in 79.3% of positive isolates (96/121), while carbapenemase genes were detected in 20.6% (25/121) of isolates, mainly *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{VIM}}$. WGS analysis revealed occurrence of clinically relevant high-risk clones, such as *K. pneumoniae* ST512/ST307 carrying *bla*$_{\text{KPC-3}}$ and *E. coli* ST10 harboring *bla*$_{\text{OXA-244}}$. These findings demonstrate widespread contamination of surface waters with clinically relevant resistant Enterobacterales and highlight the importance of integrating environmental compartments into One Health AMR surveillance frameworks.

**Keywords:** antimicrobial resistance; *Escherichia coli*; *Klebsiella pneumoniae*; ESBL; carbapenemases; surface water; WGS

## 1. Introduction

Antimicrobial resistance (AMR) has been recognised by the World Health Organization (WHO) as one of the major global health threats, driven by the rise of multidrug-resistant (MDR) bacteria and the progressive reduction of effective therapeutic options. Projections estimate that AMR could cause up to 10 million deaths annually by 2050 [1]. Among Enterobacterales, the predominant resistance

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mechanism to β-lactams is the production of enzymes capable of hydrolysing and inactivating such antimicrobials. More than 2,000 naturally occurring β-lactamase variants have been described to date, including clinically relevant families such as class A penicillinases, extended-Spectrum β-Lactamases (ESBLs; e.g., TEM, SHV, VEB, CTX-M), AmpC cephalosporinases (e.g., CMY, FOX, DHA, ACT, MOX), and carbapenemases belonging either to serine-β-lactamases (e.g., KPC, IMI, SME, GES) or metallo-β-lactamases (e.g., NDM, VIM, IMP) [2]. Environmental detection of antimicrobial-resistant bacteria (ARBs) has recently received increasing interest due to the potential health risk posed by bacteria associated with humans, livestock or wildlife at the interface with anthropogenic environments[3]. Historically, research has focused primarily on clinical, veterinary and food-producing animal settings. However, in the last decade, the environment has been increasingly recognised as playing a significant role in the development and dissemination of AMR [4]. The environment contributes to AMR through two major processes [4]. First, it serves as a vehicle for the dissemination of already resistant bacteria between humans, or between animals and humans. ARBs may be released into the environment through municipal wastewater [5], irrigation with reclaimed water [6], agricultural practices [7], and the application of treated sewage sludge as biosolids [8]. There is evidence that resistant bacteria can subsequently re-enter the human microbiome via environmental exposure [9], for example through ingestion of water contaminated by sewage during recreational activities [10], consumption of fresh produce irrigated with surface water [11,12], or more generally in settings with inadequate sanitation. Second, the environment acts as a reservoir and facilitator for the evolution of AMR. Surface waters can constitute resistance hotspots where antibiotic resistance genes (ARGs) disseminate—favoured by bacteriophages, integrons and other mobile genetic elements—and new resistant strains can be generated through horizontal gene transfer [13]. Although the presence of β-lactamase–encoding genes in bacteria isolated from surface waters and wastewater treatment plants has been reported in several geographical settings [14,15], robust and harmonised environmental surveillance data remain limited. In particular, no region-specific data are currently available for Apulia, a large region in Southern Italy characterised by high population mobility and seasonal tourist influx. This study aims to generate baseline evidence on the occurrence and distribution of ESBL-, AmpC- and carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* in surface waters of Apulia, thereby supporting environmental antimicrobial resistance surveillance, improving the identification of potential environmental exposure pathways, and contributing to public health risk assessment within a One Health framework.

## 2. Materials and Methods

### *2.1. Sampling Points*

Water samples were collected from six rivers and three canals conveying water into two lakes of the Apulia region between 2023 and 2024. The rivers included Candelaro (4 sampling points; 1A, 1B, 1C, 1D), Fortore (1 sampling point; 2A), Cervaro (3 sampling points; 3A, 3B, 3C), Carapelle (2 sampling points; 4A, 4B), Triolo (1 sampling point; 5A) and Ofanto (1 sampling point; 6A) rivers (Figure 1 and Table 1). Rivers were selected for sampling based on the annual mean E. coli concentrations recorded in 2021 at each monitoring site, as reported by ARPA Puglia (https://www.arpa.puglia.it/pagina2975_ii-ciclo-sessennale-2016-2021.html). Specifically, sampling sites with the highest average E. coli values were prioritised, given the role of E. coli as an indicator of faecal contamination in surface waters. In addition, sampling points were also chosen on the basis of their proximity to agricultural land observed using satellite imagery (Google, 2023). Sampling stations within the canals were strategically located along channels receiving wastewater discharges from adjacent settlements into the lake systems. Overall, a total of six sampling points was set for this study, three for Lake Lesina (7A, 7B and 7C) and three for Lake Varano (8A, 8B and 8C) (Figure 2 and Table 1). For Lesina lake, it was chosen to sample the waters of the Cammarata canal within which wastewater from the settlements of Lesina and Poggio Imperiale are conveyed. Of the three sampling points, two (7A, 7C) were placed along the Cammarata canal, 7A at the outlet of the wastewater

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treatment plant and 7C at the incile of the Cammarata canal into the lagoon, respectively. A third sampling point (7B) was fixed along the La Fara canal, which collects wastewater from an area close to the town of Lesina affected by various agricultural and livestock activities and discharges it into the lagoon (Figure 2). For Varano lake, it was chosen to sample the waters of the San Francesco canal within which wastewater from the town of Cagnano Varano is conveyed. The sampling point 8A is located at the outlet of the wastewater treatment plant, 8B in an area of the canal near which there is a livestock farm, and a third sampling point (8C) at the incile of the San Francesco canal in the lagoon (Figure 2). The analyzed canals constitute critical control points for the environmental quality of the lakes, as they play a pivotal role in maintaining the ecological equilibrium of the lagoon system and in safeguarding the safety and long-term sustainability of associated economic activities.

