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Limited clonality but widespread plasmid sharing of ESBL-producing *E. coli* between humans and the environment of northeastern Slovenia

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ABSTRACT

Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* is a critical antimicrobial-resistant pathogen. While well-studied in humans and animals, its presence in the environment remains underexplored. This study analyses the genotypes and plasmid composition of ESBL-producing *E. coli* from human and environmental sources in Northeastern Slovenia. Freshwater and sediment samples were collected bimonthly during one year at ten sites, including rivers, lakes, and wastewater treatment plant (WWTP) influents. Human isolates were collected during the same period and geographic area. Whole-genome sequencing and bioinformatic analyses were conducted to evaluate genetic relatedness, antimicrobial resistance genes, and plasmid composition. Among 414 sequenced isolates (230 human, 184 environmental), 108 multilocus sequence types (MLST-ST) were identified, with 20 STs shared between sources. Core-genome MLST (cgMLST) revealed 59 clonal clusters, six of which included both human and environmental isolates. The dominant ESBL gene was $bla_{CTX-M-15}$ (59 % of all ESBL isolates), and 54 % of all ESBL isolates carried ESBL genes on plasmids. Plasmid cluster AA474, found across different sources, STs, and locations, was linked to multiple ESBL genes, suggesting a key role in resistance dissemination. Despite shared STs, ESBL genes, and plasmids between humans and the environment, cgMLST analysis indicated limited clonal spread. This suggest that transmission between humans and the environment remains restricted and is more often linked to spread of plasmids than of strains.

1. Introduction

Extended-spectrum beta-lactamase (ESBL)-producing *E. coli* was recognized as critical-priority antimicrobial-resistant pathogen by the World Health Organization (WHO) in 2017 because of its significant threat to global health (Tacconelli et al., 2018). In 2024, the WHO revised its priority pathogen list and ranked ESBL-producing *E. coli* as the second most prioritized pathogen, primarily due to its antibiotic resistance, transmissibility, high incidence, and challenging prevention efforts (*WHO Bacterial Priority Pathogens List 2024*, 2024). Although the associated mortality rate is moderate, the overall health burden remains substantial.

The environment is recognized as an important factor in the transmission of antimicrobial resistance (AMR) (Larsson and Flach, 2022). While ESBL-producing *E. coli* is ubiquitous and its presence in humans and animals is well documented, its environmental reservoir remains largely underexplored. Studies have documented the presence of ESBL-producing *E. coli* in environmental sources, such as different water sources (Biggel et al., 2023; Fagerström et al., 2019a; Falgenhauer et al., 2021; Lübcke et al., 2024; Müller et al., 2016), soil, manure, agricultural products (Gekenidis et al., 2020; Tyrrell et al., 2025), and wastewater (Franz et al., 2015; Jørgensen et al., 2017a; Schmiege et al., 2021). In this study, we focused on the characterization of ESBL-producing *E. coli* isolated from a water environment and compared them to human isolates in Slovenia. Overall, little is known about the genotypes of ESBL-producing *E. coli,* or their prevalence and variability across different environmental sources in Slovenia. Most publications of ESBL-producing *E. coli* in Slovenia have focused on human isolates; for example, in 2001, *E. coli* was identified as a significant cause of hospital outbreaks in Slovenia (Klavs et al., 2003). Since then, studies have examined ESBL-producing *E. coli*, mostly in human isolates from hospitals and outpatient healthcare centers (Hrovat et al., 2024a, 2024b;

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Jeverica et al., 2024). A few Slovenian studies have also reported the presence of ESBL-producing *E. coli* from animals and products of animal origin, such as meat, meat products, and eggs (Krizman et al., 2017; Rojs et al., 2019). Two studies reported the presence of ESBL-producing *E. coli* in hospital wastewater effluent (Golle et al., 2024; Rozman et al., 2020).

The aim of this study was to investigate the presence of ESBL-producing *E. coli* genotypes in various environmental sources in a major Slovenian agricultural region, including water and sediment samples from rivers, lakes, tributaries, and wastewater treatment plant (WWTP) influent. Furthermore, environmental strains were compared to human strains (from infections and asymptomatic carriage) collected from the same region and time period to better understand their prevalence and potential transmission dynamics.

2. Materials and methods

2.1. Environmental sample collection

Environmental samples (water and sediment) were collected bimonthly at ten different sites in Northeastern Slovenia (Mura statistical region) between June 2023 and April 2024 (Fig. S1A). Four types of surface water source were sampled. A single river was included, with four distinct sampling sites: upstream of City 1 (MS2; Murska Sobota), downstream of City 1 (MS3), river site within an agricultural area (MS8) (but not in the vicinity of cities), and downstream of City 2 (MS4; Lendava). The downstream sampling sites were also located near WWTP effluents (of which only one WWTP was included in the sampling) (MS10) (Fig. S1C). Additionally, two gravel pits known for human activities, such as swimming and fishing (MS6 and MS9), were sampled along with a lake under potential agricultural influence (MS1). Furthermore, two small tributaries were sampled: one potentially affected by a pig farm and biogas plant (MS7) and another with a history of recorded pollution incidents (organic/biological pollution from biogas plant) (MS5). At each sampling site and time, both water and sediment were collected. Water samples were collected in sterile half-liter bottles, and sediment samples were sampled in 50 ml sterile centrifuge tubes. Influent water samples from the main WWTP in the region were collected between August 2023 and April 2024 (Fig. S1A). WWTP influent samples were collected as 24-hour flow-proportional composite samples in sterile half-liter bottles. All the samples were transported to the laboratory at 4 °C and processed within 24 h.

2.2. Isolation of ESBL-producing E. coli from environmental samples

For the cultivation of ESBL-producing *E. coli* from the environment, the WHO guidelines (*WHO Integrated Global Surveillance on ESBL-Producing E. coli Using a One Health Approach*, 2021) were followed, with some modifications (Fig. S2). For water samples, 200 ml of water was filtered through 0.45 μm filtration paper (Whatman™, Germany), and filter papers were cultivated on Tryptone Bile X-glucuronide agar (TBX) (Biolife, Italy) with 4 μg/mL cefotaxime sodium salt (CTX) (Sigma-Aldrich, USA). If no colonies were obtained on TBX with 4 μg/mL CTX, the filtration was repeated and the filters were cultivated on TBX containing lower concentration of CTX (1 μg/mL). After overnight incubation at 37 °C, up to five colonies were collected from each plate for species identification using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) (Bruker, Germany). In one case, six colonies were collected during the initial sampling step. ESBL production of each isolate was confirmed by the disk diffusion method according to the EUCAST guidelines (Eucast: Clinical Breakpoints and Dosing of Antibiotics. 2025) using ceftazidime + cloxacillin (CAC; 30 μg) and cefotaxime + cloxacillin (CTC; 30 μg) disks and their combination with clavulanic acid (CALC or CTLC; 30 μg/10 μg) (Liofilchem, Italy). The sediment (25 g) was transferred to a new sterile centrifuge tube and then filled with sterile water up to 50 ml. After vortexing (30 s) samples were sonicated for one minute using an ultrasonic bath BactoSonic (Bandelin Electronics, Germany) and again vortexed for 30 s, and centrifuged at low speed (800 x g) for five minutes to ease the filtration. A maximum of 10 mL of the obtained supernatant was filtered depending on the filtration success. Filters were cultivated as described above for the water samples. WWTP aliquots (100 μL) were directly inoculated onto TBX agar plates with 4 μg/mL CTX. Up to 20 presumptive *E. coli* colonies were sub-cultured and further characterized as described for the water samples. For all environmental isolates, the clonality of *E. coli* isolates from a single sample (one sample/one sampling site) was assessed using enterobacterial repetitive intergenic consensus (ERIC)-PCR (Versalovic et al., 1991). Only isolates with unique ERIC profiles from each plate were included in the further analysis. All isolates were stored at - 80 °C.

