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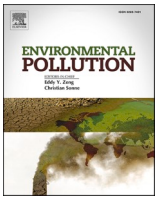

Dissemination of ESBL-producing *E. coli* ST131 through wastewater and environmental water in Switzerland☆

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ABSTRACT

The *E. coli* lineage ST131 is a major cause of multidrug-resistant urinary tract and bloodstream infections worldwide. Recently emerged ST131 sublineages spread globally within few years, but their dissemination routes are incompletely understood. In this study, we investigate the potential role of wastewater and surface water in the spread of extended-spectrum β-lactamase (ESBL)-producing ST131. Streams, lakes, and two wastewater treatment plants (WWTPs) in the canton of Zug, Switzerland, were consecutively sampled over 1.5 years. ST131 was detected in 38% of the samples taken downstream (1–5 km) of WWTP discharge sites, but usually absent in water bodies distant from urban areas or WWTP discharge. Specific strains were repeatedly isolated (≤5 pairwise cgSNP distance) from wastewater or river sites downstream of effluent discharge, indicating their repeated entry or persistence in WWTPs in large concentrations. Genetic characterization of the ESBL-producing water isolates revealed a predominance of clades A and C1 and an emerging ciprofloxacin-resistant sublineage with mutations in quinolone resistance determining regions (QRDR) within clade A. Multiple isolates belonged to internationally circulating sublineages, including C1-M27 and *papGII* + sublineages with chromosomally encoded ESBLs. This study demonstrates that the clinically relevant *E. coli* lineage ST131 pollutes river ecosystems, representing a significant challenge to public health and to technologies to minimize their entry into the water environment.

1. Introduction

The multi-drug resistant *E. coli* lineage ST131 contributes substantially to the ongoing antimicrobial resistance (AMR) crisis. After its emergence in the 1990s, ST131 has spread rapidly across the globe and is today the dominant *E. coli* clone among human cephalosporin- and ciprofloxacin-resistant bloodstream and urinary tract infections (Gladstone et al., 2021; Day et al., 2019; Wang et al., 2021). Like other extraintestinal-pathogenic *E. coli* (ExPEC), ST131 asymptomatically colonizes the human gut but can cause symptomatic infections when reaching other body sites. Asymptomatic carriage of ST131 in healthy humans ranges from 3 to 10% (Kudinha and Kong, 2022; Mohamed et al., 2020).

ST131 has a diverse gene repertoire and different subpopulations (clades, subclades, and sublineages) exhibit distinct AMR and virulence characteristics. ST131 is classified into three major clades: A, B, and C, with clade C further divided into subclades C1 and C2. Clade C isolates carry mutations in the quinolone resistance determining regions (QRDR) of *gyrA*, *parC*, and *parE*, conferring ciprofloxacin resistance (Ben Zakour et al., 2016). Resistance to third-generation cephalosporins mediated by various acquired extended-spectrum beta-lactamases (ESBL) is common as well and often maintained in specific subpopulations, such as *bla*CTX-M-15 in clade C2 and *bla*CTX-M27 in the C1 sublineage C1-M27 (Matsumura et al., 2016; Price et al., 2013). Clade C2 is overrepresented among clinical ST131 isolates suggesting enhanced virulence (Price et al., 2013; Kallonen et al., 2017). Of particular concern is the spread of ST131 sublineages carrying *papGII* pathogenicity islands due to their role in pyelonephritis and urosepsis. Multiple widespread *papGII* + sublineages have emerged within clade C2, whereas other clades contain one *papGII* + sublineage each (Biggel et al., 2022; Biggel et al., 2020).

The reason for ST131's global success, its potential niche adaptions and dissemination routes are still debated. While some genomic studies conclude that ST131 or clade C are highly adapted and possibly host-restricted to humans, others report potential ST131 reservoirs in

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poultry, pigs, wild birds, and companion animals with or without indications for human-animal transmissions (Day et al., 2019; Wu et al., 2013; Li et al., 2021; Johnson et al., 2017; Liu et al., 2018; Kidsley et al., 2020). Generally, humans acquire *E. coli* via person-to-person transmission and contaminated food. The discharge of wastewater effluent into the environment may significantly contribute to the dissemination of ST131, as surface water is used for crop irrigation and recreational activities.

In this study, we analyzed samples from raw and treated wastewater, lakes, rivers, and creeks in the canton of Zug in Switzerland for the presence of ESBL-producing ST131. We demonstrate that the pollution of environmental sites by ST131 is linked to wastewater discharge. We used whole-genome sequencing to compare wastewater and environmental isolates and to genetically characterized the local ST131 population.

2. Methods

2.1. Description of the study area

The study was conducted in the canton of Zug, an administrative district in Central Switzerland comprising 11 municipalities with approximately 130,000 inhabitants (Fachstelle für Statistik des Kantons, 2022). In addition, around 38,000 people are considered commuters to the canton of Zug. The canton has two hospitals (approximately 200 beds in total), two clinics for rehabilitation and psychiatric treatment (~350 beds), 15 nursery and elderly homes (~1100 residents), 280 human medical practices, and 16 veterinary clinics or practices. The 207 km^2 area of the canton comprises urban lands (17%), farmlands (49%) and forests (30%). Approximately 550 farms are in operation (Fachstelle für Statistik des Kantons, 2022). The main surface water system connects Lake Aegeri ("Aegerisee", 724 m above sea level) via the upper section of the river Lorze to Lake Zug ("Zugersee", 413 m) and further from Lake Zug via the lower section of the river Lorze into the river Reuss (Reussspitz, 388 m).

Wastewater from over 98% of all inhabitants of the canton of Zug (census data 2021: 129,787 individuals) is treated at the main wastewater treatment plant (WWTP), ARA Schönau (WWTP_1). Sewage from both hospitals and most care facilities is covered by this WWTP. The plant has a mean hydraulic load of ~55,000 m^3/day and discharges into the river Lorze. A second small WWTP (370 m^3/day), ARA Neuheim (WWTP_2), serves a population of 2300 and discharges into the river Sihl. The WWTPs do not have a bacterial disinfection stage (such as UV, ozonation, or chlorination) implemented. They do not receive manure