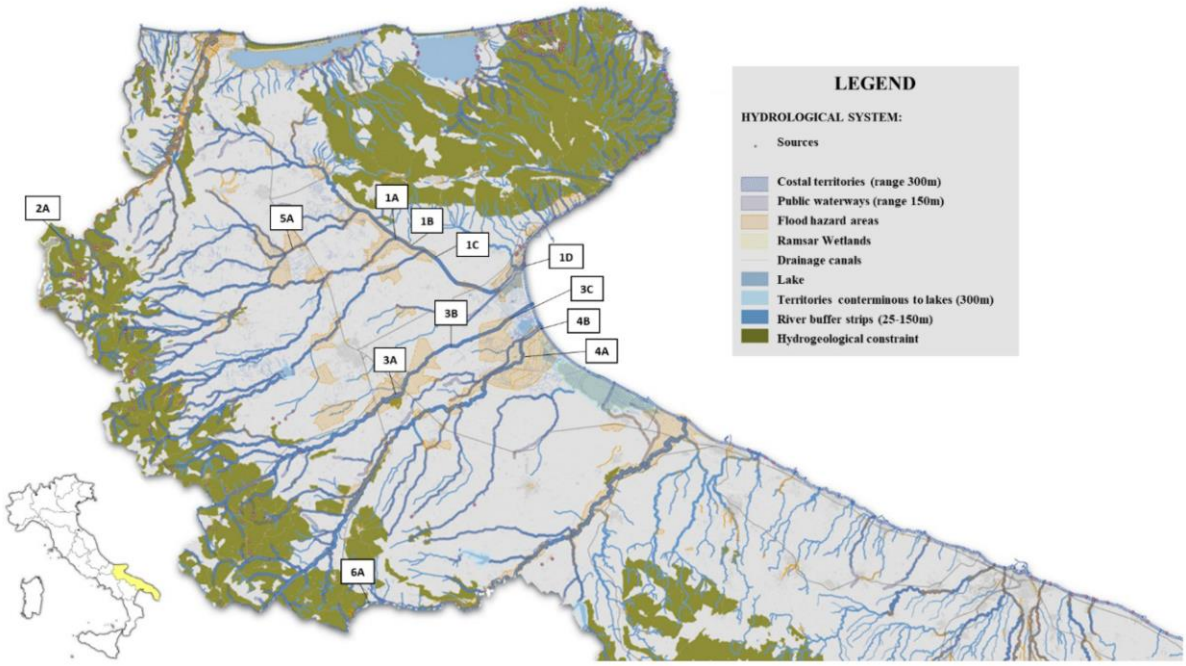


**Figure 1.** Map of rivers sampling points.

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**Figure 2.** Map of lakes sampling points.

**Table 1. Sampling points and geographical coordinates.**

| N. | Sampling points | Surface water bodies of Apulia Region | LAT (degrees, minutes, seconds-milliseconds) | LONG (degrees, minutes, seconds-milliseconds) | Annual average *E. coli* count (CFU/100 mL)[1] | Municipality |
|---|---|---|---|---|---|---|
| 1 | 1A | Candelaro River | 41°37' 34" N | 15°38' 7" E | 4715,8 | San Marco in Lamis |
| 2 | 1B | Candelaro River | 41°36' 36" N | 15°40' 4" E | 12374,8 | San Marco in Lamis |
| 3 | 1C | Candelaro River | 41°35' 58" N | 15°42' 18" E | 2240,0 | San Marco in Lamis |
| 4 | 1D | Candelaro stream | 41°34' 25" N | 15°53' 6" E | 1795,8 | Manfredonia |
| 5 | 2A | Fortore River | 41°38' 50" N | 15°2' 40" E | 505,6 | Casalnuovo Monterotaro |
| 6 | 3A | Cervaro River | 41°24' 4" N | 15°39' 8" E | 679,0 | Foggia |

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| | | | | | | |
|---|---|---|---|---|---|---|
| 7 | 3B | Cervaro River | 41°25' 37" N | 15°40' 4" E | 6228,8 | Foggia |
| 8 | 3C | Cervaro River- | 41°31' 17" N | 15°53' 55" E | 516,7 | Manfredonia |
| 9 | 4A | Carapelle Torrent | 41°23' 51" N | 15°48' 51" E | 245,1 | Cerignola |
| 10 | 4B | Carapelle Torrent | 41° 29' 26" N | 15°55' 14" E | 2420,8 | Zapponeta |
| 11 | 5A | Triolo Torrent | 41° 38' 51" N | 15°32' 44" E | 7749,2 | Rignano Garganico |
| 12 | 6A | Ofanto River | 41° 05' 29" N | 15° 34' 20" E | 1434,3 | Rocchetta S.Antonio |
| 13 | 7A | Cammarata canal (Lesina lake) | 41° 51' 34" N | 15°21' 27" E | data not available | Lesina |
| 14 | 7B | La Fara canal (Lesina lake) | 41° 51' 48" N | 15° 21' 24" E | data not available | Lesina |
| 15 | 7C | Cammarata canal (Lesina lake) | 41° 51' 54" N | 15°21'19" E | data not available | Lesina |
| 16 | 8A | San Francesco canal (Varano lake) | 41° 50 '17" N | 15°46' 32" E | data not available | Varano |
| 17 | 8B | San Francesco canal (Varano lake) | 41°50'34"N | 15°45'07" E | data not available | Varano |
| 18 | 8C | San Francesco canal (Varano lake) | 41°50'37" N | 15°46'25"E | data not available | Varano |

[1]·A limit of < 5,000 CFU/100 mL is recommended.

*2.2. Sampling Procedure*

From May 2023 to December 2024, a total of 148 surface water samples were collected from surface water bodies in the Apulia region, including six rivers and three canals conveying water into two lakes (Figure 1, Figure 2, Table 1). For each surface water body, a minimum of ten samples were collected and analysed (Table 1). On each sampling occasion, a volume of 500 mL was collected at each site directly by filling a sterile glass container with surface water. The samples were transferred under refrigerated conditions to the laboratories of the Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata (IZS PB, Foggia, Italy). The samples were stored at 4 °C and analysed within 24 h.

