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A comparative study on antibiotic resistant *Escherichia coli* isolates from Austrian patients and wastewater-influenced Danube River water and biofilms

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ABSTRACT

Antimicrobial resistance (AMR) poses a major threat to human health worldwide. AMR can be introduced into natural aquatic ecosystems, for example, from clinical facilities via wastewater emissions. Understanding AMR patterns in environmental populations of bacterial pathogens is important to elucidate propagation routes and develop mitigation strategies. In this study, AMR patterns of *Escherichia coli* isolates from urinary tract infections and colonised urinary catheters of inpatients and outpatients were compared to isolates from the Danube River within the same catchment in Austria to potentially link environmental with clinical resistance patterns. Susceptibility to 20 antibiotics was tested for 697 patient, 489 water and 440 biofilm isolates. The resistance ratios in patient isolates were significantly higher than in the environmental isolates and higher resistance ratios were found in biofilm in comparison to water isolates. The role of the biofilm as potential sink of resistances was reflected by two extended-spectrum beta-lactamase (ESBL) producing isolates in the biofilm while none were found in water, and by higher amoxicillin/clavulanic acid resistance ratios in biofilm compared to patient isolates. Although, resistances to last-line antibiotics such as carbapenems and tigecycline were found in the patient and in the environmental isolates, they still occurred at low frequency.

1. Introduction

In Europe, annually more than 35,000 people die from infections caused by antimicrobial-resistant microbes (ECDC, 2020), which is an alarmingly high number. Especially in human medicine but also in the treatment of animals, a large amount of antibiotics is used (Van Boeckel et al., 2014; Monahan et al., 2022). Although it is known since decades that the usage of antibiotics causes the acquirement and development of new resistance mechanisms in pathogens (Normark and Normark, 2002; Davies and Davies, 2010), to date antibiotics are still often over-used, specifically in countries with poor antimicrobial stewardship (Van Boeckel et al., 2014; Nandi et al., 2023). The clinical environment in all countries worldwide is known to be the main hotspot of AMR development and spread (Aleem et al., 2021). Here, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are transferred between patients, and in particular the clinical wastewater path is a potential sink of ARB and ARGs (Sib et al., 2019; Voigt et al., 2019). Clinical wastewaters are released to wastewater treatment plants (WWTPs) wherein a mixture of nutrients and harmful substances, different microbiomes from humans and animals and numerous other factors affect the establishment or removal of ARB and ARGs (Manaia et al., 2018). The wastewater is discharged to rivers and lakes, representing a potential transmission route of clinical ARB into the water environment. Not only the water itself, but also biofilms are known to harbour ARB and ARGs (Schwartz et al., 2003) in different aquatic ecosystems and it has been shown that the discharge of wastewater

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promotes the establishment of ARB and ARGs also in riverine biofilms (Aubertheau et al., 2017; Matviichuk et al., 2023).

For Europe, there are only few studies that have directly investigated the potential linkage between clinical and environmental resistances in pathogens. Already in 2013, a comparative investigation of sewage sludge and clinical *Escherichia coli* (*E. coli)* showed an increase of multi-resistance in both sample sets over one decade (Reinthaler et al., 2013). In a Polish study, a high proportion of extended spectrum beta lactamase (ESBL) producers were found in *E. coli* populations isolated from a river influenced by clinical wastewater (Korzeniewska et al., 2013). A Swedish study showed that clinical and river *E. coli* populations exhibited a similar diversity and that the distribution of ESBL genes was similar with a dominance of $bla_{\text{CTX-M-15}}$ (Fagerstrom et al., 2019). Lepuschitz et al. demonstrated that ESBL and carbapenem resistant *Klebsiella* isolates from Austrian rivers genetically clustered with clinical isolates from the cities which discharged their wastewater into these rivers (Lepuschitz et al., 2019). Specifically, ESBL producing bacteria which evolved after the introduction of cephalosporins as new beta-lactam antibiotics are of high relevance when investigating the link between environmental with clinical isolates (Grundmann et al., 2010).