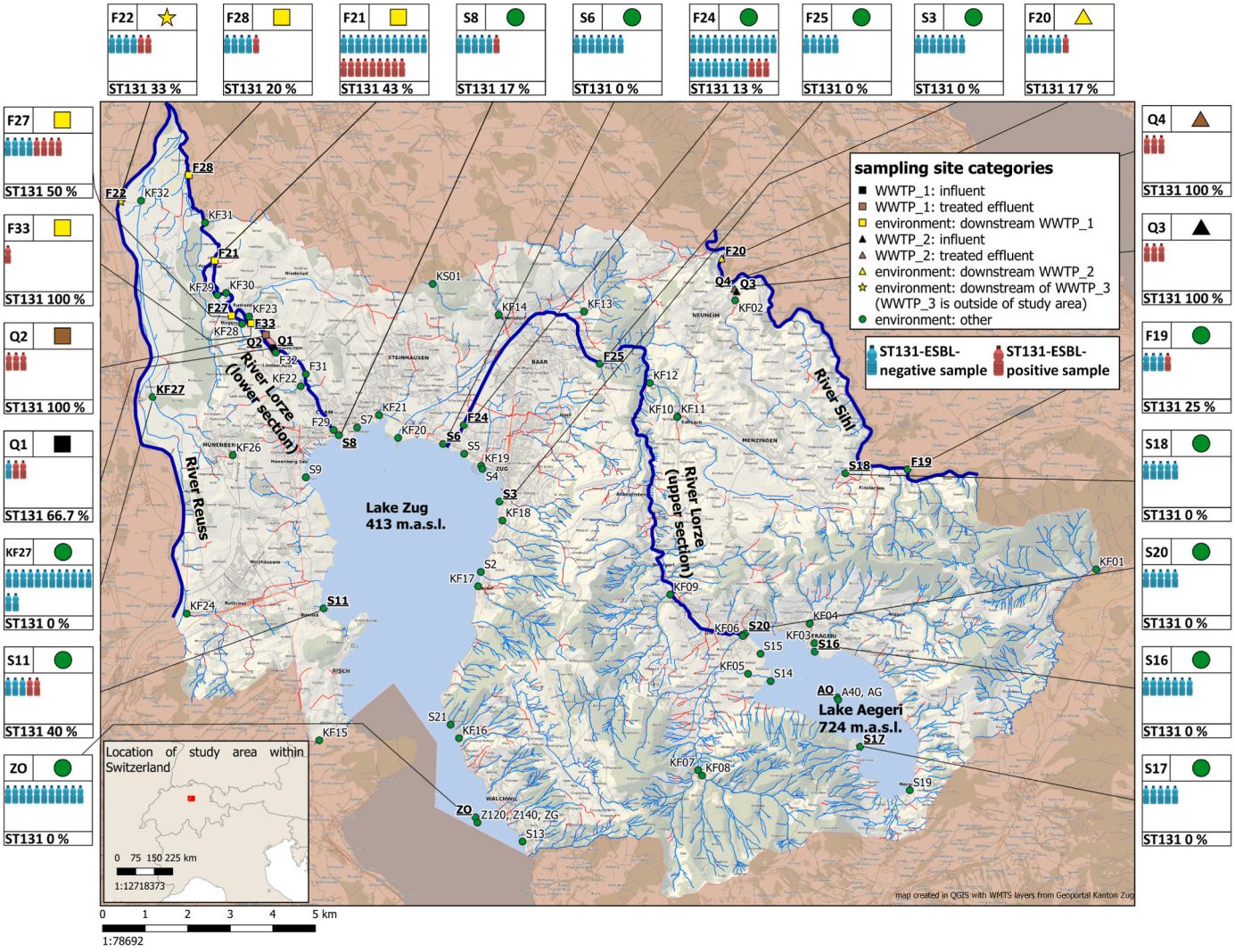

Fig. 1. Map of the study area and ST131-positivity rates of selected sampling sites. Sampling sites are indicated according to the legend and labelled by site ID. For WWTP influent and effluent sites and environmental sites with at least one positive sample or sampled at least 5 times, ST131-positivity rates are shown in boxes and IDs in the map are highlighted in bold.
ESBL: extended-spectrum beta-lactamase, m.a.s.l.: meters above sea level, WWTP: wastewater treatment plant.

from livestock farming. As rainwater run-off and sewage are combined and collected in a single wastewater system, untreated wastewater can overflow from various sections of the system into Lake Zug and the upper and lower sections of the river Lorze during heavy rainfall. With its main surface water and wastewater system, an established water surveillance platform, and a diverse population and geography, the canton of Zug is a potential model area for antimicrobial resistance (AMR) surveillance.

2.2. Description of the sampling sites

This surveillance program included 7 sampling sites in the canton's two WWTPs and 69 environmental surface water sampling sites. Samples were taken between August 2020 and January 2022. WWTP sampling sites included the influent (raw wastewater), activated sludge, retention basin wastewater, and treated effluent, and were sampled 1 to 3 times each. Environmental sampling sites were selected to represent the two major lakes in the area (Lake Zug and Lake Aegeri), the major rivers (Lorze, Sihl, and Reuss), as well as small lakes and creeks covering urban, rural, and farming areas (Fig. 1). The environmental sampling sites included popular swimming spots and six sites near (1–5 km) WWTP effluent discharge (Fig. 1 and Supplementary Table S1). Four sites (F21, F27, F28, F33) at the river Lorze were within 5 km downstream of WWTP_1. One of those (F28) may also be affected by discharge from two WWTPs located in a neighbouring canton (ARA Maschwanden, 600 individuals, 160 m^3/day, and ARA Knonau, 8500 individuals, 3500 m^3/day). One sampling site (F20) at the river Sihl was located within 5 km downstream of WWTP_2. An additional sampling site at the river Reuss (F22) was within 5 km downstream of WWTP ARA Sins (WWTP_3), which is in a neighbouring canton and serves 12,000 individuals (2300 m^3/day). While sites at major rivers and lakes were sampled up to 23 times, most small creeks were sampled once (Supplementary Table S1). Creeks were usually tested after rainfall, increasing the chance of containing manure and soil runoff.

2.3. Sampling procedure

All surface water samples were collected using sterile 1 L glass bottles containing sodium-thiosulfate. Surface water samples represent single time point samples. Sampling poles were used to collect samples from the shoreline or difficult-to-reach sampling spots, usually 10 cm below the water surface. Water samples from rivers and creeks were collected from the shoreline or via boat from free-flowing areas. Samples were transported at ambient temperature to the laboratory and analyzed the same day, usually within 4 h after sampling.

Samples from the WWTPs were collected in sterile 1 L plastic flasks containing sodium thiosulfate. Raw sewage and treated WWTP effluent samples consisted of flow-proportional 24-h and 48-h composite samples. These samples were stored at 4–8 °C until being transported to the laboratory for analysis within 4 h. Sewage sludge and retention basin wastewater samples represent single time point samples.

2.4. Bacterial isolation from water samples

All samples were first homogenized by vigorous shaking. Surfaces water and effluent wastewater aliquots (100 mL) were filtered through a 0.45 μm membrane (diameter 47 mm, cellulose ester with grid lines, Millipore) and membranes transferred into 10 mL *Enterobacteriaceae* enrichment broth (EE Broth Mossel, Becton Dickinson). Influent and retention basin wastewater sample aliquots (100 mL) were filtered through an 8.0 μm pre-filter followed by a 0.45 μm main filter. Due to particle clogging, some aliquots were split into two sets of 50 mL and filtered independently. All pre- and main filters were then transferred into a single EE broth tube. Activated sludge sample aliquots (400 μL) were added directly into 10 mL EE broth. After incubation for 24 h at 37 °C without shaking, one inoculation loop (approximately 1 μL) of the enriched EE broth was streaked on Brilliance ESBL agar (Oxoid) and incubated for 24 h at 37 °C.

Due to a changed focus of the cantonal surveillance program, a modified protocol was used for the last sample set collected in January 2022. Lake water (1 L), river water (1 mL and 10 mL), and WWTP effluent samples (10 mL) were filtered (0.45 μm) and membranes were placed directly on Brilliance ESBL agar plates before incubation for 24 h at 37 °C. For WWTP effluent samples, undiluted 1 mL aliquots were additionally spread directly on Brilliance ESBL agar (instead of filtering). For WWTP influent samples, 10^{-1} and 10^{-2} dilutions in sterile PBS were plated for qualitative analysis on Brilliance ESBL agar before incubation for 24 h at 37 °C.