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*2.3. Microbiological Screening, Isolation and Identification of Target Bacteria*

The isolation procedure aimed to selectively detect resistant bacteria from the examined samples. For the isolation of target microorganisms, 100 mL of each water sample was filtered (0.45 µm; Sartorius Stedim Biotech GmbH, Goettingen, Germany). To select for ESBL-producing Enterobacterales, the filter was placed in 100 mL of Buffer Peptone Water (BPW) (Thermo Fisher Scientific, Rhone, MI) and incubated at 42 °C for 18-24 hours. Subsequently, 100 µL of each dilution (up to 10-3) was plated on CHROMagar ESBL plates (CHROMagar, Paris, France) and incubated at 42 °C for 18-24 hours. For the isolation of carbapenemase-producing Enterobacterales (CPE), the filter was placed in 100 mL of Mossel broth (EE Broth) (Scharlab S.L., Sentmenat, Spain) and the enrichment incubated at 42 °C for 18-24 hours. Subsequently, 100 µL of each dilution (up to 10 -2) was plated on CHROMagar mSuperCARBA plates (CHROMagar, Paris, France) and incubated at 42°C for 18-24 hours (Savin et al., 2020). After incubation, up to three presumptive colonies of E. coli and Klebsiella spp. per sample were subcultured onto Blood Agar plates (Liofilchem, Roseto degli Abruzzi, Italy), incubated at 37 °C for 24 hours and subjected to identification by MALDI-TOF mass spectrometry (Bruker Daltonics, Milan, IT). The isolates were cryopreserved in Microbank (PRO-LAB DIAGNOSTICS, Richmond Hill, ON, Canada) and stored at –80°C.

*2.4. Phenotypic Detection of Antimicrobial Resistance*

Phenotypic antimicrobial susceptibility was assessed using the broth microdilution method, following EUCAST guidelines (version 15.0; www.eucast.org) [16]. GN4F and EUVSEC2 panels (Thermo Fisher Scientific Diagnostics, Netherlands) were used. Plates were incubated at 37 °C for 24 h, and MICs were read using the Thermo Scientific™ Sensititre™ Vizion™ system. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 served as quality control strains. Results were interpreted according to epidemiological cut-off values (ECOFFs) provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) [16]. Multidrug resistance (MDR) was defined as non-susceptibility to at least one agent in three or more antimicrobial classes, according to Magiorakos et al. [17].

*2.5. Genotypic Detection of ESBL- and Carbapenemase-Encoding Genes and Characterization by Whole-Genome Sequencing*

Automated bacterial DNA extraction was performed on the Maxwell RSC Extraction System (Promega, USA) using the Maxwell RSC PureFood Pathogen kit (Promega, USA). The quantity (ng/µL) and purities (260/280 and 260/230 ratios) of the extracted DNA were measured spectrophotometrically using the IMPLEN NanoPhotometer N60 (Implen GmbH, München, Germany). DNA from all isolates was used for real-Time PCR using the Allplex EnteroDR Assay kit for the detection of *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{CTX-M}$ genes (Seegene Inc, Seoul, Republic of Korea). DNA from isolates that tested positive in PCR for carbapenemase genes was also characterised by whole-genome sequencing (WGS). Genomic DNA was sequenced using Illumina short-read technology on a NextSeq platform with 2 × 151 bp reads. Short-read assembly was performed with SPAdes v3.15.2. The quality of raw sequencing and assembly data was assessed using the analytical tool BIFROST (https://github.com/ssi-dk/bifrost/). For *K. pneumoniae* strains, species identification, multilocus sequence typing (MLST), resistance gene detection and plasmid replicon typing were performed using the Pathogenwatch platform (https://pathogen.watch). For *E. coli*, all bioinformatics analyses were performed on the public ARIES Galaxy server [18].

*2.6. Data Availability and Sequence Deposition*

Whole-genome sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1358062.

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## 3. Results

### *3.1. Selective Isolation of Presumptive ESBL- and Carbapenemase-Producing Enterobacterales from Surface Water*

A total of 148 water samples were cultured to isolate ESBL- and/or carbapenemase-producing Enterobacterales. All surface water sites tested positive, on at least one sampling occasion, for presumptive ESBL- and/or carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae*, as indicated by growth on selective media. Isolates were recovered on CHROMagar ESBL and/or mSuperCARBA selective media from 67.6% (100/148) of the samples, yielding 176 strains, one strain per sample: 57.4% (101/176) *E. coli* and 42.6% (75/176) *K. pneumoniae*.

### *3.2. Detection of ESBL- and Carbapenemase-Encoding Genes by Multiplex Real-Time PCR*

A total of 176 isolates (75 *K. pneumoniae* and 101 *E. coli*) were analysed by PCR, revealing that 31.2% (55/176) of isolates were negative for all tested genes (Supplementary data) and 68.8% (121/176) of isolates carried at least one of the investigated resistance genes.

The most prevalent resistance gene was *bla*$_{\text{CTX-M}}$, detected in 79.3% (96/121) of alone or in combination with carbapenemase-encoding genes. Carbapenemase genes were detected in 20.6% (25/121) of isolates, including ***bla***$_{\textbf{OXA-48}}$ (3.3%, 4/121), ***bla***$_{\textbf{KPC}}$ **(1.7%, 2/121),** ***bla***$_{\textbf{VIM}}$ (9.1%, 11/121), or in combination with other genes (*bla*$_{\text{CTX-M}}$ + *bla*$_{\text{KPC}}$, 2.5%, 3/121; *bla*$_{\text{CTX-M}}$ + *bla*$_{\text{VIM}}$, 2.5%, 3/121; *bla*$_{\text{CTX-M}}$ + *bla*$_{\text{OXA-48}}$ 1.7%, 2/121) (Table 1). Among *E. coli*, 30.7% (31/101) were negative for all antibiotic resistance genes tested by PCR (11 isolated from CHROMagar mSuperCARBA, 20 from CHROMAgar ESBL) (Supplementary data). Moreover, 80% (56/70) of *E. coli* isolates positive for resistance genes were recovered from CHROMagar ESBL, whereas the remaining 20% (14/70) were obtained from CHROMagar mSuperCARBA.