The Danube River, the second longest river in Europe and an important receiving water for wastewater emissions, has been intensively studied concerning its faecal pollution status (Kirschner et al., 2009, 2017) and the occurrence of antibiotic resistance in different clinically relevant bacteria (Kittinger et al., 2016a, 2016b, 2017). For Enterobacteriaceae, *E. coli* was investigated as a representative indicator of faecal pollution and AMR. A comparison of the resistance proportions over the whole length of the Danube River showed that in the upper stretch (including Austria) already one third of the sampled isolates were resistant to one or two antibiotic classes and more than 10% were multi-resistant (Kittinger et al., 2016a).

The aim of this study was the comparison of the resistance patterns in *E. coli* isolates collected from patients with isolates from the Danube River in Austria considering in total 1626 isolates. We chose *E. coli* as model organism as it is known to be the most relevant agent causing urinary tract infections (UTIs) (Zagaglia et al., 2022) and is also frequently found in indwelling urethral catheters as contamination (Albu et al., 2018). Furthermore, in environmental waters *E. coli* is often used as indicator for AMR and recommended as indicator of ESBL in the environment by the WHO (Matheu and Aidara-Kane, 2017). *E. coli* isolates were collected from stationary patients in a large hospital and from outpatients living in the same geographical area to cover potential differences between the general population and hospital patients. To elucidate whether the resistances in the patient-associated *E. coli* population are reflected in the environmental *E. coli* population, river isolates were collected from two environmental compartments, water and biofilm, upstream and downstream of the discharge site of the municipal WWTP receiving the wastewaters from the respective hospital and the general population studied.

2. Material and methods

2.1. Isolation of E. coli

During the period from October 2020 till October 2021, *E. coli* were isolated from in- and outpatients at the University Clinic St. Pölten suffering either from UTI or from colonized urethral catheters. Urine samples were streaked onto agar plates selective for both gram-positive and gram-negative bacteria (CHROMID® CPS® Elite/Columbia CNA + 5% sheep blood (Biomérieux, France)). After 24 h of incubation at 37 °C, *E. coli* were streaked onto Müller-Hinton agar plates (Becton Dickinson, Germany) for an immediate antibiogram to guide the patients' treatment. Aliquots of those isolates were also stirred into 50% glycerol and stored at −20 °C. Every four to six weeks all collected isolates were transported to the laboratory at Karl Landsteiner University and stored at −80 °C until further processing.

During the same period, water and biofilm samples were taken on five occasions in quarterly (exact dates see Supplementary Table S1) intervals on the right river side of the Danube River around the WWTP Traismauer (280,000 P.E., tertiary treatment). This WWTP receives the wastewater of the university clinic and the regional population and discharges into the Danube River. On all dates, samples were taken directly upstream (48°22′29.8″ N, 15°46′40.8″ E) and downstream (48°22′31.2″ N, 15°46′45.5″ E) of the outlet of the WWTP which is located 1.5 m below the water surface. During the sampling campaign the discharge varied between 1080 and 4390 m^3/s on the sampling dates, corresponding to a range from base-flow to one-year flood conditions. River water was taken in sterile 500 ml glass flasks approximately 30 cm below the water surface which is the same depth where we collected stones for biofilm sampling. Biofilm was scratched from 5 × 5 cm areas on at least nine submerged stones or branches with a sterile toothbrush, which was then combined and suspended in Ringer solution (1x, prepared with Thermo Scientific™ Oxoid™ Ringer Solution Tablets, Thermo Fisher Scientific, Austria). All samples were transported in a cooled box to the laboratory at the Karl Landsteiner University by car within 1 h.

For quantification of faecal pollution, the Colilert-18 system (IDEXX, Germany) was used according to manufacturer's instructions and most probable numbers (MPN) of *E. coli* were determined. Depending on the expected pollution level, water samples up to 100 ml were investigated, while always using 10 ml of the biofilm suspensions.