Subsequent isolation of presumptive ESBL-producing bacteria was performed by picking isolates matching the ESBL-producing colony morphology according to the manufacturer's instructions and further purified on Tryptic Soy Agar (TSA, Biolife). Once purified, isolates were first tested for oxidase activity using Bactident Oxidase strips (Merck). Isolates matching ESBL-producing morphologies on Brilliance ESBL agar and showing an oxidase-negative phenotype were considered as presumptive ESBL-producers and transferred into cryo tubes containing 1 mL BHI broth (Biolife) with 30% glycerol for storage at −80 °C.

2.5. Identification of E. coli ST131 isolates

Isolates considered presumptive ESBL producers were subjected to matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS, Bruker Daltonics) followed by either multilocus sequence typing (MLST) or an ST131-specific colony PCR assay (Matsumura et al., 2017). For each observed morphotype on Brilliance ESBL agar one representative colony was tested for ST131 affiliation. From January 2022, approximately 30 colonies were screened for each wastewater sample irrespective of their morphotype, including at least one colony of each distinct morphotype. MLST was conducted by amplification and sequencing of the seven housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* (Wirth et al., 2006). For the ST131-specific assay, primers ST131_R19-YF1 und ST131_R19-YR1 (final concentration 0.15 μM; Microsynth) were used in combination with a 2x Taq Master Mix (Dye plus, Vazyme). The PCR conditions were 2 min at 95 °C for initial denaturation, 35 cycles of 15 s at 95 °C, 30 s at 57 °C, and 35 s at 72 °C followed by a final extension of 7 min at 72 °C. As controls, an MLST-confirmed ST131 strain, sterile water, and non-ST131 *E. coli* DNA were included. Electrophoresis was performed using a 1.5% agarose gel (Agarose basic, AppliChem). The expected DNA amplicon size was 580 bp. Antimicrobial susceptibility testing for ciprofloxacin was performed using the disc diffusion method (5 μg; BD). Selected isolates were additionally tested for meropenem, imipenem, and ertapenem susceptibility using Etests (0.002–32 μg/mL each; bioMérieux). Breakpoints were interpreted according to EUCAST guidelines (The European Committee on Antimicrobial Susceptibility Testing, 2022).

2.6. Whole genome sequencing

Genome sequences were determined using short- and long-read sequencing. Genomic DNA was isolated using the New England Biolabs HMW DNA extraction kit T3060L (New England Biolabs). For short-read sequencing, libraries were prepared using the Nextera DNA Flex Library Preparation Kit (Illumina) and sequenced on the Illumina MiniSeq platform (2 × 150 bp). Read trimming and quality control were performed with fastp v0.20.1 (Chen et al., 2018). For long-read sequencing, libraries were prepared using the SQK-LSK109 kit paired with the EXP-NBD104 and EXP-NBD114 native barcoding system and the flow cell priming kit EXP-FLP002 (all Oxford Nanopore Technologies). The libraries were sequenced on a MinION Mk1B device using MinION R9.4.1 flow cells (Oxford Nanopore Technologies). Basecalling (high accuracy), adapter trimming, and demultiplexing were performed

using MinKNOW (version 22.03.6) with default settings.

2.7. Genome analysis

Hybrid assemblies were generated using the Unicycler v0.5 pipeline (Wick et al., 2017) and annotated with Bakta v1.6.1 (Schwengers et al., 2021). Sequence types were confirmed using mlst 2.22.0 (https://github.com/tseemann/mlst). Assembly-based core genome alignments were generated with SKA 1.0 (Harris, 2018) and recombinant regions in the alignments identified using gubbins 2.4.1 (Croucher et al., 2015). Maximum-likelihood phylogenetic trees were constructed from the recombinant-free cgSNP alignment using IQ-TREE 2.0.3 (Nguyen et al., 2015) with the generalized time-reversible (GTR) model with gamma distribution, 100 bootstraps, and the number of invariant sites (-fconst option), which were determined from the core genome alignment using SNP-sites 2.5.1 (-C option) (Page et al., 2016). For the identification of SNP clusters, pairwise high-quality cgSNP distances were determined separately for each clade using the CFSAN SNP pipeline 2.2.1 (Davis et al., 2015). Genetically identical isolates (≤5 pairwise cgSNPs) originating from the same sample were excluded from the study. ST131 clade affiliation was determined based on phylogenetic clustering with previously assigned isolates (reference collection; listed in Supplementary Table S3) and assignment supported by the isolates' *fimH* type and serotype determined with FimTyper v1.1 (Roer et al., 2017) and ECTyper 1.0.0 (Bessonov et al., 2021), respectively.

Antimicrobial resistance genes and mutations in quinolone-resistance determining regions (QRDRs) were identified using AMRFinderPlus (Feldgarden et al., 2021). Virulence-associated genes were identified using ABRicate 1.0.0 (https://github.com/tseemann/abricate) (minimum sequence coverage/identity 70%/90%) in conjunction with the EcVGDB database (Biggel et al., 2020). IncF family plasmid replicon alleles were identified using the pubMLST RESTful API v1.27.0 (Jolley et al., 2017) (IncF RST scheme) and replicon types of other families with ABRicate v0.9.3 (Seemann) in conjunction with the PlasmidFinder database (Carattoli et al., 2014) (minimum sequence coverage/identity 70%/90%). Chromosomal insertion sites of *bla*CTX-M transposition units were identified by manually comparing the surrounding region in the assembly with the respective region of phylogenetic neighbours. Pre-assembled genomes of global ST131 isolates collected between 2020 and 2022 were retrieved from Enterobase (Alikhan et al., 2018) accessed in January 2023. Assemblies with N50 < 25 kb, coverage <30x, assembly length >5.6 Mb, or >200,000 low-quality bases were excluded. Details on assemblies are provided in Supplementary Table S3.

2.8. Data availability

Genome assemblies and sequencing data generated as part of this study are available under NCBI Bioproject No. PRJNA961666. Individual accession numbers of assemblies included in this study are listed in Supplementary Tables S2 and S3.

3. Results

3.1. Surface water downstream of WWTP effluent is frequently polluted by E. coli ST131

In this study, we screened wastewater, lakes, rivers, and creeks in the canton of Zug over 18 months (August 2020 to January 2022) for ESBL-producing ST131 (Fig. 1). The canton's two WWTPs were sampled at the influent (n = 6), retention basin (n = 2), activated sludge (n = 3), and treated effluent (n = 6). Overall, 16/17 (94.1%) WWTP samples tested positive for ST131, including all treated effluent samples. A total of 219 surface water samples were collected at 69 environmental sites (1–23 samples per site) (Supplementary Table S1). Six of those were WWTP-associated, i.e., located between 1 and 5 km downstream of WWTP effluent sites. For an initial cross-sectional evaluation, only the first sample of each site was considered. Among those, four (5.8%) tested positive: two (33.3%) WWTP-associated and two (3.2%) of the remaining environmental sites. Overall, 25/219 (11.4%) of the environmental samples were positive: 18/47 (38.3%) from WWTP-associated sites and 7/172 (4.1%) from non-WWTP-associated sites. Of the 7 positive samples from non-WWTP-associated sites, 6 were collected near sewage overflow sites. Data on overflow events was available for one of these sites (F24). Notably, two of the three positive samples from this site were collected within 3 days of an overflow event.