*bla*$_{\text{CTX-M}}$ was the most prevalent gene (84.3%, 59/70), mainly *bla*$_{\text{CTX-M}}$ alone (80%, 56/70), followed by combinations with *bla*$_{\text{VIM}}$ (1.4%, 1/70) and *bla*$_{\text{OXA-48}}$ (2.9%, 2/70). Carbapenemase genes were identified in 20% (14/70), including *bla*$_{\text{OXA-48}}$ (4.3%, 3/70), *bla*$_{\text{VIM}}$ (8.6%, 6/70), and combined patterns mentioned above. Among *K. pneumoniae*, 32% (24/75) were PCR negative (11 isolated from CHROMagar mSuperCARBA, 13 from CHROMAgar ESBL) (Supplementary data). *bla*$_{\text{CTX-M}}$ was again the most common determinant (88.2%, 45/51), either alone (78.4%, 40/51) or in combination with *bla*$_{\text{KPC}}$ (5.8%, 3/51) or or bla$_{\text{VIM}}$ (5.8%, 3/51). Carbapenemases were detected in 21.6% (11/51), including *bla*$_{\text{OXA-48}}$ (2%, 1/51), *bla*$_{\text{VIM}}$ (9.8%, 5/51), and combined profiles (Table 1).

### *3.3. Phenotypic and Genotypic Detection of Antimicrobial Resistance*

Antimicrobial susceptibility testing was performed on all 176 isolates (Table 2, Figure 3). Among the 31 *E. coli* strains that tested negative for all resistance genes by PCR, 19 isolates were susceptible to all antibiotics tested, 11 showed resistance to up to three antibiotic classes, and only one isolate was multidrug-resistant (Supplementary Data). For *K. pneumoniae*, 24 isolates tested negative in the PCR analysis, and their antimicrobial profiles are reported in the Supplementary Data. Overall, resistance patterns varied between *E. coli* and *K. pneumoniae*, with multidrug resistance observed in both species (Table 3 and Figure 3).

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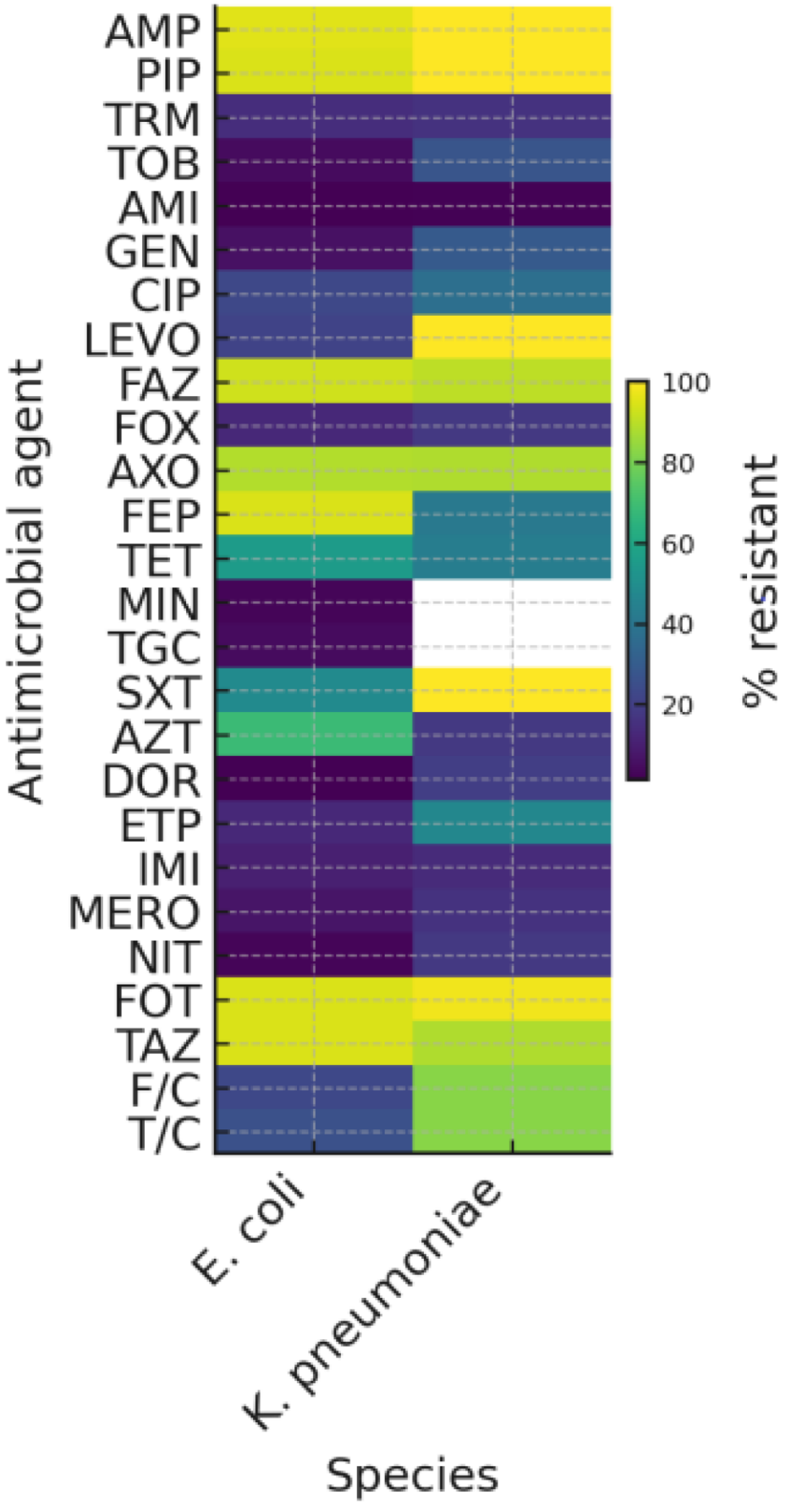