To obtain single *E. coli* isolates from water samples, a volume of five to 100 ml was filtered through a 45 μm pore-size cellulose nitrate filter (47 mm diameter; Sartorius, Germany) which was then placed onto Tryptone Bile X-Glucuronide (TBX) agar plates (Thermo Fisher Scientific, Austria), the recommended agar for quantifying and isolating *E. coli* from environmental water samples (Kemper et al., 2023). To isolate *E. coli* from the biofilm suspensions, 500 μl of the sample matrix was spread with a sterile spatula on the TBX agar plates. After overnight incubation (16–18 h) at 37 °C, blue presumptive *E. coli* colonies were streaked onto MacConkey agar plates (prepared in-house, according manufacturer's instructions on the agar powder, Thermo Fisher Scientific, Austria) for biomass enrichment. Sufficient biomass was taken from each *E. coli* culture, stirred into 50% glycerol and stored at −80 °C until further processing. About 10% of the environmental isolates for each sampling date were randomly selected and confirmed by Matrix Assisted Laser Desorption Ionisation - Time of Flight (MALDI-TOF).

2.2. Antibiotic susceptibility testing (AST)

All *E. coli* isolates (both environmental and patient) were tested for their susceptibility to 20 antibiotics from seven antibiotic classes (beta-lactams: ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefalexin, cefuroxime, cefoxitin, cefotaxime, ceftazidime, cefepime, imipenem, meropenem; aminoglycosides: gentamicin, amikacin; quinolones: moxifloxacin, ciprofloxacin; tetracyclines: tetracycline, tigecycline; chloramphenicols: chloramphenicol; folic acid antagonists: trimethoprim/sulfamethoxazole; polymyxins: colistin) with the disk-diffusion method. The isolates were thawed and streaked on MacConkey or Müller-Hinton agar plates (prepared in-house, according manufacturer's instructions of the agar powder, Thermo Fisher Scientific, Austria), from which colonies were stirred into 0.85% NaCl solution (prepared in-house). The McFarland turbidity, determined with a DensiChek (bioMérieux, France), of the bacterial suspensions was adjusted to 0.5 and ranged for all samples between 0.45 and 0.55. The bacterial suspension was plated with a cotton swab evenly on Müller-Hinton plates using a rotator and antibiotic disks (Thermo Fisher Scientific, Austria) were applied with a stamp (Oxoid, Germany). For quality control, on every day one plate with the NaCl solution to prove sterility and reference *E. coli* strain ATCC 25922, for antibiotic susceptibility testing was plated. Samples and controls were incubated overnight (16–18 h) at 35 °C. As breakpoints, the European Committee on

Antimicrobial Susceptibility Testing (EUCAST) criteria from 2020 (EUCAST, 2020) were used with two exceptions; colistin and tetracycline were evaluated according the guideline of the Clinical and Laboratory Standards Institute (CLSI) M100 (CLSI, 2020). All isolates were identified as susceptible or resistant according to EUCAST (2020) or CLSI M100, respectively.

2.3. ESBL identification

E. coli isolates that were resistant to antibiotics of the beta-lactam class were analysed for ESBL via the double-disk synergy test using cefotaxime and ceftazidime and these two antibiotics in combination with clavulanic acid (CLSI, 2020). A difference >5 mm between the pairs was considered as positive for ESBL-production. For identification of the ESBL genes, DNA from the isolates was extracted by boiling a colony in 50 µl double-deionized water for 10 min. Five different beta-lactamase families, $bla_{\text{CTX-M-1-group}}$, $bla_{\text{CTX-M-2-group}}$, $bla_{\text{CTX-M-9-group}}$, bla_{TEM} and bla_{SHV}, were screened by PCR under the conditions and protocols as described previously (Kittinger et al., 2016a). After a positive PCR result the isolates were sent to sequencing to identify the specific ESBL-gene.

2.4. Statistical analysis

Statistical analysis was performed with the R® Version 4.2.3 using RStudio (both from www.r-project.org). For all antibiotics and resistance patterns, the numbers of resistant and non-resistant isolates between groups were compared with Fisher's exact test using Kassambara (2023) package with a confidence interval of 0.95.