2.3. Collection of human strains

Human ESBL-producing *E. coli* isolates were collected every month between June 2023 and May 2024 (Fig. S1A) from the Department of Medical Microbiology of the National Laboratory of Health, Environment, and Food (NLZOH) serving a hospital and general practitioners in the same geographical region as used for environmental sampling. Ethical approval was obtained from the Committee of Medical Faculty, University of Maribor (038/2023/6-513; 2023.3.23). Human samples were collected sequentially, regardless of hospital or community testing request. Primary isolates were collected from clinical specimens, such as urine, blood cultures, soft tissue infections, and from screening specimens of asymptomatic carriers. Because of the high number of isolates obtained from the screening specimens, we limited the collection to the first 10 isolates in each month. The collected human isolates of ESBL-producing *E. coli* had already been identified and confirmed for ESBL production during routine practice and were stored at −80 °C.

2.4. DNA extraction and whole genome sequencing (WGS)

Genomic DNA of *E. coli* isolates was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's protocol for isolating DNA from Gram-negative bacteria. The extracted DNA was stored at −20 °C. Libraries were prepared using the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England Biolabs, UK) and subjected to 2 × 150 or 2 × 300 bp paired-end sequencing on the NextSeq2000 platform (Illumina, US). A subset of isolates containing the widespread plasmid cluster AA474 ($n = 79$) was also sequenced on the GridION platform (Oxford Nanopore Technologies, ONT, United Kingdom). In this case, genomic DNA was extracted using the same kit and protocol as described for the Illumina-sequenced isolates, and libraries were prepared using the Rapid Barcoding Kit 96 SQK-RBK110.96 (ONT, United Kingdom).

2.5. Genomic analysis

Short read sequences were trimmed with Trimmomatic version 0.39 (Bolger et al., 2014), using the following settings: ILLUMINACLIP: NEBPE-PE.fa:2:30:10, LEADING:10, TRAILING:10, SLIDINGWINDOW:4:20, MINLEN:40. The trimmed reads were assembled *de novo* into contigs using SPAdes version 3.13.0 (Prjibelski et al., 2020) with the -careful option, while retaining all other settings at their default values. Ridom SeqSphere+ 10.0.5 (Jünemann et al., 2013) was used to perform multilocus sequence typing (MLST) (Wirth et al., 2006) and core-genome MLST (cgMLST) analysis to assess genetic relatedness of isolates. A minimum spanning tree (MST) was constructed to assess the clustering of the isolates based on cgMLST. Clonal clusters included isolates that differed by no >10 allelic differences. In addition, as part of the Ridom SeqSphere+, the following tools were used: AMRFinderPlus version 3.11.26 with database 3.11 (Feldgarden et al., 2021) for antimicrobial resistance gene (ARG) detection and MOBSuite version 3.1.8

(Robertson and Nash, 2018) for plasmid detection and classification. For long-read sequencing data, we used NanoFilt 2.8.0 (De Coster et al., 2018) to filter long-read sequences based on a Q score above 10 and a read length of at least 5000 bases. Hybrid assembly of Illumina and Nanopore reads was performed using Unicycler v0.5.1 (Wick et al., 2017). Phylogenetic trees, data, and networks were visualized using iTOL 7 (Letunic and Bork, 2007), R 4.4.1 (R Core Team, 2023) and Cytoscape 3.10.1 (Shannon et al., 2003).

2.6. Plasmid network analysis

For 79 isolates, both short-read and long-read sequencing were performed to construct hybrid genomes for detailed plasmid reconstruction and similarity analysis. Plasmid network analysis was conducted using Mash similarity distances, and all plasmids were compared against each other (Matlock et al., 2021). The results were visualized as a network, where each circle (node) represents a plasmid, and the connections (edges) indicate similarity between plasmids. Edge density was reduced through a process called 'sparsification,' which involved removing all edges below a specified Mash similarity threshold (threshold details in Fig. S4). This approach produced distinct clusters of highly similar plasmids. To validate the plasmid networks and clustering into communities, the homogeneity index (HI) and purity of our communities were calculated using different attributes: replicon types and primary and secondary clusters provided by MOBSuite plasmid classification (Supplementary Table 5 and Fig. S5). All values were high in all cases were high, suggesting good clustering.

2.7. Data availability

The Illumina short-read sequences for all isolates can be found in the European Nucleotide Archive (ENA) under accession number PRJEB87212. Nanopore long-read sequences for 65 isolates can be found in the Sequence Read Archive (SRA) under the accession number PRJNA1248674.

2.8. Statistical analysis

To investigate the diversity of MLST sequence types (STs), phylogroups and plasmid clusters across sources, the richness index was calculated as a standard measure of diversity. To compare diversity between sources, ANOVA and Student's *t*-test with Benjamini-Hochberg (BH) correction for multiple comparisons were performed. Additionally, the composition of STs, phylogroups and plasmid clusters was assessed using PERMANOVA based on Jaccard dissimilarity. A significance level of 0.05 was applied for all statistical tests. Statistical analyses were performed using R version 4.4.1 (R Core Team, 2023). Packages utilised for statistical analysis include vegan v2.6.8 (Oksanen et al., 2025), rstatix v0.7.2 (Kassambara, 2023), RVAideMemoire v0.9.83.7 (HERVE, 2023), EcolUtils v 0.1 (Salazar, 2015/2024).