**Figure 3.** Heatmap of AMR profiles of Escherichia coli and K. pneumoniae isolated.

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**Table 2. Occurrence of ESBL- and carbapenemase-encoding genes in E. *coli* and *K. pneumoniae* isolated on selective agar plates.**

| Organism | No. of PCR-positive isolates | Resistance genes in Enterobacterales isolates | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | CRE* (25/121, 20.6%) | | | | | |
| | | *bla*CTX-M | *bla*OXA-48 | *bla*KPC | *bla*VIM | *bla*CTXM + *bla*KPC | *bla*CTX-M + *bla*VIM | *bla*CTX-M-+ *bla*OXA-48 |
| *K. pneumoniae* | 51/75 (68%) | 40/51 (78.4%) | 1/51 (2%) | - | 5/51 (9.8%) | 3/51 (5.9%) | 2/51 (3.9%) | - |
| *E. coli* | 70/101 (69.3%) | 56/70 (80%) | 3/70 (4.3%) | 2/70 (2.9%) | 6/70 (8.6%) | - | 1/70 (1.4%) | 2/70 (2.9%) |
| Total No. | 121/176 (68.8%) | 96/121 (79.3%) | 4/121 (3.3%) | 2/121 (1.7%) | 11/121 (9.1%) | 3/121 (2.5%) | 3/121 (2.5%) | 2/121 (1.7%) |

* CRE, carbapenem-resistant Enterobacterales.

**Table 3. Antimicrobial susceptibility profiles of presumptive ESBL- and carbapenemase-producing Enterobacterales isolates.**

| | Molecules[a] | No. of *E. coli* categorized as resistant (%) | No. of *K. pneumoniae* categorized as resistant (%) | MIC* (mg/L) *E. coli* | MIC* (mg/L) *K. pneumoniae* |
|---|---|---|---|---|---|
| Penicillins | AMP | 67/70 (95.71) | - | >8 | ** |
| | PIP | 67/70 (94.28) | - | >8 | ** |
| Penicillin derivatives*** | TRM | 10/70 (14.28) | 8/51 (15.68) | >16 | >8 |
| Aminoglycosides | TOB | 3/70 (4.28) | 14/51 (27.45) | >4 | >2 |
| | AMI | 1/70 (1.42) | 1/51 (1.96) | >8 | >8 |
| | GEN | 4/70 (5.71) | 15/51 (29.41) | >2 | >2 |
| Fluoroquinolones | CIP | 16/70 (22.85) | 19/51 (37.25) | >0.06 | >0.125 |
| | LEVO | 16/70 (21.42) | 11/51 (21.56) | >0.125 | >0.25 |
| First generation cephalosporins | FAZ | 65/70 (92.85) | 46/51 (90.19) | >4 | >4 |
| Second-generation cephalosporins | FOX | 9/70 (12.85) | 9/51 (17.64) | >1 | >8 |

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| | | | | | |
|---|---|---|---|---|---|
| **Third- and fourth-generation** | **AXO** | 62/70 (88.57) | 45/51 (88.23) | >2 | >2 |
| | **FEP** | 66/70 (94.28) | 21/51 (41.17) | >0.25 | >0.125 |
| **Tetracyclines** | **TET** | 39/70 (55.71) | 22/51 (43.13) | >8 | >8 |
| | **MIN** | 2/70 (2.85) | 8/51 (15.68) | >4 | >8 |
| | **TGC** | 3/70 (4.28) | 2/51 (3.92) | >0.5 | >2 |
| **Sulfonamides** | **SXT** | 34/70 (48.57) | 36/51 (70.58) | >4 | >4 |
| **Monobactam** | **AZT** | 48/70 (68.57) | 9/51 (17.64) | >0.25 | >4 |
| **Carbapenems** | **DOR** | 1/70 (1.42) | 10/51 (19.60) | >0.06 | >0.125 |
| | **ETP** | 9/70 (12.85) | 24/51 (47.05) | >0.06 | >0.03 |
| | **IMI** | 7/70 (10.00) | 7/51 (13.72) | >0.5 | >1 |
| | **MERO** | 7/70 (7.14) | 8/51 (15.68) | >0.06 | >0.125 |
| **Nitrofurans** | **NIT** | 2/70 (2.85) | - | >64 | ** |
| **ESBL confirmatory tests** | **FOT** | 67/70 (94.28) | 50/51 (98.03) | >0.25 | >0.25 |
| | **TAZ** | 67/70 (94.28) | 45/51 (88.23) | >1 | >1 |
| | **F/C** | 16/70 (22.85) | 42/51 (82.35) | >0.25 | >0.25 |
| | **T/C** | 18/70 (25.71) | 42/51 (82.35) | >1 | >0.5 |

[a] Abbreviations: AMP, ampicillin; PIP, piperacillin; TRM, temocillin; TOB, tobramycin; AMI, amikacin; GEN, gentamicin; CIP, ciprofloxacin; LEVO, levofloxacin; FAZ, cefazolin; FOX, cefoxitin; AXO, ceftriaxone; FEP, cefepime; TET, tetracycline; MIN, minocycline; TGC, tigecycline; SXT, trimethoprim-sulfamethoxazole; AZT, aztreonam; DOR, doripenem; ETP, ertapenem; IMI, imipenem; MERO, meropenem; NIT, nitrofurantoin. ESBL confirmatory tests: FOT, cefotaxime; TAZ, ceftazidime; F/C, cefotaxime-clavulanic acid; T/C, ceftazidime-clavulanic acid. * The MIC values reported in the table represent the epidemiological cut-off values (ECOFFs) used for categorisation, except for ESBL confirmatory tests, which were interpreted according to EUCAST phenotypic criteria. **For *K. pneumoniae*, no ECOFFs were applied for ampicillin, piperacillin and nitrofurantoin, as this species is considered intrinsically resistant or not a target organism according to EUCAST. *** Temocillin susceptibility was interpreted as an ESBL screening marker according to EUCAST recommendations.