3. Results

3.1. Wastewater influence on E. coli abundance in the Danube River

At the Danube River sampling site, the *E. coli* abundance data was very consistent throughout the sampling campaign, except for the flood event in July (Supplementary Table 1). No difference between the upstream and downstream site of the WWTP was observed. Therefore, we assumed that the downstream samples were not significantly influenced by the local wastewater inflow of the WWTP Traismauer. This assumption is further supported by electrical conductivity measurements (Supplementary Table 1) performed on-site which did also show no significant differences. An explanation might be that the WWTP effluent site is situated at a higher water depth (1.5 m) than the depth at which the samples were taken (20–30 cm) and likely the downstream sample was not located within the wastewater plume. Furthermore, there is a high dilution of the wastewater discharged from the WWTP (mean discharge: 0.74 m^3/s, ranging from 0.52 m^3/s to 1.55 m^3/s) into the large Danube River (mean discharge: 1685 m^3/s, ranging from 1080 m^3/s to 4390 m^3/s during the sampling campaign) preventing an observable difference between upstream and downstream sites.

3.2. Classification of isolates into wildtype, resistant and multi-resistant

In total, 697 *E. coli* isolated from human urine samples (424 from inpatients, 273 from outpatients) were compared to 440 *E. coli* isolated from biofilm (220 from upstream and 220 from downstream) and 489 isolates from water samples (241 from upstream and 248 from downstream of the WWTP) of the Danube River (Fig. 1). Isolates without any phenotypic resistance to the tested antibiotics were defined as wildtype and the differentiation into resistance and multi-resistance was defined with resistance to antibiotic classes. Isolates which were defined as resistant show phenotypic resistance to one or two antibiotic classes and multi-resistant ones to three or more.

Patient isolates – When the isolates from the inpatients were compared with those from outpatients, we observed similar resistance

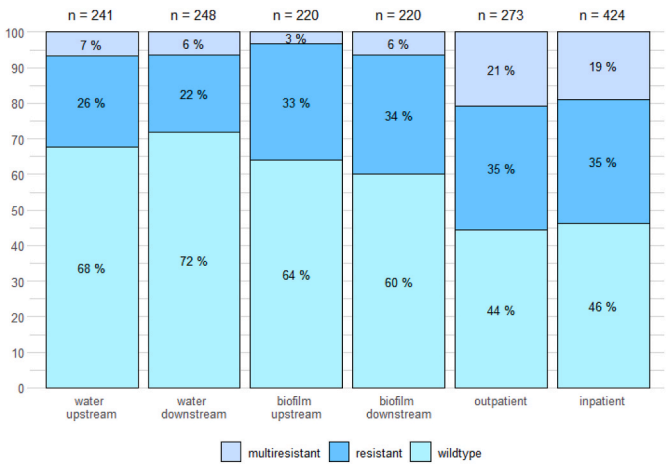

Fig. 1. Distribution of the percentages of wildtype (no phenotypic resistance), resistant (phenotypic resistance to one or two antibiotic classes), and multi-resistant (phenotypic resistance to three or more antibiotic classes) isolates in the six different groups.

proportions for both patient groups for the three resistance categories (Fisher's exact test, $p > 0.05$, $n = 679$). Thus, for further analysis all isolates retrieved from patients were combined. Of the patient isolates, 45.5% (317/697) were classified as wildtype, which was significantly lower in comparison to the environmental isolates (Fisher's exact test, $p < 0.001$ for water ($n = 489$) and biofilm ($n = 440$)). 19.7% (137/697) of the tested *E. coli* isolates from patients were multi-resistant and significantly more than the environmental isolates (5.5%; Fisher's exact test, $p < 0.001$). The proportions of resistant patient isolates (34.9%; 243/697) were not significantly different from the biofilm isolates (33.2%; 146/440) while the proportions of resistant water isolates (23.7%; 116/489) were significantly lower (Fisher's exact test, $p < 0.01$).