3. Results

Altogether 414 successfully sequenced ESBL-producing *E. coli* isolates were included in the final collection, including 230 human and 184 environmental isolates (Fig. S3A). The human isolates were obtained from hospitalized patients (n = 153), outpatients (n = 74), and long-term healthcare facilities (n = 3). Most human isolates were obtained from screening specimens (n = 119), while clinically relevant strains were obtained from urine (n = 96), soft tissues (n = 11), and blood (n = 4). At 10 different environmental sampling sites (including WWTP), a total of 113 samples were collected. ESBL-producing *E. coli* was detected in 30 out of 54 water samples and 22 out of 54 sediment samples (Fig. S1A, Fig. S3B), as well as in all five samples from the WWTP. The highest number of presumptive ESBL-producing *E. coli*, determined as average colony-forming unit (CFU) counts per volume or weight, was obtained from WWTP, followed by sediment and water samples (Fig. S3B). When the number of presumptive ESBL-producing *E. coli* was compared between the upstream and downstream sites, no notable difference was observed in water samples, whereas a clear decrease in presumptive sediment isolates was recorded from upstream (4 CFU/g) to downstream (1,8 CFU/g) (Fig. S3C). Altogether, 350 presumptive isolates were subcultivated from the environmental samples. Following the confirmation of ESBL production and ERIC profiling to eliminate clonal isolates, the final collection consisted of 184 environmental isolates: 78 from water, 59 from sediments, and 47 from the WWTP. Most ESBL-producing environmental *E. coli* isolates (n = 126, 68 %) were obtained from all four sampling sites along the river, all of which were under direct anthropogenic influence. These included upstream (MS2; n = 22) and downstream river sites of the cities (MS3; n = 33 and MS4; n = 34), and a river site in an agricultural area (MS8; n = 37) (Fig. S1B). Fewer isolates were obtained from lake (MS1; n = 5), gravel pit (MS6; n = 2), and tributaries (MS5; n = 1 and MS7; n = 3). No ESBL-producing *E. coli* isolates were obtained from the sampling site MS9 (gravel pit). No distinct seasonal patterns were observed. Furthermore, no correlation was found between the presence of ESBL-producing isolates and factors such as water temperature, atmospheric temperature, or water levels. Metadata for all collected isolates is provided in Supplementary Table 1.

3.1. Diversity and overlap of multilocus sequence types and phylogroups between human and environmental strains

A total of 108 distinct STs were identified across all 414 isolates using the Wirth/Warwick MLST scheme. Among these, 62 different STs were identified in 230 human isolates and 66 different STs in 184 environmental isolates. Forty-two (39 %) STs were found only in human isolates and 46 (43 %) STs were found only in environmental isolates. Several STs (n = 20, 19 %) were shared between human and environmental isolates (Figs. 1, 2A). Detailed information regarding the presence of STs across humans and different environmental sources is provided in Supplementary Table 2.

The most frequently shared STs between human and environmental sources were ST131, ST38, ST69, and ST16433. No significant difference in ST diversity (measured as richness) was observed between the sources (ANOVA, F = 1.066, Pr(>F) = 0.381) (Supplementary Table 5). Additionally, no statistical difference was found in the composition of STs across the different sources (PERMANOVA, R^2 = 0.115, F = 1.083, Pr(>F) = 0.222). No seasonal patterns in ST distribution were observed across the sampling months (Fig. S6).

Eleven novel STs were identified across all samples, including three STs found in the environment, seven STs in human isolates and one shared between humans and the environment (Supplementary Table 1). One of the novel STs, ST16433, could represent a locally emerging type, as these isolates (n = 19) belonging to the same clonal cluster were found across all sources and were detected in different locations in rivers and a small tributary. ST16433 was determined to be a single-locus variant of ST1193 with a SNP mutation in the *adk* gene, potentially representing a new lineage derived from ST1193. Based on short-read sequences, the ESBL $bla_{CTX-M-15}$ gene was detected in all ST16433 isolates; in 18 of these, the gene was located on the chromosome, while in one isolate (ESBLEC388), it was carried on a plasmid.

All isolates were also assigned to phylogroups, with the majority belonging to phylogroup B2 (47 %), followed by phylogroups A (22 %), D (12 %), and B1 (11 %). Phylogroups A and B1 were more prevalent in the environment, whereas phylogroup B2 was considerably more frequent in humans (Supplementary Table 1). A significant difference in phylogroup diversity between human and environment was observed (ANOVA, F = 18.29, Pr(>F) = 0.000579; Supplementary Table 5). Similarly, a significant difference in phylogroup composition between the humans and environment was detected (PERMANOVA, R^2 = 0.17, F = 3.36, Pr(>F) = 0.016).