### *3.4. Whole Genome Sequencing (WGS) of Isolates Positive for Carbapenemase-Encoding Genes by PCR*

All (25/121) of Enterobacterales PCR-positive for carbapenemase-encoding were subjected to WGS. Table 4 summarizes genomic data for the 25 isolates (56% (14/25) *E. coli* and 44% (11/25) *K. pneumoniae)*, including detected β-lactamase genes (carbapenemases, ESBL/AmpC), additional antimicrobial resistance (AMR) determinants, multilocus sequence types (MLST), and plasmid replicon types. WGS confirmed the presence of carbapenemase-encoding genes in 68% (17/25) of isolates, of which 58.8% (10/17) were *E. coli* and 41.2% (7/17) *K. pneumoniae*. Among *E. coli*, carbapenemase genes were *bla*$_{\text{OXA-244}}$ (4/10, 40%), followed by *bla*$_{\text{VIM-4}}$ (3/10, 30%), *bla*$_{\text{OXA-181}}$ (1/10, 10%)

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and *bla*KPC-3 (2/10, 20%). In *K. pneumoniae*, carbapenemase genes included *bla*KPC-3 (3/7, 42.85%) and *bla*VIM-1 (4/7, 57.14%). ESBL/AmpC genes were detected in 36.0% (9/25) of isolates, including five *E. coli* and four *K. pneumoniae*. The most frequently detected ESBL/AmpC genes were *bla*CTX-M-15, *bla*SHV-12, *bla*SHV-28, *bla*TEM-52B, and *bla*DHA-1. Multilocus sequence typing (MLST) revealed considerable diversity. High-risk clones such as *K. pneumoniae* ST512 and ST307 carrying *bla*KPC-3 and *E. coli* ST10 carrying *bla*OXA-244 were identified (Table 4). In addition to β-lactam resistance determinants, multiple acquired resistance genes were detected: 72% of isolates carried aminoglycoside-modifying enzymes (*aac*, *aph*), 64% harboured sulfonamide resistance genes (*sul1* or *sul2*), 60% carried *dfrA* variants (trimethoprim resistance), 44% had macrolide resistance genes (*mph(A)*), and 40% possessed tetracycline resistance genes (tet(A/B/D)). The isolates showed marked genomic diversity, encompassing 12 distinct MLST types (e.g. ST10, ST43, ST307, ST512, ST540, ST746, ST1721), and multiple plasmid incompatibility groups, most frequently IncFIB (56%), IncFIA (48%), IncX1 (28%), IncR (20%), and Col-type plasmids (32%).

**Table 4. Whole-genome sequencing results of 25 presumptive carbapenemase-producing *Enterobacterales*.**

| **No.** | **Sampling points** | **Date** | **ID** | **Species** | **Multiplex PCR results** | **Carbapenemase** | **ESBL_AmpC** | **Other AMR genes** | **MLST** | **Plasmid type** |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1B | 11/23 | IZSPB_EC01 | *E. coli* | *bla*CTX-M *bla*OXA-48 | *bla*OXA-244 | *bla*CTX-M-15 | *bla*TEM-1B, *qnrS1*, *aph(6)-Id*, *aph(3'')-Ib*, *sul2*, *dfrA14*, *tet(A)* | ST10 | IncFIB(K), IncFIB(AP0019) |

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|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 1B | 06/24 | IZSPB_KP01 | *K. pneumoniae* | *bla*$_{CTX-M}$ *bla*$_{OXA-48}$ | *bla*$_{KPC-3}$ | - | *bla*$_{TEM-1A}$, *bla*$_{OXA-9}$, *aadA3*, *aac(3)-IIa*, *aadA2*, *aph(3')-Ia*, *aac(6')-Ib*, *mph(A)*, *sul1*, *dfrA12*, *catA1* | ST512 | IncFIB(K), ColRNAI, IncQ1, IncFIB(pQil) |
| 3 | 1B | 07/24 | IZSPB_EC02 | *E. coli* | *bla*$_{VIM}$ | - | *bla*$_{TEM-52B}$ | *ant(3'')-Ia*, *lnu(F)* | ST602 | IncX1, IncI1-I(Alpha) |
| 4 | 1D | 02/24 | IZSPB_EC03 | *E. coli* | *bla*$_{OXA-48}$ | *bla*$_{OXA-244}$ | - | - | ST43 | IncFIB(AP0019) |
| 5 | 1D | 05/24 | IZSPB_EC04 | *E. coli* | *bla*$_{KPC}$ | *bla*$_{KPC-3}$ | - | - | ST1139 | IncI(Gamma), IncFIB(pQil) |

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|---|---|---|---|---|---|---|---|---|---|---|
| 6 | 1D | 05/24 | IZSPB_KP02 | *K. pneumoniae* | *bla*CTX-M *bla*KPC | *bla*KPC-3 | *bla*SHV-28, *bla*CTX-M-15 | *bla*TEM-1A, *bla*OXA-1, *bla*OXA-9, *qnrB1*, *aac(3)-IIa*, *aph(6)-Id*, *aph(3'')-Ib*, *sul2*, *dfrA14*, *aac(6')-Ib-cr* | ST307 | IncFIB(K), IncN4, IncFIB(pQil) |
| | | | | | | | | | | |
| 7 | 2A | 02/24 | IZSPB_KP03 | *K. pneumoniae* | *bla*OXA-48 | - | - | - | ST3442 | IncFIB(K)(pCA), RepB |
| 8 | 3B | 02/24 | IZSPB_KP04 | *K. pneumoniae* | *bla*VIM | *bla*VIM-1 | *bla*SHV-12 | *qnrS1*, *aph(3')-XV*, *mph(A)*, *sul1*, *dfrA14*, *catB2* | ST1537 | IncA |
| 9 | 3B | 07/24 | IZSPB_KP05 | *K. pneumoniae* | *bla*CTX-M *bla*KPC | *bla*KPC-3 | | *bla*TEM-1A, *bla*OXA-9, *tet(D)* | ST45 | IncFIB(K), Col440II, ColRNAI, IncFIB(pQil) |