Both WWTPs discharge into rivers. Consequently, when categorized by water body type, river samples had higher positivity rates (22/84 [26.2%]) than surface water samples from lakes (3/89 [3.4%]) or creeks (0/46 [0%]). The two most contaminated environmental sites were located 1 km and 3 km downstream of the effluent of the region's main WWTP (WWTP_1), yielding positivity rates of 50% (4/8) and 43% (9/21), respectively (Fig. 1 and Supplementary Table 1). One of the two highly contaminated sites downstream of WWTP_1 is a public bathing site. Among 69 samples from 19 bathing sites not located downstream of WWTP effluents, three (4.3%) samples from two sites tested positive.

3.2. SNP analyses suggest repeated environmental contamination with long-term resident clones

Following whole-genome sequencing of all recovered isolates (n = 58) and exclusion of identical same-sample isolates (n = 1), genome assemblies of 57 ST131 isolates were available for genetic characterization. These included 26 isolates from WWTP samples, 27 isolates from WWTP-associated environmental sites, and 4 isolates from other environmental sites. Some samples yielded more than one and up to six genetically distinct ST131 isolates.

To investigate a potential recent shared source of the isolates, pairwise cgSNP distances were determined. Out of the 57 isolates, 24 (42%) belonged to one of five SNP clusters (≤5 pairwise cgSNP distance; cl. 1 to 5) with 2–10 isolates each (Fig. 2). Three of the clusters (cl. 3, 4, and 5) comprised isolates from environmental sites that matched genetically with isolates from the respective upstream WWTP. Isolates within these clusters were collected up to 12 months apart (Supplementary Table S2). Cluster 2 comprised an isolate from WWTP_1 that matched with an environmental isolate (AVS0501) obtained two months earlier downstream of an overflow basin connected to WWTP_1. Cluster 1 comprised two isolates obtained on the same day from WWTP_1's influent and effluent, respectively. Our data suggest residency or repeated entry of ST131 clones in WWTPs with frequent spillover into the environment.

3.3. Population structure and genetic characteristics

The 5 representative isolates from each SNP cluster and 33 unique isolates were further genetically characterized. Most of the isolates belonged to ST131 clades A (n = 16) or C1 (n = 16) (Fig. 2). Clade B and C2 were represented by one and 5 isolates, respectively.

As expected, all C1 and C2 isolates harboured five chromosomal mutations in QRDRs (*gyrA* D87N and S83L; *parC* E84V and S80I; *parE* I529L) and were highly resistant to ciprofloxacin (inhibition zone diameter [IZD] 6 mm) (Fig. 2). The five QRDR mutations and high-level ciprofloxacin resistance were also detected in three clade A isolates. A fourth clade A isolate, AVS0662, contained four QRDR mutations (*gyrA* S83L; *parC* E84V and S80I; *parE* I529L) and was also classified as resistant (IZD 19 mm).

All isolates possessed at least one ESBL-encoding *bla*CTX-M gene, which were chromosomally integrated (n = 14), plasmidal (n = 23), or both (n = 1; isolate AVS0747) (Fig. 2). The 16 **clade A** isolates contained either *bla*CTX-M-15 or *bla*CTX-M-27. Six of these isolates belonged to the internationally circulating *papGII*-positive sublineage in clade A, which is characterized by the stable chromosomal integration of a *bla*CTX-M-27 transposition unit into the *gspD* gene and a pathogenicity island (PAI)

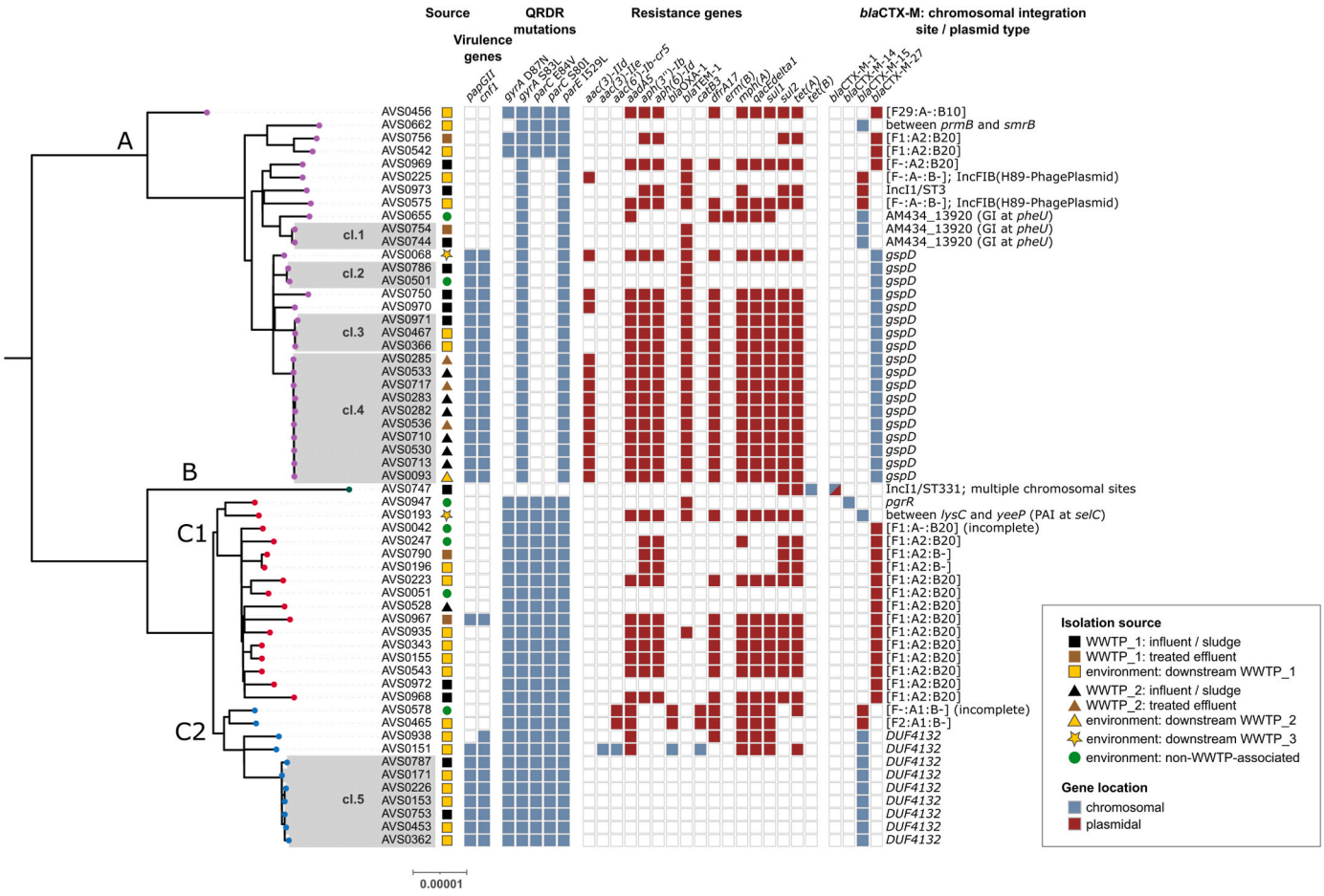

Fig. 2. Phylogenetic tree and genetic characteristics of 57 ESBL-producing ST131 water isolates. The 57 isolates were recovered from 41 water samples. Genetically identical same-sample isolates were excluded from the study. Branch tips indicate ST131 clade affiliations (clade A: purple; B: green; C1: red; C2: blue). The presence of selected virulence genes, QRDR (quinolone resistance determining region) mutations, and acquired antimicrobial resistance genes is indicated according to their location in blue (chromosomal) or red (plasmid-located). The genetic context of ESBL-encoding genes (i.e., the plasmid type or chromosomal insertion site of transposition units) is described on the right. Isolates belonging to SNP clusters (≤5 pairwise cgSNPs) are shaded in grey. The maximum likelihood tree is based on 1376 recombination-free variable sites identified in a 4.0 Mbp core genome alignment. The number of substitutions per core genome alignment site is indicated by the scale bar. The tree was visualized in iTOL v6.7 (Letunic and Bork, 2019).