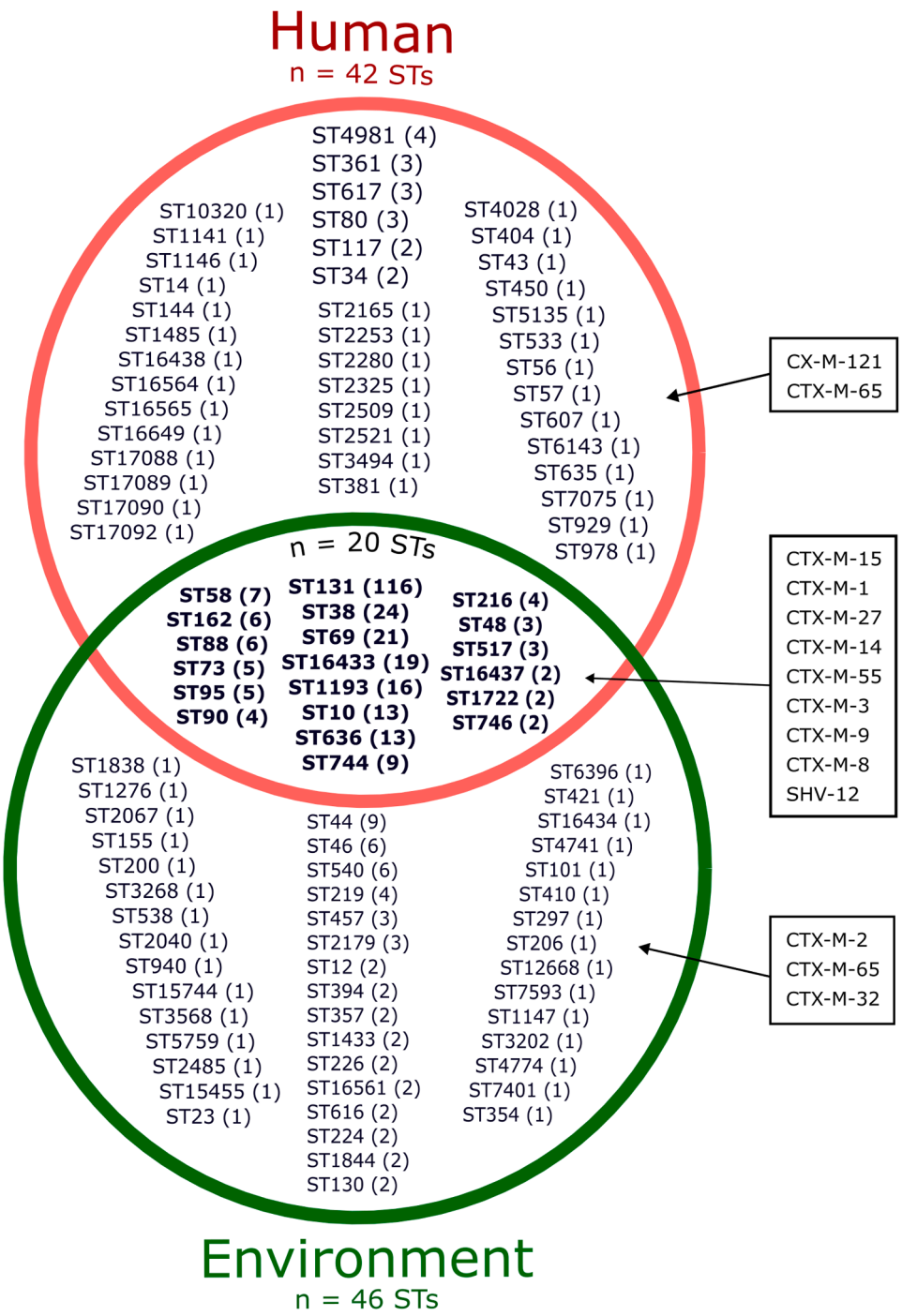

Fig. 1. Venn diagram of ESBL-producing *E. coli* MLST-STs found in humans and the environment. The MLST-STs identified in human (red) and the environment (green). The number of isolates is shown in parentheses next to each ST. Additionally, the ESBL genes detected in each source are listed.

3.2. Genomic relatedness of isolates from different samples

Out of the 414 isolates, 212 (51 %) were assigned to 59 distinct clonal clusters, whereas 202 isolates (49 %) were classified as singletons (Fig. 3). The four largest clonal clusters (cluster 1–4) contained 19, 18, 12 and nine isolates, respectively. Three clonal clusters included eight isolates, one cluster contained six isolates, and another included five isolates. Five, nine, and 36 clusters contained four, three and two isolates, respectively.

Out of the 59 clusters, 28 contained isolates from different sources. The largest clonal cluster (cluster 1) consisted exclusively of the 19 ST16433 isolates present across all sources: humans ($n = 8$), water ($n = 6$), sediment ($n = 3$) and WTTP ($n = 2$). Two clusters (cluster 3 and cluster 10) contained isolates from three different sources: humans, sediment, and water. Cluster 3 consisted of 12 ST636 isolates from humans ($n = 8$), sediment ($n = 1$), and water ($n = 3$). Cluster 10 contained four ST131 isolates: human ($n = 1$), sediment ($n = 1$), and water ($n = 2$). Twenty-five clusters contained isolates from two different sources. Only six clusters contained isolates from humans and the environment. Detailed information regarding the presence of clonal clusters is provided in Supplementary Table 2.

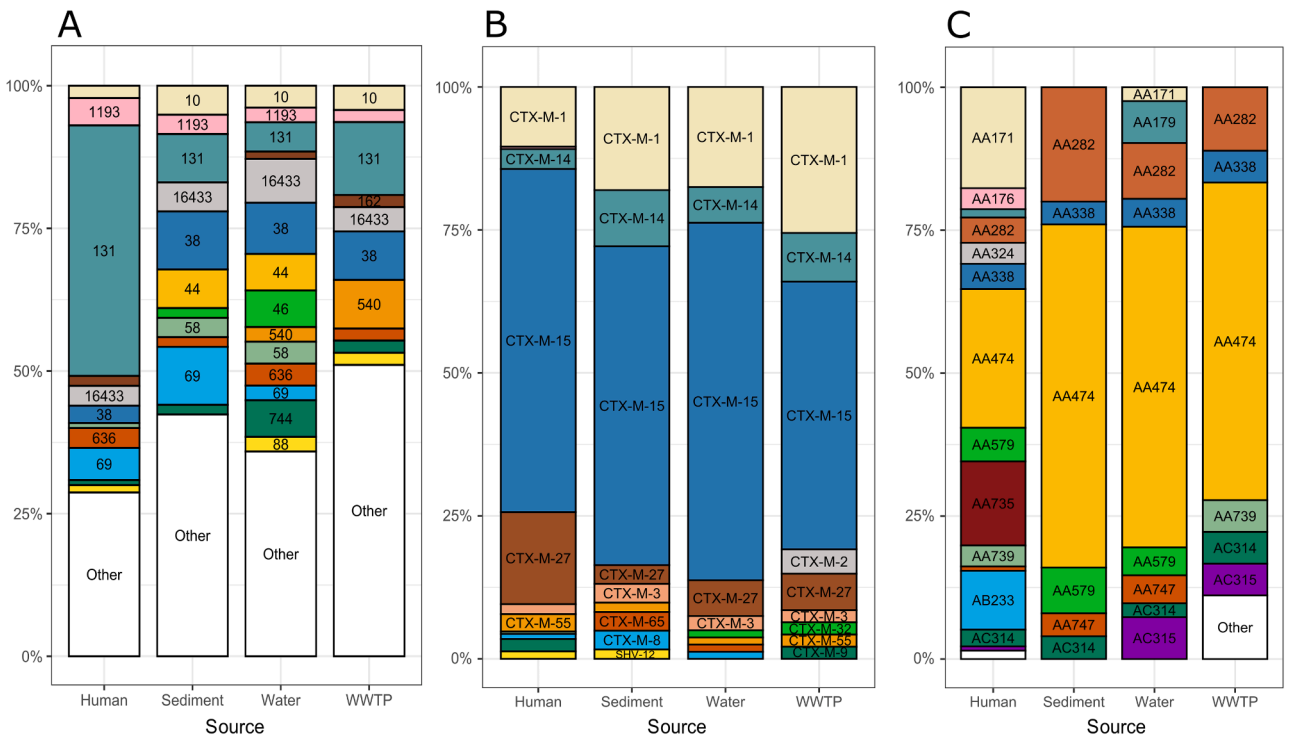

Fig. 2. The relative proportions of STs, ESBL genes and MOBSuite's plasmids primary clusters found in each source. (A) The relative proportions of STs, which are colored and labeled according to the STs found in each source, with only the most common STs shown. Those represented by fewer than 5 occurrences are grouped and labeled as "Other". (B) The relative proportion of ESBL genes found in each source. Bars are colored and labeled according to the ESBL genes. (C) The relative proportion of MOBSuite's plasmids primary cluster, found in each source. Bars are colored according to the plasmid clusters, with only the most common one shown. Those found in fewer than 2 isolates are signed as "Other." Labels inside present specific plasmid cluster. Please note that each figure represents a separate entity, and the colors used do not indicate any connection between the figures A, B or C.