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|---|---|---|---|---|---|---|---|---|---|---|
| 10 | 3B | 08/24 | IZSPB_KP06 | *K. pneumoniae* | *bla*VIM | - | *bla*DHA-1 | *bla*OKP-B-2, *qnrB4*, *sul1*, *dfrA1* | ST2059 | IncFIB(K), IncR, Col440I |
| 11 | 3B | 09/24 | IZSPB_KP07 | *K. pneumoniae* | *bla*VIM | - | - | - | ST983 | negative |
| 12 | 3B | 09/24 | IZSPB_EC05 | *E. coli* | *bla*VIM | - | - | *bla*TEM-1B, aph(3'')-Ib, tet(B) | ST Novel* | IncFIB (AP0019) |
| 13 | 4A | 03/24 | IZSPB_EC06 | *E. coli* | *bla*OXA-48 | *bla*OXA-181 | - | *bla*TEM-1B, *aac(3)-IId*, *aph(6)-Id*, *aph(3')-Ia*, *aph(3'')-Ib*, *aadA5*, *mph(A)*, *erm(B)*, *sul1*, *sul2*, *dfrA17*, *floR*, *catA1* | ST542 | IncFII, IncX1, IncX1, Col (BS512), Col156, IncQ1 |
| 14 | 5A | 02/24 | IZSPB_EC07 | *E. coli* | *bla*OXA-48 | *bla*OXA-244 | - | - | ST746 | negative |

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|---|---|---|---|---|---|---|---|---|---|---|
| 15 | 5A | 02/24 | IZSPB_EC08 | *E. coli* | *bla*$_{VIM}$ | *bla*$_{VIM-4}$ | - | - | ST Novel* | IncFIB(K), IncFIA(HI1) |
| 16 | 5A | 02/24 | IZSPB_KP08 | *K. pneumoniae* | *bla*$_{VIM}$ | *bla*$_{VIM-1}$ | - | *aph(3'')-XV*, *mph(A)*, *sul1*, *catB2* | ST Novel* | repB(R1701), IncFIB(K)(pCA), IncFIB(pKPHS1, Col440I |
| 17 | 5A | 03/24 | IZSPB_KP09 | *K. pneumoniae* | *bla*$_{VIM}$ | *bla*$_{VIM-1}$ | - | *qnrS1*, *aph(3'')-XV*, *mph(A)*, *sul1*, *dfrA14*, *catB2* | ST34 | IncFIB(K), IncN, IncR |
| 18 | 5A | 03/24 | IZSPB_EC09 | *E. coli* | *bla*$_{OXA-48}$ | *bla*$_{OXA-244}$ | *bla*$_{CTX-M-15}$ | *bla*$_{TEM-1B}$, *qnrS1*, *aph(6)-Id*, *aph(3'')-Ib*, *sul2*, *dfrA14*, *tet(A)* | ST540 | IncFIB(K), IncFIB(AP0019) |

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|---|---|---|---|---|---|---|---|---|---|---|
| 19 | 5A | 03/24 | IZSPB_KP10 | *K. pneumoniae* | *bla*CTX-M *bla*VIM | - | *bla*CTX-M-15 | *qnrS1, aph(6)-Id, aadA2, aph(3')-Ia, aph(3'')-Ib, mph(A), sul1, sul2, dfrA12, catA2* | ST469 | IncFIB(K)(pCA), IncFIB(pKPHS1, RepB |
| 20 | 5A | 04/24 | IZSPB_EC10 | *E. coli* | *bla*CTX-M *bla*VIM | *bla*VIM-4 | - | - | ST1721 | negative |
| 21 | 5A | 04/24 | IZSPB_KP11 | *K. pneumoniae* | *bla*VIM | *bla*VIM-1 | - | *aph(3')-XV, sul1, catB2* | ST Novel* | IncFIB(K), IncFIA(HI1), IncX1, IncX1, IncR, Col(pHAD28), Col440I, Col(pHAD28) |
| 22 | 5A | 06/24 | IZSPB_EC11 | *E. coli* | *bla*VIM | *bla*VIM-4 | - | - | ST1721 | IncFIB(pHCM2) |
| 23 | 8A | 08/24 | IZSPB_EC12 | *E. coli* | *bla*VIM | - | *bla*SHV-12 | qnrS1, aph(3'')-Ib, tet(A), floR | ST2144 | IncFIA, IncFIA, IncX1, IncX1, ColpVC, IncFIB(AP0019) |

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|---|---|---|---|---|---|---|---|---|---|---|
| 24 | 8B | 02/24 | IZSPB_EC13 | *E. coli* | *bla*$_{KPC}$ | *bla*$_{KPC-3}$ | - | - | ST141 | IncN |
| 25 | 8B | 08/24 | IZSPB_EC14 | *E. coli* | *bla*$_{VIM}$ | - | *bla*$_{SHV-12}$ | qnrS1, aph(3'')-Ib, tet(A), floR | ST2144 | IncFII(29), IncFIA, IncFIA, IncX1, IncX1, ColpVC, IncFIB(AP0019) |