at the tRNA-*pheU* locus containing the virulence factors *papGII*, *cnf1* (cytotoxic necrotizing factor 1), *ucl* (Ucl fimbriae), and *hly* (alpha-hemolysin) (Biggel et al., 2022). Two clade A isolates carried a *bla*CTX-M-15 transposition unit integrated into an uncharacterized gene (AM434_13,920 in assembly CP021935.1; hypothetical protein) located in a genomic island at the tRNA-*pheU* locus. Clade A isolate AVS0662 had *bla*CTX-M-15 integrated between the *smrB* and *prmB* genes. The remaining clade A isolates possessed *bla*CTX-M-15 or *bla*CTX-M-27 on plasmids. AVS0225 and AVS0575 carried *bla*CTX-M-15 on highly similar 111 kb phage-like plasmids typed as IncFIB(H89-PhagePlasmid). pAVS0225-A was near identical (one SNP and one complex mutation [CTT - > ACTTA], no structural variation) to pECOH89 (Genbank accession HG530657.1) described in 2014 (Falgenhauer et al., 2014), suggesting unusually high sequence conservation. Notably, clade A isolate AVS0542 carried *bla*CTX-M-27 on an F1:A2:B20 plasmid and contained the M27PP1 prophage-like region; both features are typically associated with the C1-M27 subclade (Matsumura et al., 2016).

The only **clade B** isolate (AVS0747) contained eight *bla*CTX-M-1 transposition units distributed across the chromosome and an additional unit on an IncI1/ST331 plasmid. One of the chromosomal units was integrated into the regulatory region (−11) of *ompF*. OmpF is a porin involved in the passive uptake of carbapenems, as is a second porin encoded by *ompC* (Pagès et al., 2008), which in AVS0747 was disrupted by an IS*10L*-like element. The simultaneous defects of OmpC and OmpF did however not result in carbapenem resistance (MIC imipenem: 0.125 mg/L, meropenem: 0.016 mg/L, ertapenem: 0.008 mg/L).

Of the 16 **cla**de C1 isolates, 14 harboured blaCTX-M-27 on F1:A2:B- or F1:A2:B20 plasmids. All 14 carried the C1-M27 subclade-specific M27PP1 prophage-like region, and three of those additionally carried the M27PP2 region (Supplementary Table S2). Of the two C1 isolates with chromosomally encoded ESBLs, AVS0193 had a *bla*CTX-M-14 transposition integrated between *lysC* and *yeeP*, which are part of a 50 kb PAI at the tRNA-*selC* site containing an *afa/dra* operon; and AVS0947 had a *bla*CTX-M-14 transposition unit integrated into *pgrR*, encoding a regulator of MppA (murein peptide permease A). The *pgrR-mppA* operon was previously described as the integration site of *bla*CTX-M-15 in a clade C2 sublineage (Biggel et al., 2022; Ludden et al., 2020).

All five **clade C2** isolates possessed *bla*CTX-M-15. Three of those belonged to the internationally circulating *papGII* + sublineage L3, characterized by the chromosomal integration of *bla*CTX-M-15 into a *DUF4132* gene (located upstream of *yehI/yehK*) and the presence of a *papGII*- and *cnf1*-containing pathogenicity island (PAI) (Biggel et al., 2022). The two remaining C2 isolates possessed *bla*CTX-M-15 on plasmids.

We subsequently investigated the phylogenetic diversity of the 38 de-duplicated study isolates within the context of 1155 global isolates that had assemblies available on Enterobase and were collected during a similar period (2020–2022). The global isolates were almost equally

divided into clades A, B, C1, and C2 (Fig. 3). While the Swiss water isolates did not show pronounced clustering, they were overrepresented in clade A and the subclade C1-M27. Three of the ciprofloxacin-resistant clade A isolates were part of a monophyletic branch consisting of 44 global isolates, of which 40 harboured at least four QRDR mutations in variable combinations, including combination *gyrA* (S83L, D87N) + *parC* (S80I, E84V) + *parE* (I529L) (n = 18) and combination *gyrA* (S83L, D87N) + *parC* (S80I) + *parE* (E460D) (n = 15) (Supplementary Table S3).

4. Discussion

The discharge of wastewater from human and animal sources into the environment can lead to the bacterial contamination of water bodies, soil, and food products, thereby facilitating their spread. WWTPs substantially reduce bacterial loads in wastewater and therefore have an imperative role in minimizing the impact on the water ecosystem and human health (Cutler and Miller, 2005). Nevertheless, our analyses of WWTP and environmental water samples in the canton of Zug suggest that strains of the multi-drug resistant lineage ST131 are continuously released via wastewater effluent. All effluent samples and 38% of the samples from rivers taken within 5 km downstream of discharge sites were ST131-positive. Incomplete removal of ESBL-producing *E. coli* from wastewater has previously been recognized as a public health issue: studies in France, Sweden, and the Netherlands estimated that 10^{11}–10^{12} ESBL-producing *E. coli* were released daily from the investigated WWTPs via treated effluent (Kwak et al., 2015; Bréchet et al., 2014; Blaak et al., 2014).

The release of untreated wastewater from WWTPs or the sewage system during intense rainfall is known to contribute significantly to environmental contamination with bacteria and antimicrobial resistance genes (Lee et al., 2022; Honda et al., 2020). Sewage overflow may explain the occasional detection of ST131 in environmental water from areas with sewage infrastructure (but distant from WWTP discharge sites) in our study. A systematic analysis of overflow events for one site indeed revealed a correlation with sample positivity.

Given the widespread carriage in humans, we expected to recover heterogeneous ST131 strains from the wastewater samples. However, specific clonal strains appeared to be dominant and were repeatedly isolated over up to 12 months and at different sites along the water flow (WWTP influent – sludge – effluent – river). This increased prevalence of specific strains may be explained by their circulation in the community or healthcare institutions. Outbreaks of ST131 have for example been reported in long-term care facilities and hospitals (Ludden et al., 2020; Boll et al., 2020; Woksepp et al., 2017). We also cannot rule out the possibility that these strains permanently reside in wastewater systems, for example as biofilms. Previous longitudinal studies suggest that specific *E. coli* clones may persist in wastewater drainage over several years