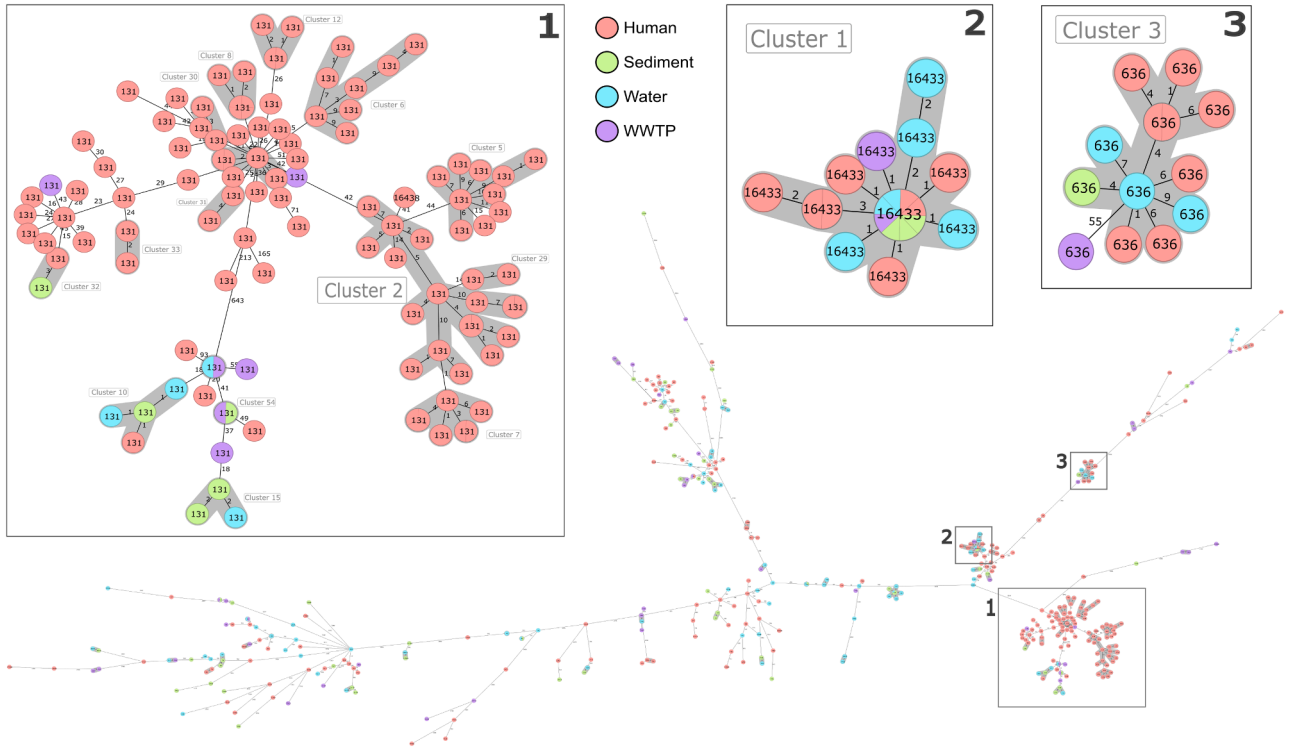

Fig. 3. Minimum spanning tree (MST) of 414 ESBL-producing *E. coli* isolates. The clonal clusters with isolates from various sources are highlighted within three boxes. Circles are color-coded according to their sources, and the numbers inside the circles represent MLST-STs.

3.3. Diversity of ESBL genes in E. coli isolates

Among the 414 ESBL-producing isolates, 14 distinct ESBL genes were identified (Figs. 1, 2B, Supplementary Table 3). Human isolates contained 11 distinct ESBL genes and environmental isolates contained 12 distinct ESBL genes. The most prevalent ESBL genes were shared between the human and environmental isolates, with most isolates encoding $bla_{CTX-M-15}$ ($n = 244$, 59 %), followed by $bla_{CTX-M-1}$ ($n = 62$, 15 %), $bla_{CTX-M-27}$ ($n = 47$, 11 %), and $bla_{CTX-M-14}$ ($n = 23$, 6 %). A total of nine ESBL genes were found in both human and environmental isolates, two ESBL genes were found exclusively in humans, and three ESBL genes were found only in the environment (Fig. 1). Six ESBL genes were found in all four sources: humans, water, sediment, and WWTP (Supplementary Table 3). Based on short-read sequences, four ST38 isolates encoded two ESBLs, $bla_{CTX-M-14}$ on chromosome and $bla_{CTX-M-15}$ on plasmid, and one ST457 isolate encoded two copies of $bla_{CTX-M-15}$ on the chromosome. The presence of co-resistance genes is summarized in Supplementary Table 3.

3.4. Prevalence of ESBL-encoded plasmids

In more than half of the isolates ($n = 222$; 54 %), ESBL genes were predicted to be carried on the plasmids. Using MOBSuite tools, we clustered the plasmids reconstructed from the draft genomes into MOB primary clusters. These clusters group similar plasmids based on MASH distances, using a clustering threshold of 0.06. In total, 18 distinct plasmid clusters were identified (Supplementary Table 4). Plasmid cluster diversity differed significantly between the human and environmental sources (ANOVA, $F = 25.87$, $Pr(>F) = 0.000000078$) (Supplementary Table 5). However, no significant differences in diversity were observed when only different environmental sources were compared one to another. Similarly, the composition of plasmid clusters was statistically different between the human source and each environmental source (PERMANOVA, $R^2 = 0.30$, $F = 3.64$, $Pr(>F) = 0.001$), but no significant differences were found between the environmental sources. Four plasmid clusters (AA474, AA282, AA338, and AC314) were identified across all four sources (Fig. 2C). The plasmid cluster AA474 was the most prevalent, detected in 34 (25 %) human isolates and 48 (57 %) environmental isolates. Most AA474 plasmids contained replicon type IncI/gamma/K1 ($n = 59$, 73 %) and IncI1/B/O ($n = 18$, 22 %). Detailed information regarding the replicon types for all plasmid clusters is provided in Supplementary Table 1. The ESBL genes encoded on plasmids from that cluster (AA474) were $bla_{CTX-M-1}$, $bla_{CTX-M-15}$, $bla_{CTX-M-8}$, $bla_{CTX-M-27}$, $bla_{CTX-M-3}$, $bla_{CTX-M-64}$, and bla_{SHV-12}. The presence of ESBLs in other primary clusters is shown in Fig. 4. The majority of the plasmids ($n = 114$, 51 %) encoded $bla_{CTX-M-15}$.