## 4. Discussion

Antimicrobial resistance (AMR) represents a major global public health threat, and the World Health Organisation (WHO) classifies ESBL- and carbapenemase-producing Enterobacterales among the highest priority pathogens [19]. The environment is increasingly recognised as a key compartment in the transmission and persistence of AMR [20], with anthropogenic activities considered major drivers of carbapenem resistance dissemination [21,22]. This study provides evidence of widespread contamination of surface waters in the Apulia Region (southern Italy) with ESBL- and carbapenemase-producing *E. coli* and *K. pneumoniae*. Using selective culture methods, 176 non-duplicate isolates were recovered from 148 samples collected across eight surface water bodies, with ESBL- or carbapenemase-producing *E. coli* or *K. pneumoniae* detected in 68.8% of the analysed samples. These findings confirm that surface waters can function as important reservoirs and dissemination pathways for AMR, particularly in areas influenced by wastewater discharge and agricultural activities [23,24] and support the integration of environmental surveillance into One Health AMR monitoring frameworks. Marked spatial heterogeneity in *E. coli* concentrations was observed across surface waters, indicating variable faecal contamination from anthropogenic sources, including untreated or inadequately treated wastewater, agricultural runoff and livestock effluents. According to previous reports [25,26], *E. coli* was the most frequently isolated species (57.4%), in line with its recognised ubiquity and persistence in aquatic environments [27], where it can act as a reservoir and vector for horizontally transferable resistance genes [28]. The co-occurrence of *K. pneumoniae* (42.6%) further suggests faecal contamination and potential contributions from both human and animal sources [10]. Notably, all sampling sites yielded presumptive ESBL- and carbapenemase-producing strains on at least one sampling occasion, suggesting widespread environmental dissemination of resistant clones. Phenotypic antimicrobial susceptibility revealed high occurrence of resistance to multiple antibiotic classes. The heatmap highlights clear species-specific differences in the distribution of phenotypes across antimicrobial classes. The high proportions of resistance observed in both *E. coli* and *K. pneumoniae* to aminopenicillins and extended-spectrum cephalosporins should be interpreted in light of the study design, as isolates were obtained using selective culture media. This targeted isolation approach was intentionally applied to enhance the recovery of resistant Enterobacterales and, by definition, enriches for resistance to the corresponding antimicrobial classes. Therefore, the observed resistance profiles do not reflect the background prevalence of antimicrobial resistance in the sampled environments but rather the phenotypic characteristics of the selected isolate collection. Nevertheless, the identified resistance patterns are in line with findings from EU-level surveillance reports documenting the widespread occurrence of ESBL-producing Enterobacterales in environmental and food-production settings [16]. The detection of isolates growing on selective chromogenic media but testing negative for the ESBL- and carbapenemase-encoding genes targeted by PCR can be explained by the intrinsic characteristics of the screening approach. Chromogenic media such as CHROMagar ESBL and CHROMagar mSuperCARBA are designed to maximise sensitivity and may therefore allow the growth of

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Enterobacterales with reduced β-lactam susceptibility due to intrinsic or low-level resistance mechanisms, even in the absence of classical ESBL or carbapenemase genes. Similar limitations, including reduced specificity and occasional growth of PCR-negative isolates, have been reported previously [29,30]. In addition, phenotypic resistance patterns not explained by the PCR targets may reflect the presence of alternative β-lactam resistance mechanisms not covered by the multiplex assay, such as non-CTX-M ESBLs, chromosomal AmpC overexpression, porin alterations or efflux mechanisms [30]. These findings highlight the complementary value and limitations of selective culture and targeted molecular assays in environmental AMR surveillance and support the use of broader genomic approaches when detailed characterisation of resistance determinants is required [31]. Comparison between phenotypic antimicrobial resistance and genomic sequencing-based predictions showed a high degree of concordance. Among ESBL genes, *bla*$_{\text{CTX-M}}$ was the most frequently detected (79.3%), in agreement with its global predominance in both clinical and environmental settings [32,33]. Its similar prevalence in *E. coli* and *K. pneumoniae* suggests sustained selective pressure in the environment, potentially driven by antimicrobial residues and/or untreated wastewater inputs [34]. Carbapenemase genes were detected in 20.6% (25/121) of isolates carrying at least one of the investigated resistance genes, mainly *bla*$_{\text{VIM}}$, *bla*$_{\text{KPC}}$ and *bla*$_{\text{OXA-48}}$-like variants, which are widely disseminated in Europe [35–37]. The observed co-occurrence of multiple resistance determinants, including combinations of ESBL- and carbapenemase-encoding genes, indicates the presence of complex resistance profiles within the analysed isolates. While such genetic constellations are commonly associated with mobile genetic elements and horizontal gene transfer in aquatic environments [38], the present findings should be interpreted cautiously, as no specific analyses of plasmids or other mobile genetic elements were performed, and no conclusions can therefore be drawn regarding the underlying mechanisms of gene dissemination. WGS of 25 presumptive carbapenemase-producing isolates confirmed carbapenemase genes in 68%, supporting the reliability of PCR screening but also highlighting occasional discrepancies between phenotypic and genotypic methods [31]. Detected carbapenemase variants included *bla*$_{\text{KPC-3}}$, *bla*$_{\text{VIM-1}}$, *bla*$_{\text{VIM-4}}$, *bla*$_{\text{OXA-244}}$ and *bla*$_{\text{OXA-181}}$, confirming that surface waters can act as convergence points for multiple resistance genes [39]. WGS also revealed substantial clonal diversity. The detection of high-risk clinical lineages such as *K. pneumoniae* ST512 and ST307 carrying *bla*$_{\text{KPC-3}}$, and *E. coli* ST10 carrying *bla*$_{\text{OXA-244}}$, is particularly noteworthy, as these lineages are commonly associated with healthcare-associated outbreaks in Italy and Europe [40–43]. Their presence in rivers and canals suggests possible bidirectional exchange between clinical environments and natural ecosystems [42,44]. In addition, several sequence types (STs) typically associated with environmental or zoonotic reservoirs were identified, including *E. coli* ST602, ST746 and ST1721 and *K. pneumoniae* ST34, ST45 and ST1537, which have been increasingly reported in surface waters, wastewater effluents and animal-associated settings [4,45–49]. Freshwater ecosystems receiving inputs from wastewater treatment plants, agricultural effluents and stormwater runoff are recognised as hotspots of microbial evolution, where multiple selective pressures can promote the emergence and maintenance of resistant strains [13,34]. Plasmid replicon typing showed a predominance of IncF, IncX and IncN plasmids, which are key vectors for ESBL and carbapenemase dissemination [50,51]. However, due to availability of short-read data only, we could not associate ESBL and carbapenemase genes to plasmid replicons. Taken together, these findings indicate that surface waters in the Apulia Region act both as reservoirs of clinically relevant high-risk clones and as dynamic environments supporting the persistence and evolution of environmentally adapted Enterobacterales. This highlights the permeability of ecological boundaries between human, animal and environmental compartments and reinforces the importance of integrating environmental surveillance into One Health AMR monitoring systems. Targeted interventions addressing wastewater treatment, agricultural runoff and environmental contamination are urgently needed to mitigate the environmental spread of carbapenemase-producing Enterobacterales in Italy.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Antibiotic resistance profiles of *E. coli* isolates negative for all genes

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tested by PCR.; Table S2: Antibiotic susceptibility testing of *Klebsiella pneumoniae* isolates negative for antimicrobial resistance genes tested by PCR.

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