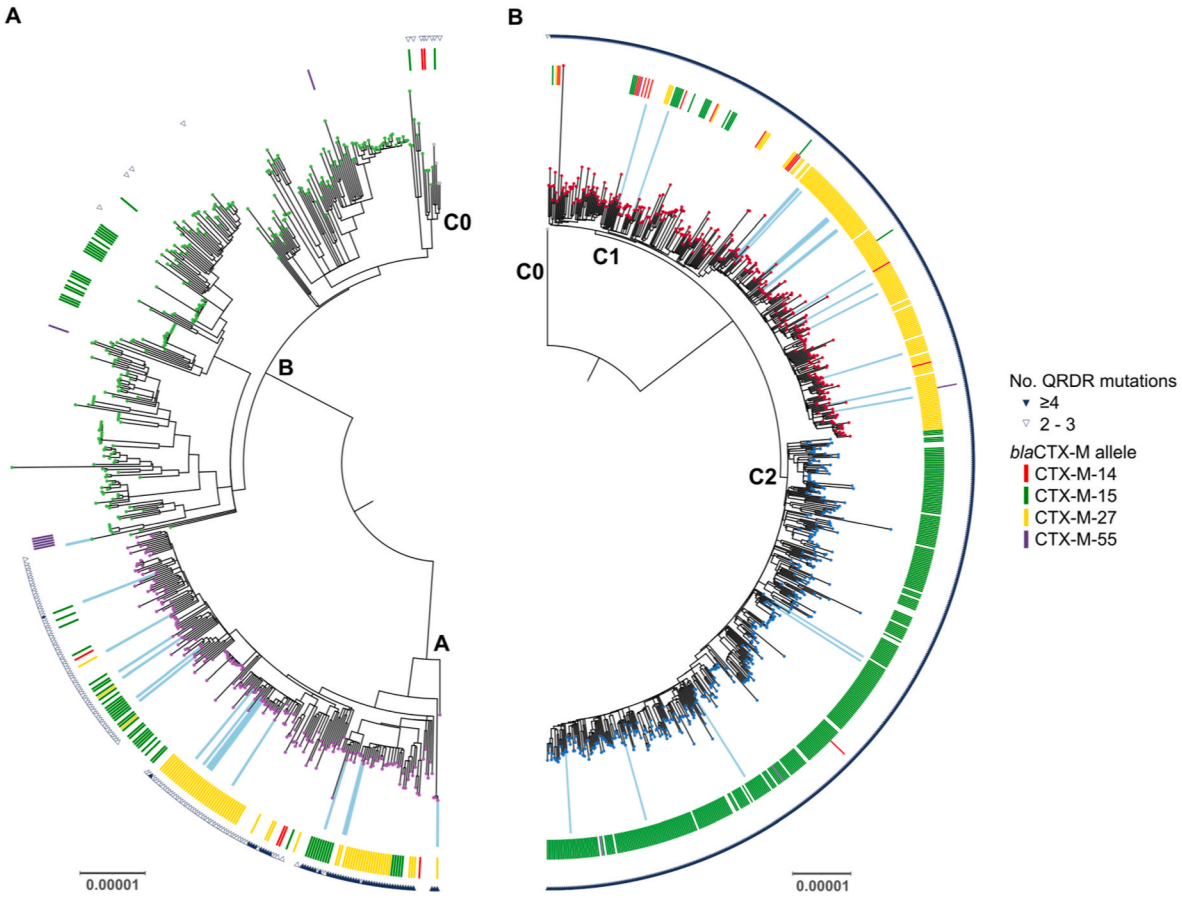

Fig. 3. Phylogeny of the 38 deduplicated ST131 study isolates and 1168 global ST131 isolates. Global isolates include 1155 assemblies from Enterobase collected between 2020 and 2022; and 13 reference assemblies with previously determined clade affiliations. Bright blue shades indicate the positions of the 38 water isolates obtained as part of this study. The number of mutations in quinolone resistance determining region (QRDRs)* and the presence of dominant *bla*CTX-M alleles is labelled in the outer rings. **A.** Phylogenetic tree of 506 isolates from clades A (purple branch tips), B (green), and C0 (grey). The tree is based on 9763 nonrecombinant variable sites in a 3.2 Mbp core genome alignment. **B.** Phylogenetic tree of 701 isolates from clades C1 (red) and C2 (blue), including a clade C0 isolate (grey) as an outgroup. The tree is based on 11,526 nonrecombinant variable sites in a 2.9 Mbp core genome alignment. The number of substitutions per core genome alignment site is indicated by the scale bar. The trees were visualized in iTOL v6.7 (Letunic and Bork, 2019).
*considered mutations include *gyrA* D87G, D87N, D87Y, and S83L; *parC* A108T, E84A, E84V, S57T, S80I, and S80R; *parE* E460D, I529L, L445H, and S458A.

(Carlsen et al., 2023; Paulshus et al., 2023).

Compared to ST131 isolates obtained in other countries between 2020 and 2022, the Swiss water isolates were strongly overrepresented in clades A and C1. Because international travel was restricted during most of the study period due to the COVID-19 pandemic, we assume that our collection represents carriage of ESBL-producing ST131 in the local community. Nevertheless, many isolates belonged to known internationally distributed ST131 sublineages. These included the C1-M27 lineage and two C2 *papGII* + sublineages with characteristic chromosomal *bla*CTX-M integration sites. Notably, several clade A isolates were resistant to ciprofloxacin and belonged to an emerging sublineage that contained four or five QRDR mutations. Ciprofloxacin-resistant clade A isolates were recently reported to be common in wastewater in Canada (Finn et al., 2020) and isolated from various sources in Australia (Li et al., 2021).

A limitation of this study was that quantitative data on the abundance of ST131 in the samples were not available, precluding conclusions on the efficiency of the wastewater treatment process. Previous studies reported a 98–99.9% reduction of ESBL-producing *E. coli* during treatment to 20–120 CFU/mL in the effluent (Kwak et al., 2015; Bréchet et al., 2014).

In conclusion, our study suggests that effluent discharge and wastewater overflow represent important sources of environmental ST131 contamination. The implementation of disinfection steps during wastewater treatment such as membrane filtration, chlorination, or ozonation and improved infrastructure such as separate stormwater and sewage systems to prevent untreated wastewater overflow may reduce the spread of pathogens and antimicrobial resistance.

CRediT authorship contribution statement

Michael Biggel: Formal analysis, Writing – original draft, Visualization. **Sarah Hoehn:** Investigation, Resources, Writing – review & editing, Visualization. **Andrea Frei:** Investigation. **Kira Dassler:** Investigation. **Christoph Jans:** Conceptualization, Writing – review & editing, Supervision. **Roger Stephan:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

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

Data availability

Genome assemblies and sequencing data generated as part of this study are available under NCBI Bioproject No. PRJNA961666.

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Appendix A. Supplementary data

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References

Alikhan, N.-F., Zhou, Z., Sergeant, M.J., Achtman, M., 2018. A genomic overview of the population structure of *Salmonella*. In: Casadesús, J. (Ed.), PLOS Genet [Internet] 14 (4), e1007261. https://dx.plos.org/10.1371/journal.pgen.1007261.

Ben Zakour, N.L., Alsheikh-Hussain, A.S., Ashcroft, M.M., Khanh Nhu, N.T., Roberts, L. W., Stanton-Cook, M., et al., 2016. Sequential acquisition of virulence and fluoroquinolone resistance has shaped the evolution of *Escherichia coli* ST131. MBio [Internet] 7 (2), 1–11. http://mbio.asm.org/lookup/doi/10.1128/mBio.00347-16.

Bessonov, K., Laing, C., Robertson, J., Yong, I., Ziebell, K., Gannon, V.P.J., et al., 2021. ECTyper: in silico Escherichia coli serotype and species prediction from raw and assembled whole-genome sequence data. Microb Genomics [Internet] 7 (12). https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000728.

Biggel, M., Xavier, B.B., Johnson, J.R., Nielsen, K.L., Frimodt-Møller, N., Matheeussen, V., et al., 2020. Horizontally acquired *papGII*-containing pathogenicity islands underlie the emergence of invasive uropathogenic *Escherichia coli* lineages. Nat Commun [Internet 11 (1), 5968. https://doi.org/10.1038/s41467-020-19714-9.