3.5. Plasmid network of AA474 plasmid cluster

Due to the high prevalence of plasmids from the AA474 cluster across all sources, sampling locations (Fig. 5), and STs, we conducted a detailed analysis and characterization of these plasmids to better understand their similarity and prevalence. The 79 isolates with presumptive ESBL-AA474 plasmids detected in the short-read Illumina data underwent long-read sequencing using Nanopore Sequencing technology. Of these, 65 were included in the comprehensive analysis, while 14 were excluded due to the absence of reconstructed plasmids ($n = 11$) or because the ESBL genes were located on the chromosome rather than on plasmids ($n = 3$). Out of 65 plasmids, we identified five isolates with ESBL genes located on plasmids from a different primary cluster (three isolates from primary cluster AA735, one from AA171 and one from AA324); these isolates were included in subsequent analyses. The plasmid network analysis identified eight distinct communities of closely related plasmids (Fig. 6). The two largest plasmid communities, community 1 and community 2, encoded $bla_{CTX-M-1}$, and included plasmids from all four sample sources. Communities 3 and 6 consisted of plasmids from humans, water, and sediment encoding $bla_{CTX-M-15}$ and $bla_{CTX-M-8}$, respectively. Communities 4 and 7 contained plasmids from humans and sediment samples, encoding $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$, respectively. Hybrid assembly of Illumina and Nanopore sequencing data revealed that the plasmids in community 5 belonged to primary cluster AA735. These plasmids were exclusively found in humans and encoded

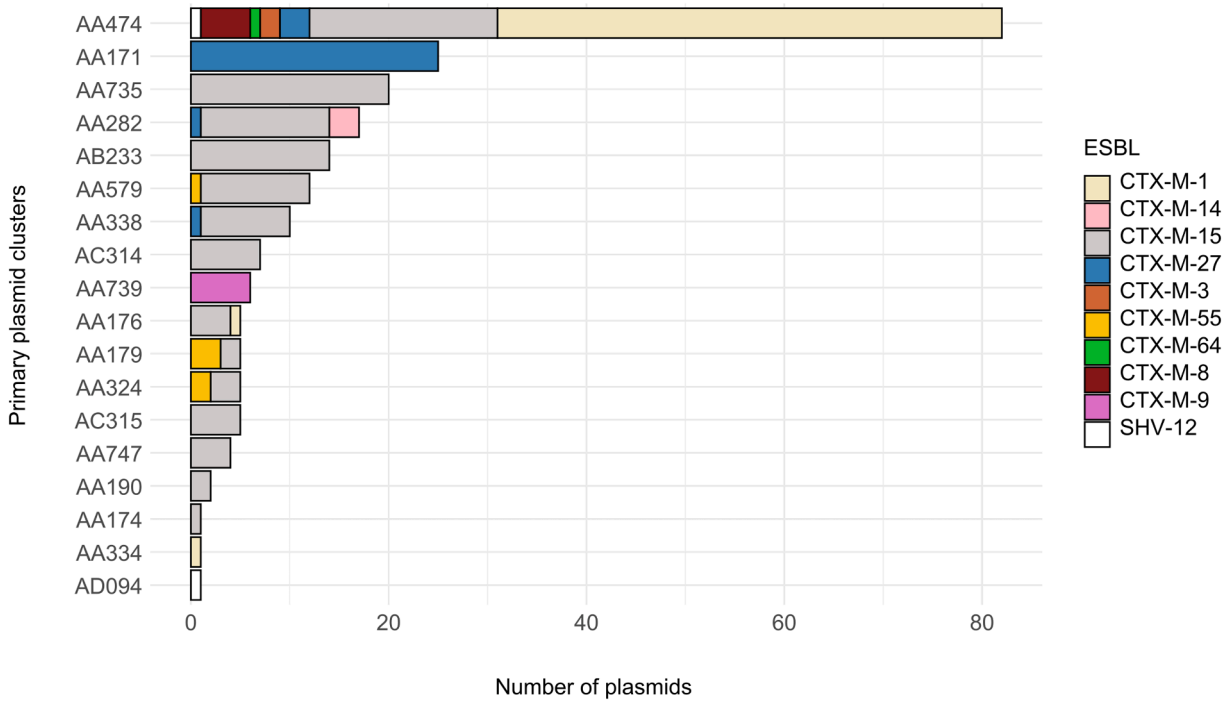

Fig. 4. Presence of ESBL genes across primary plasmid clusters. The figure presents the number of ESBL-encoding plasmids (X-axis) within each plasmid cluster (Y-axis). Bars are color-coded according to the specific ESBL encoded on plasmids.

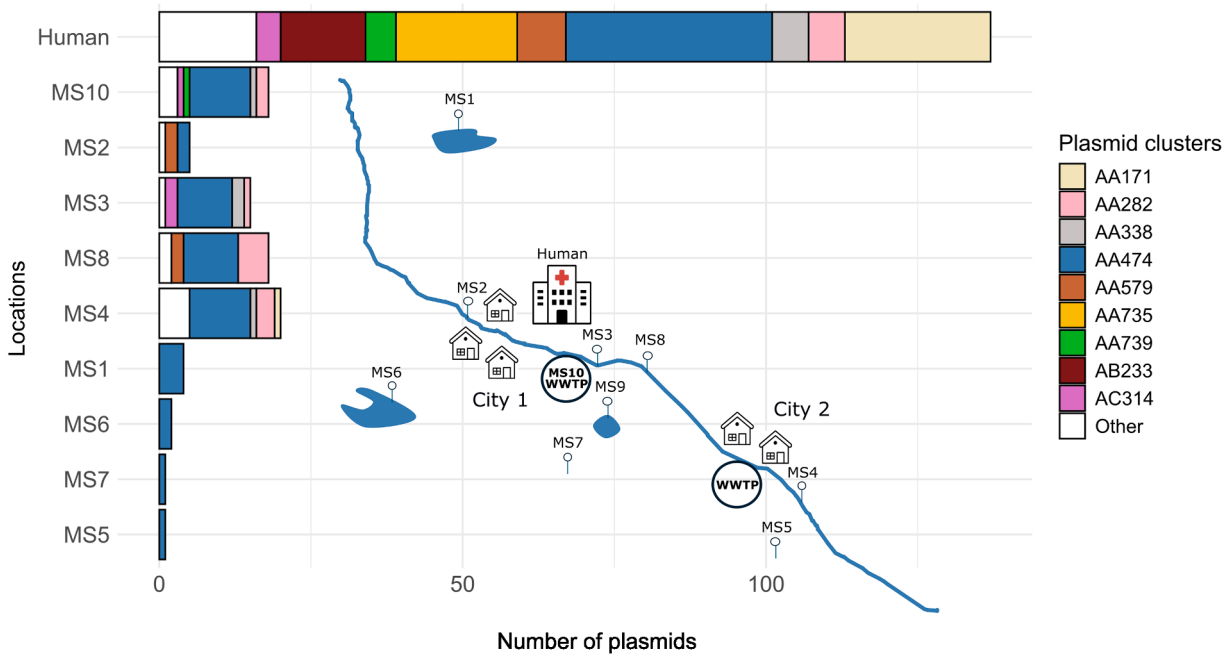

Fig. 5. Diversity of plasmid clusters across human isolates and environmental sampling sites. Environmental sampling sites are schematically shown together with anthropogenic influences. The left side of the chart (Y-axis) represents the sampling locations, while X-axis displays the number of ESBL-encoded plasmids found at each location. Each bar color corresponds to a specific plasmid cluster according to legend. Plasmid cluster present in no more than 5 isolates are categorized as "Other." The river map shows the distribution of sampling points: MS1 – lake under potential agricultural influence; MS2 – upstream of the first city; MS3 and MS4 – downstream of the first and second city, respectively; MS8 – river in an agricultural area; MS6, and MS9 – lakes known for human activities; MS5 – tributary with history of recorded pollution incidents; MS7 – tributary potentially affected by a pig farm and biogas plant; MS10 – WWTP.

bla$_{CTX-M-15}$. Finally, community 8 comprised three *bla*$_{CTX-M-1}$-encoded plasmids detected in water.