Environmental isolates – The comparison of upstream and downstream isolates concerning their classification in wildtype, resistant or multi-resistant did not give a significant difference for both water and biofilm (Fisher's exact test, $p > 0.05$, $n = 489$ (water), $n = 440$ (biofilm)) (Fig. 1). This was expected considering the *E. coli* abundance data (Supplementary Table 1) and the low local wastewater influence at the downstream sampling site. Therefore, upstream and downstream *E. coli* isolates were combined and referred to as water and biofilm. Overall, 69.7% (341/489) of all tested *E. coli* isolates from water were classified as wildtype, showing no phenotypic resistance to any of the tested antibiotics. In the biofilm, we found 62% wildtype isolates (273/440), resulting in a significant difference between water and biofilm (Fisher's exact test, $p < 0.05$). The number of resistant isolates, showing phenotypic resistance to one or two antibiotic classes, was significantly higher in biofilm (33.5%) than in water (24%, Fisher's exact test, $p < 0.01$), while multi-resistance levels, indicating resistance to three or more antibiotic classes, did not differ significantly between the two groups and were below 8% in both environmental compartments.

3.3. Resistances to individual antibiotics

In all six data sets, highest resistance levels were found to ampicillin (patient more than 41% (289/697), water 18.8% (92/489) and biofilm 25.9% (114/440)) and no resistances to colistin (Supplementary Table S2). For all antibiotics tested, a significant difference between inpatient and outpatient isolates was found only for amikacin (Supplementary Table S2, Fisher's exact test, $p < 0.05$, $n = 697$). Concerning the environmental *E. coli,* the comparison of upstream and downstream water isolates did not show any statistically significant difference (Supplementary Table S2, Fisher's exact test, $p > 0.05$, $n = 489$). However, for the *E. coli* isolated from biofilm, a significant difference

was observed for tetracycline with 8.2% resistance in the upstream isolates compared to 19.6% of the downstream isolates (Supplementary Table S2, Fisher's exact test, $p < 0.01$, $n = 440$). The higher tetracycline resistance in the downstream biofilm may be explained by a picked clone, as all the resistant isolates were from the sample taken in October 2020 (Supplementary Table S2).

Due to the low number of significant differences between inpatient/outpatient and between upstream/downstream isolates, for further statistical analysis the isolates of all three pairs were taken together and referred to as water, biofilm and patient (Fig. 2). For the two environmental sample sets, most of the evaluated antibiotics did not exhibit a significant difference. However, three antibiotics, namely ampicillin, amoxicillin/clavulanic acid and cefoxitin, showed significantly higher ($p < 0.05$) resistance proportions in biofilm than in water (Table 1). The comparison of the water isolates to those from patients showed that 12 out of the tested 20 antibiotics had significantly higher resistance proportions in the patients set (Table 1). The ampicillin resistance proportion in isolates from patient was with 41.9% approximately twice as high as in those from the water (18.8%). In addition, the patient isolates had significantly higher resistance proportions in some beta-lactam antibiotics (ampicillin, cefalexin, cefuroxime, cefotaxime, ceftazidime, cefepime), both quinolones (moxifloxacin and ciprofloxacin), trimethoprim/sulfamethoxazole as representative of folic acid antagonists and chloramphenicol. Another significant difference was detected for tetracycline (patient: 23.7% (165/697), water: 11.72% (57/489) resistance), although the second representative of the tetracycline class, tigecycline, did not show any resistance in both data sets. In the aminoglycoside class we saw a significantly higher resistance to gentamicin in the patient isolates and low resistance proportions around 1% for amikacin in both data sets. When comparing the biofilm to the patient isolates, similar results as for the water set were obtained, with two exceptions (Table 1). First, resistance proportions of amoxicillin/clavulanic acid where significantly higher in the biofilm in comparison to the patient isolates. Second, no significant difference was found for cefalexin.