Biggel, M., Moons, P., Nguyen, M.N., Goossens, H., Van Puyvelde, S., 2022. Convergence of virulence and antimicrobial resistance in increasingly prevalent *Escherichia coli* ST131 *papGII*+ sublineages. Commun. Biol. [Internet 5 (1), 752. https://www.nature.com/articles/s42003-022-03660-x.

Blaak, H., de Kruijf, P., Hamidjaja, R.A., van Hoek, A.H.A.M., de Roda Husman, A.M., Schets, F.M., 2014. Prevalence and characteristics of ESBL-producing *E. coli* in Dutch recreational waters influenced by wastewater treatment plants. Vet. Microbiol. [Internet 171 (3–4), 448–459. https://doi.org/10.1016/j.vetmic.2014.03.007.

Boll, E.J., Overballe-Petersen, S., Hasman, H., Roer, L., Ng, K., Scheutz, F., et al., 2020. Emergence of enteroaggregative *Escherichia coli* within the ST131 lineage as a cause of extraintestinal infections. Kline KA. MBio. [Internet]. 11 (3), 1–13. http://mbio.asm.org/lookup/doi/10.1128/mBio.00353-20.

Bréchet, C., Plantin, J., Sauget, M., Thouverez, M., Talon, D., Cholley, P., et al., 2014. Wastewater treatment plants release large amounts of extended-spectrum β-lactamase–producing *Escherichia coli* into the environment. Clin. Infect. Dis. [Internet 58 (12), 1658–1665. https://academic.oup.com/cid/article-lookup/doi/10.1093/cid/ciu190.

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., et al., 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob. Agents Chemother. [Internet] 58 (7), 3895–3903. http://aac.asm.org/lookup/doi/10.1128/AAC.02412-14.

Carlsen, L., Büttner, H., Christner, M., Cordts, L., Franke, G., Hoffmann, A., et al., 2023. Long time persistence and evolution of carbapenemase-producing *Enterobacterales* in the wastewater of a tertiary care hospital in Germany. J. Infect. Publ. Health [Internet]. https://doi.org/10.1016/j.jiph.2023.05.029.

Chen, S., Zhou, Y., Chen, Y., Gu, J., 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics [Internet] 34 (17), i884–i890. https://academic.oup.com/bioinformatics/article/34/17/i884/5093234.

Croucher, N.J., Page, A.J., Connor, T.R., Delaney, A.J., Keane, J.A., Bentley, S.D., et al., 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic. Acids Res. [Internet] [cited 2021 Feb 15]; 43(3):e15–e15. https://academic.oup.com/nar/article/43/3/e15/2410982.

Cutler, D., Miller, G., 2005. The role of public health improvements in health advances: the twentieth-century United States. Demography. [Internet] 42 (1), 1–22. https://read.dukeupress.edu/demography/article/42/1/1/170249/The-role-of-public-health-improvements-in-health.

Davis, S., Pettengill, J.B., Luo, Y., Payne, J., Shpuntoff, A., Rand, H., et al., 2015. CFSAN SNP Pipeline: an automated method for constructing SNP matrices from next-generation sequence data. PeerJ. Comput. Sci. [Internet] 1 (8), e20. https://peerj.com/articles/cs-20.

Day, M.J., Hopkins, K.L., Wareham, D.W., Toleman, M.A., Elviss, N., Randall, L., et al., 2019. Extended-spectrum β-lactamase-producing *Escherichia coli* in human-derived and foodchain-derived samples from England, Wales, and Scotland: an epidemiological surveillance and typing study. Lancet. Infect. Dis. [Internet] 19 (12), 1325–1335. https://doi.org/10.1016/S1473-3099(19)30273-7.

Fachstelle für Statistik des Kantons Zug und Zuger Kantonalbank. Der Kanton Zug in Zahlen 8, 2022. https://www.zg.ch/behoerden/gesundheitsdirektion/statistikfachstelle/themen/zug-in-zahlen.

Falgenhauer, L., Yao, Y., Fritzenwanker, M., Schmiedel, J., Imirzalioglu, C., Chakraborty, T., 2014. Complete genome sequence of phage-like plasmid pECOH89, encoding CTX-M-15. Genome Announc. 2 (2), 2–3.

Feldgarden, M., Brover, V., Gonzalez-Escalona, N., Frye, J.G., Haendiges, J., Haft, D.H., et al., 2021. AMRFinderPlus and the Reference Gene Catalog facilitate examination of the genomic links among antimicrobial resistance, stress response, and virulence. Sci. Rep. [Internet 11 (1), 12728. https://doi.org/10.1038/s41598-021-91456-0.

Finn, T.J., Scriver, L., Lam, L., Duong, M., Peirano, G., Lynch, T., et al., 2020. A comprehensive account of *Escherichia coli* sequence type 131 in wastewater reveals an abundance of fluoroquinolone-resistant clade A strains. Stams AJM. Appl. Environ. Microbiol. [Internet] 86 (4). https://journals.asm.org/doi/10.1128/AEM.01913-19.

Gladstone, R.A., McNally, A., Pöntinen, A.K., Tonkin-Hill, G., Lees, J.A., Skytén, K., et al., 2021. Emergence and dissemination of antimicrobial resistance in *Escherichia coli* causing bloodstream infections in Norway in 2002–17: a nationwide, longitudinal, microbial population genomic study. Lancet. Microbe. [Internet] 2 (7) e331–41. https://linkinghub.elsevier.com/retrieve/pii/S2666524721000318.

Harris, S.R., 2018. SKA: split kmer analysis toolkit for bacterial genomic epidemiology. bioRxiv. [Internet], 453142. https://www.biorxiv.org/content/10.1101/453142v1%0A. https://www.biorxiv.org/content/10.1101/453142v1.abstract.

Honda, R., Tachi, C., Yasuda, K., Hirata, T., Noguchi, M., Hara-Yamamura, H., et al., 2020. Estimated discharge of antibiotic-resistant bacteria from combined sewer overflows of urban sewage system. npj Clean Water [Internet] 3 (1), 15. https://doi.org/10.1038/s41545-020-0059-5.

Johnson, J.R., Porter, S.B., Johnston, B., Thuras, P., Clock, S., Crupain, M., et al., 2017. Extraintestinal pathogenic and antimicrobial-resistant Escherichia coli, including sequence type 131 (ST131), from retail chicken breasts in the United States in 2013. In: Schaffner, D.W. (Ed.), Appl. Environ. Microbiol. [Internet] 83 (6), 1–9. https://journals.asm.org/doi/10.1128/AEM.02956-16.

Jolley, K.A., Bray, J.E., Maiden, M.C.J., 2017. A RESTful application programming interface for the PubMLST molecular typing and genome databases. Database [Internet] 2017, 1–7. https://academic.oup.com/database/article/doi/10.1093/database/bax060/4079979.

Kallonen, T., Brodrick, H.J., Harris, S.R., Corander, J., Brown, N.M., Martin, V., et al., 2017. Systematic longitudinal survey of invasive *Escherichia coli* in England demonstrates a stable population structure only transiently disturbed by the emergence of ST131. Genome. Res. [Internet 27 (8), 1437–1449. http://genome.cshlp.org/lookup/doi/10.1101/gr.216606.116.