4. Discussion

ESBL-producing *E. coli* isolates from environmental niches are less studied than those obtained from animals and humans. We performed genome and plasmid analysis of ESBL-producing *E. coli* isolates from humans, surface waters, sediments, and WWTP influents obtained in Northeastern Slovenia. Our findings show that the most prevalent ESBL genes, STs, and plasmid clusters are shared across human and environmental sources, with a possible but limited overlap of clonal isolates but with highly similar plasmids.

A high prevalence of ESBL-producing *E. coli* isolates was observed in samples from river sites, including upstream and downstream locations in two cities and an agricultural area. Surface waters face continuous anthropogenic impacts such as wastewater from municipal, industrial, and hospital sources (Larsson and Flach, 2022). Therefore, it is not surprising that a substantial number of ESBL-producing *E. coli* isolates were found in WWTPs, where both the general community and one hospital contribute to the occurrence of antibiotic-resistant bacteria (Schmiege et al., 2021; Tanabe et al., 2024). However, in our study tributary close to pig farm and biogas plant had low isolate numbers, presumably because wastewater from a pig farm and a biogas plant were cleaned on an own small-scale WWTP before being released to the tributary. Fewer or no ESBL-producing *E. coli* isolates were also found in gravel pits or lake, which in our case serve for recreational purposes and sport fishing. However, some studies have reported UTI-related transmission and infection in water and swimmers (Leonard et al., 2018; Søraas et al., 2013).

A substantial number of ESBL-producing *E. coli* isolates were found not only in water, but also in sediments. While many studies have reported a high prevalence of ESBL-producing *E. coli* in surface water and WWTP, very few studies have focused on water sediments, despite their potential role as AMR reservoirs (Cho et al., 2023; Davidova-Gerzova et al., 2023; Lu et al., 2010). For example, bacteria in sediments form biofilms that contain diverse species from multiple taxonomic groups, contributing to the diversity of shared ARG. Additionally, the close physical proximity of bacteria within biofilms can make sediments a significant AMR reservoir (Abbassi et al., 2022).

As noted in previous small-scale study, ST diversity did not significantly differ between human and environmental isolates, and no seasonal patterns were observed (Fagerström et al., 2019b). This emphasizes the widespread presence of resistant *E. coli* STs across different sources throughout the year. Sediment and water shared the most STs ($n = 18$, 17 %), followed by human and water ($n = 16$, 15 %), with the least overlap observed between sediment and WWTP ($n = 10$, 9 %). Human and environmental isolates shared 20 STs (19 %), with seven STs detected across all four sources (ST131, ST38, ST16433, ST1193, ST10, ST636, and ST744). Except for the newly described ST16433, these One Health relevant STs are among the most common globally, and some of them (ST131, ST38, ST10, ST1193) are considered high-risk clonal lineages responsible for severe human infections. (Fagerström et al., 2019b; Haenni et al., 2018; Nicolas-Chanoine et al., 2014; Pitout et al., 2022; Wyrsch et al., 2022a). Our finding of the new endemic ST16433 further emphasizes that while some STs are prevalent globally, others could be of local importance.

In our study, phylogroups A and B1 were more prevalent in the environment, while B2 predominated in humans, consistent with previous studies (Chaisaeng et al., 2024; Cookson et al., 2024). Phylogroups A and B1 mostly include commensal *E. coli* from the environment and animals, whereas B2 is often linked to human pathogens (Clermont et al., 2000). B2 strains appear better adapted to colonize the human gut and may not persist long in environment. However, some studies warn that A and B1 strains, though less virulent, can carry higher antibiotic resistance (Chakraborty et al., 2015; Johnson et al., 2003).

Of the 59 clonal clusters identified, only six contained both human and environmental isolates. Cluster 1 was the only cluster that contained