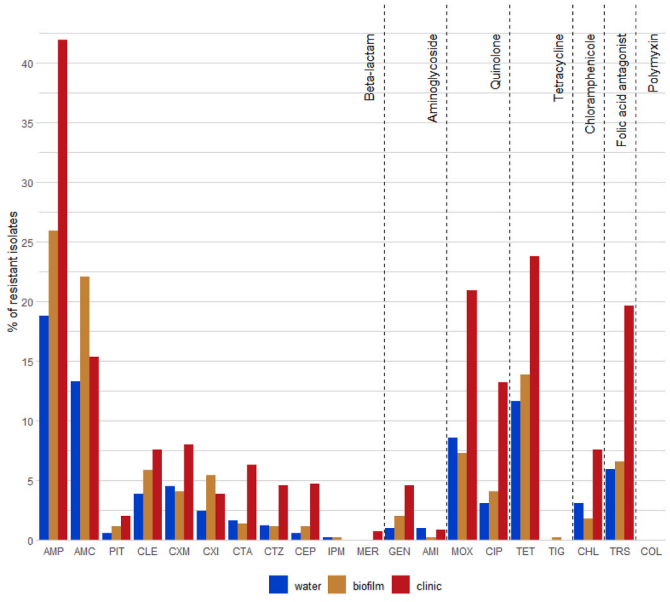

Fig. 2. Percentage of isolates resistant to the 20 tested antibiotics in the water, the biofilm and the patients isolate sets. Beta-lactams: AMP – ampicillin, AMC – amoxicillin/clavulanate, PIT – piperacillin/tazobactam, CLE – cefalexin, CXM – cefalexin, CXI – cefoxitin, CTA – cefotaxime, CTZ – ceftazidime, CEP – cefepime, IPM – imipenem, MER – meropenem,; aminoglycoside: GEN – gentamicin, AMI – amikacin; quinolones: MOX – moxifloxacin, CIP – ciprofloxacin; tetracyclines: TET – tetracycline, TIG – tigecycline; chloramphenicoles: CHL – chloramphenicol; folic acid antagonists: TRS – trimethoprim/sulfamethoxazole; polymyxins: COL – colistin; dashed lines separate the different antibiotic classes.

Surprisingly, resistance to the last-line antibiotic tigecycline was found in biofilm but not in the patient data set, but the difference was not significant as it was only one isolate which carried the resistance (Table 1, Supplementary Table 3).

3.4. Resistances of single isolates and characterization of ESBL

Environmental isolates – At single isolate level, one *E. coli* resistant to 12 out of the 20 analysed antibiotics and to six out of the seven antibiotic classes was found in the water upstream of the WWTP. In the biofilm, one isolate from the upstream sample set and two isolates from the downstream sample set showed resistances to at least ten individual antibiotics (Table 2). All of them were multi-resistant, but were still susceptible to the two last-line carbapenem antibiotics imipenem and meropenem as well as to amikacin and colistin. Furthermore, among the biofilm isolates, two isolates (one from upstream and one from downstream) which were phenotypically suspected ESBL-isolates, showed in the genetic analysis that both of them harbour the $bla_{\text{CTX-M-15}}$ gene (Table 2).

Patient isolates – From the patients, 16 out of the 697 analysed isolates were resistant to ten or more individual antibiotics and all of them belonged to the multi-resistant group. All of these isolates shared resistance to ampicillin and were susceptible to the last-line antibiotics amikacin, colistin, imipenem and tigecycline (Table 2). All seven outpatient isolates also shared resistance to trimethoprim/sulfamethoxazole while the nine inpatient ones were all resistant to several beta-lactams (cefalexin, cefuroxime, cefotaxime, ceftazidime and cefepime) and to the tested quinolones. Overall, from the patient isolates, seven were suspected to harbour ESBL genes. Six from these seven belong to the inpatient data set and all of them were positive for $bla_{\text{CTX-M-15}}$. In contrast, the one, which was isolated from an outpatient, harboured the $bla_{\text{CTX-M-14}}$ gene (Table 2).