Kidsley, A.K., White, R.T., Beatson, S.A., Saputra, S., Schembri, M.A., Gordon, D., et al., 2020. Companion animals are spillover hosts of the multidrug-resistant human extraintestinal *Escherichia coli* pandemic clones ST131 and ST1193. Front. Microbiol. [Internet] 1–10. https://www.frontiersin.org/article/10.3389/fmicb.2020.01968/full.

Kudinha, T., Kong, F., 2022. Possible step - up in prevalence for *Escherichia coli* ST131 from fecal to clinical isolates : inferred virulence potential comparative studies within phylogenetic group. J. Biomed. Sci. [Internet 1–13. https://doi.org/10.1186/s12929-022-00862-7.

Kwak, Y.-K., Colque, P., Byfors, S., Giske, C.G., Möllby, R., Kühn, I., 2015. Surveillance of antimicrobial resistance among *Escherichia coli* in wastewater in Stockholm during 1 year: does it reflect the resistance trends in the society? Int. J. Antimicrob. Agents [Internet] 45 (1), 25–32. https://doi.org/10.1016/j.ijantimicag.2014.09.016.

Lee, J., Beck, K., Bürgmann, H., 2022. Wastewater bypass is a major temporary point-source of antibiotic resistance genes and multi-resistance risk factors in a Swiss river. Water Res. [Internet], 117827. https://doi.org/10.1016/j.watres.2021.117827.

Letunic, I., Bork, P., 2019. Interactive Tree of Life (iTOL) v4: recent updates and new developments. Nucleic. Acids Res. [Internet] 47. W1):W256–9. Available from: https://academic.oup.com/nar/article/47/W1/W256/5424068.

Li, D., Wyrsch, E.R., Elankumaran, P., Dolejska, M., Marenda, M.S., Browning, G.F., et al., 2021. Genomic comparisons of Escherichia coli ST131 from Australia. Microb Genomics [Internet] 7 (12). https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000721.

Liu, C.M., Stegger, M., Aziz, M., Johnson, T.J., Waits, K., Nordstrom, L., et al., 2018. Escherichia coli ST131-H22 as a foodborne uropathogen. In: Mobley, H.L.T., Ravel, J. (Eds.), MBio [Internet] 9 (4). https://journals.asm.org/doi/10.1128/mBio.00470-18.

Ludden, C., Decano, A.G., Jamrozy, D., Pickard, D., Morris, D., Parkhill, J., et al., 2020. Genomic surveillance of *Escherichia coli* ST131 identifies local expansion and serial replacement of subclones. Microb Genomics [Internet] 6 (4). https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000352.

Matsumura, Y., Pitout, J.D.D., Gomi, R., Matsuda, T., Noguchi, T., Yamamoto, M., et al., 2016. Global *Escherichia coli* sequence type 131 clade with *bla*CTX-M-27 gene. Emerg. Infect. Dis. [Internet] 22 (11). http://wwwnc.cdc.gov/eid/article/22/11/16-0519_article.htm.

Matsumura, Y., Pitout, J.D.D., Peirano, G., DeVinney, R., Noguchi, T., Yamamoto, M., et al., 2017. Rapid identification of different *Escherichia coli* sequence type 131 clades. Antimicrob. Agents Chemother. [Internet] 61 (8). https://journals.asm.org/doi/10.1128/AAC.00179-17.

Mohamed, M., Clabots, C., Porter, S.B., Bender, T., Thuras, P., Johnson, J.R., 2020. Large fecal reservoir of Escherichia coli sequence type 131-H30 subclone strains that are shared within households and resemble clinical ST131-H30 isolates. J. Infect. Dis. Immun. 221 (10), 1659–1668. https://academic.oup.com/jid/article/221/10/1659/5680216.

Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. [Internet] 32 (1), 268–274. https://academic.oup.com/mbe/article-lookup/doi/10.1093/molbev/msu300.

Page, A.J., Taylor, B., Delaney, A.J., Soares, J., Seemann, T., Keane, J.A., et al., 2016. SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. Microb Genomics [Internet] 2 (4), e000056. https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000056.

Pagès, J.M., James, C.E., Winterhalter, M., 2008. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat. Rev. Microbiol. 6 (12), 893–903.

Paulshus, E., Colque, P., Kühn, I., Tauhid, T., Hu, Y.O.O., Zhou, Y., et al., 2023. *Escherichia coli* ST2797 is abundant in wastewater and might Be a novel emerging extended-spectrum beta-lactamase *E. coli*. Jun S-R. Microbiol. Spectr. [Internet]. https://journals.asm.org/doi/10.1128/spectrum.04486-22.

Price, L.B., Johnson, J.R., Aziz, M., Clabots, C., Johnston, B., Tchesnokova, V., et al., 2013. In: Parkhill, J. (Ed.), The Epidemic of Extended-Spectrum-β-Lactamase-Producing *Escherichia coli* ST131 Is Driven by a Single Highly Pathogenic Subclone, H30-Rx, vol. 4. MBio [Internet]. https://journals.asm.org/doi/10.1128/mBio.00377-13.

Roer, L., Tchesnokova, V., Allesøe, R., Muradova, M., Chattopadhyay, S., Ahrenfeldt, J., et al., 2017. In: Diekema, D.J. (Ed.), Development of a Web Tool for *Escherichia coli* Subtyping Based on *fimH* Alleles, vol. 55, pp. 2538–2543. J Clin Microbiol [Internet]. http://jcm.asm.org/lookup/doi/10.1128/JCM.00737-17.

Schwengers, O., Jelonek, L., Dieckmann, M.A., Beyvers, S., Blom, J., Goesmann, A., 2021. Bakta: rapid and standardized annotation of bacterial genomes via alignment-free sequence identification. Microb. Genom. 7 (11).

Seemann, T.. Abricate. https://github.com/tseemann/abricate.

The European Committee on Antimicrobial Susceptibility Testing, 2022. Breakpoint Tables for Interpretation of MICs and Zone Diameters [Internet], Version 12.0. http://www.eucast.org.

Wang, J.-L., Ko, W.-C., Hung, C.-H., Cheng, M.-F., Wang, H.-Y., Shiau, Y.-R., et al., 2021. Temporal trend of ST131 clone among urinary *Escherichia coli* isolates in the community: a taiwan national surveillance from 2002 to 2016. Microorganisms [Internet] 9 (5), 963. https://www.mdpi.com/2076-2607/9/5/963.

Wick, R.R., Judd, L.M., Gorrie, C.L., Holt, K.E., 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLOS Comput. Biol. [Internet] 13 (6), e1005595. https://dx.plos.org/10.1371/journal.pcbi.1005595.

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., et al., 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol. Microbiol. [Internet] 60 (5), 1136–1151. https://onlinelibrary.wiley.com/doi/10.1111/j.1365-2958.2006.05172.x.

Woksepp, H., Ryberg, A., Berglind, L., Schön, T., Söderman, J., 2017. Epidemiological characterization of a nosocomial outbreak of extended spectrum β-lactamase *Escherichia coli* ST-131 confirms the clinical value of core genome multilocus sequence typing. APMIS [Internet] 125 (12), 1117–1124. https://onlinelibrary.wiley.com/doi/10.1111/apm.12753.

Wu, G., Day, M.J., Mafura, M.T., Nunez-Garcia, J., Fenner, J.J., Sharma, M., et al., 2013. Comparative analysis of ESBL-positive *Escherichia coli* isolates from animals and humans from the UK, The Netherlands and Germany. PLoS One 8 (9), 1–10.