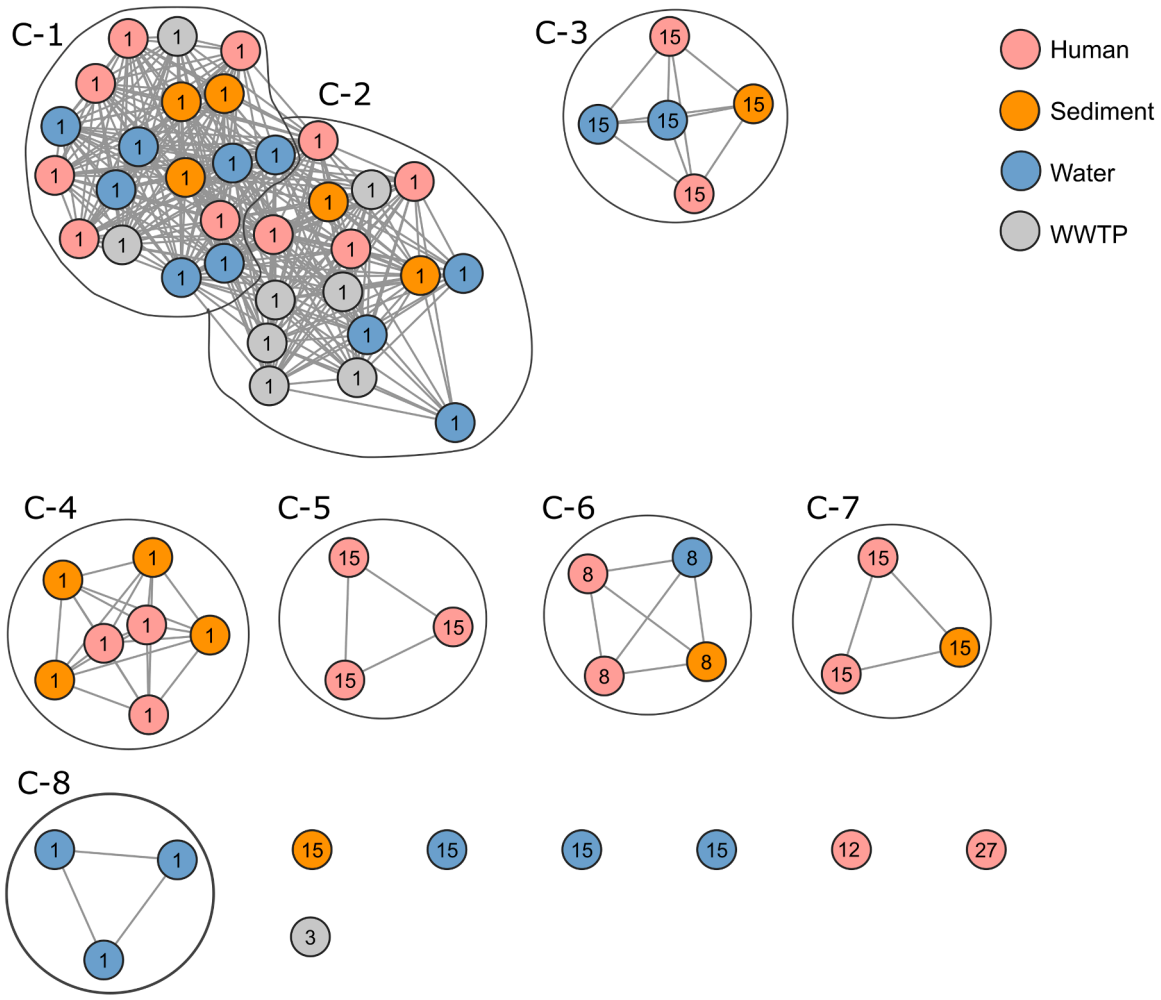

Fig. 6. The plasmid network of ESBL-encoded plasmids across various sources. Each node (circle) in the network represents an individual plasmid. The nodes are color-coded according to the corresponding source, as indicated in the legend. Numbers inside the nodes indicate specific ESBL: 1 - $bla_{CTX-M-1}$; 3 - $bla_{CTX-M-3}$; 8 - $bla_{CTX-M-8}$; 12 - $bla_{CTX-M-12}$; 15 - $bla_{CTX-M-15}$; 27 - $bla_{CTX-M-27}$. Communities are labeled from C-1 to C-8.

isolates from all four sources and included the newly identified ST16433, which is likely derived from ST1193. Studies have highlighted the importance of ST1193 as this rapidly emerging ST is a major cause of urinary and bloodstream infections in humans. While ST1193 is predominantly found in human infections, a few studies have reported its presence in animals and the environment (Pitout et al., 2022; Kidsley et al., 2020; Wyrsch et al., 2022b). Our data further support this, with the majority of ST1193 isolates originating from human samples (n = 11, 69 %) but were also detected in environmental samples (n = 5, 31 %). We also found clonal clusters containing isolates from humans, sediments and water (n = 2), humans-sediments (n = 1) and humans-WWTP (n = 2). These findings suggest limited clonal transmission of ESBL *E. coli* between humans and the environment. High ST diversity and limited clonal spread indicate that *E. coli* likely originates from multiple sources and has diverse transmission routes (Larsson and Flach, 2022). Despite our results indicating limited clonal transmission, it is important to consider that our study was conducted in a high-income country (Slovenia) and the situation in low- and middle-income countries (LMIC) may involve a higher degree of clonal transmission between humans and the environment. To date, relatively few studies have investigated clonality of ESBL-producing *E. coli* between humans and environment in LMIC (Flatgard et al., 2024; Gay et al., 2023; Gray et al., 2025; Jørgensen et al., 2017b; Romdhani et al., 2023).

Similar to STs, the most prevalent ESBL genes were detected in both human and environmental isolates, indicating a significant overlap and widespread occurrence of ESBLs in different environments. Many ESBL genes (n = 6, 43 %) were found across all sources. The most prevalent ESBL genes in our data ($bla_{CTX-M-15}$, $bla_{CTX-M-1}$, $bla_{CTX-M-27}$, $bla_{CTX-M-14}$) have been widely reported in previous studies and found across human, animal, food, and environmental sources (Day et al., 2019; Mughini-Gras et al., 2019). Conversely, $bla_{CTX-M-121}$ and $bla_{CTX-M-64}$ were exclusive to human isolates, whereas $bla_{CTX-M-2}$, $bla_{CTX-M-32}$, and $bla_{CTX-M-65}$ were found only in environmental samples. The ESBL genes $bla_{CTX-M-32}$ and $bla_{CTX-M-64}$ have been documented in humans, animals, and the environment (Flatgard et al., 2024; Mughini-Gras et al., 2019; Tanaka et al., 2019; Zhang et al., 2019), whereas $bla_{CTX-M-121}$, $bla_{CTX-M-2}$, and $bla_{CTX-M-65}$ have been reported in humans or animals, but not in the environment (Chen et al., 2016; Chmelnitsky et al., 2005; Su et al., 2020; Zhang et al., 2014).

AMR dissemination between human and environment is more likely to occur at the plasmid level rather than through clonal spread. The clonal spread of AMR appears limited, as only six (out of 59, 10 %) clonal clusters that overlapped between human and environment were detected, comprising 41 isolates. In contrast, plasmid-mediated spread was more extensive, with 11 (out of 18, 61 %) plasmid clusters shared between human and environment, with a higher number of isolates (n = 175). All plasmid clusters, except for one plasmid cluster (AA334), found in the environment were also present in humans.

Although many of plasmids overlapped, our analysis revealed

distinct plasmid composition and diversity between human and environmental sources. The plasmid cluster AA474 was consistently found across all sources, and encoded various ESBL genes, with $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$ being the most common. This makes it a potentially important backbone for the spread of AMR in *E. coli*. Hybrid sequencing analysis confirmed the presence of highly similar plasmid communities of AA474 plasmid clusters across different sources. Most plasmids from the AA474 cluster contained the IncI/gamma/K1 and IncI1/B/O replicon regions. Plasmids from the IncI complex are recognized as key ESBL gene carriers and are predominantly found in environmental sources, although a significant proportion is also present in humans (Flatgard et al., 2024; Rozwandowicz et al., 2018; Zhang et al., 2019).

One of the limitations of our study was the lack of isolates from animal sources, which could help bridge the gap in understanding the transmission of ESBL-producing *E. coli* in the context of One-Health. For example, human exposure to surface water is limited; however, animals exhibit greater exposure to contaminated water and may act as intermediaries in the transmission cycle (Mughini-Gras et al., 2019). However, surface water still presents a high risk of human infections due to the high prevalence of ESBL-producing *E. coli.* Another limitation of our study is that plasmid analysis for all isolates was conducted using short-read sequences, with a small subset undergoing long-read sequencing to construct hybrid assemblies, providing more accurate results. Based on the hybrid assemblies, we observed minor inconsistencies with the short-read data. While short-read-based plasmid analysis serves as a general indicator, long-read data are crucial for the accurate representation of plasmids.

In summary, our findings revealed a high prevalence of shared STs, ESBL genes, and plasmid clusters between human and environmental sources, indicating a significant connection of ESBL-producing *E. coli* between these reservoirs. However, in-depth cgMLST analysis demonstrated limited clonal spread, suggesting a restricted transmission of strains between humans and the environment. The spread of ESBL genes in *E. coli* is more likely driven by plasmids, since highly similar ESBL-encoding plasmid communities have been found to be widespread across different environments.

CRediT authorship contribution statement

Leon Maric: Conceptualization, Formal analysis, Methodology, Writing – original draft, Visualization. **Sandra Janezic:** Conceptualization, Methodology, Data curation, Writing – review & editing. **Camilla Wiuff Coia:** Methodology, Writing – review & editing. **Louise Roer:** Methodology, Writing – review & editing. **Maja Rupnik:** Conceptualization, Writing – original draft, Funding acquisition.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2025.100408.

Data availability

Data will be made available on request.

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