4. Discussion

4.1. Inpatient and outpatient isolates have similar resistance patterns

Interestingly, we did not find significant differences between resistances in *E. coli* isolates from in- and outpatients except for amikacin. This finding is in contrast to studies where resistances in inpatients were significantly higher compared to outpatients (Karki et al., 2001; Demir and Kazanasmaz, 2020). This difference to other countries (Estonia, Turkey) might be explained with generally lower resistance levels in Austria. Moreover, it is an indication of good hygiene standards in the clinical environment in Austria, although the hospital is still a hotspot of resistances when compared to the water environment. Resistance to amikacin was observed in a very low proportion (1.9%; 8/424) of the inpatient isolates but never in an outpatient isolate. Low resistance proportions to amikacin in comparison to other aminoglycosides have been found in *E. coli* (and other Enterobacteriaceae) throughout Europe (Haldorsen et al., 2014; Ojdana et al., 2018). A large study containing data from different European countries, e.g., Germany, Sweden and Poland, evaluated the resistance patterns of *E. coli* isolated from uncomplicated UTIs (Ny et al., 2019). The comparison of their combined European results with our outpatient results showed similar resistance proportions, where ampicillin was the highest reported resistance, followed by trimethoprim/sulfamethoxazole and amoxicillin/clavulanic acid. The ciprofloxacin resistance in Austria was at a similar level than in Sweden (Ny et al., 2019). Concerning urethral catheters, Albu et al. (2018) showed that most *E. coli* were resistant to ampicillin (~77%), followed by tetracycline (nearly 64%) and highlighted the risk of a UTI through the catheter due to biofilm formation.

Table 1
Differences in resistance levels of combined data sets (upstream and downstream; inpatients and outpatients) of water, biofilm and patient isolates on single antibiotic level were tested with Fisher's exact test, significance level 0.95. Beta-lactams: AMP – ampicillin, AMC – amoxicillin/clavulanate, PIT – piperacillin/tazobactam, CLE – cefalexin, CXM – cefalexin, CXI – cefoxitin, CTA – cefotaxime, CTZ – ceftazidime, CEP – cefepime, IPM – imipenem, MER – meropenem,; aminoglycoside: GEN – gentamicin, AMI – amikacin; quinolones: MOX – moxifloxacin, CIP – ciprofloxacin; tetracyclines: TET – tetracycline, TIG – tigecycline; chloramphenicoles: CHL – chloramphenicol; folic acid antagonists: TRS – trimethoprim/sulfamethoxazole; polymyxins: COL – colistin.

	water	biofilm	p-value	water	patient	p-value	biofilm	patient	p-value
beta-lactam									
AMP	18.81	25.91	<0.05	18.81	41.89	<0.01	25.91	41.89	<0.01
AMC	13.29	22.05	<0.01	13.29	15.35	ns	**22.05**	**15.35**	**<0.01**
PIT	0.61	1.14	ns	0.61	2.01	ns	1.14	2.01	ns
CLE	3.89	5.91	ns	3.89	7.60	<0,05	5.91	7.60	ns
CXM	4.50	4.09	ns	4.50	8.03	<0.05	4.09	8.03	<0.05
CXI	2.45	5.45	<0.05	2.45	3.87	ns	5.45	3.87	ns
CTA	1.64	1.36	ns	1.64	6.31	<0.01	1.36	6.31	<0.01
CTZ	1.23	1.14	ns	1.23	4.59	<0.01	1.14	4.59	<0.01
CEP	0.61	1.14	ns	0.61	4.73	<0.01	1.14	4.73	<0,01
IMI	0.20	0.23	ns	0.20	0.00	ns	0.23	0.00	ns
MER	0.00	0.00	ns	0.00	0.72	ns	0.00	0.72	ns
aminoglycoside									
GEN	1.02	2.05	ns	1.02	4.59	<0.01	2.05	4.59	<0.05
AMI	1.02	0.23	ns	1.02	0.86	ns	0.23	0.86	ns
quinolones									
MOX	8.59	7.27	ns	8.59	20.95	<0.01	7.27	20.95	<0.01
CIP	3.07	4.09	ns	3.07	13.20	<0.01	4.09	13.20	<0.01
tetracyclines									
TET	11.66	13.86	ns	11.66	23.82	<0.01	13.86	23.82	<0.01
TIG	0.00	0.23	ns	0.00	0.00	ns	0.23	0.00	ns
chloramphenicol									
CHL	3.07	1.82	ns	3.07	7.60	<0.01	1.82	7.60	<0.01
folic acid antagonist									
TRS	5.93	6.59	ns	5.93	19.66	<0.01	6.59	19.66	<0.01
polymyxin									
COL	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns

Table 2
Isolates with ten or more resistances to individual antibiotics (R_{sum}) and ESBL-harboring ones (shown in italics) of all six data sets. R indicates resistance to the antibiotic and S susceptibility. Red (resistant) and green (susceptible) background shows similarities of the different data sets (patient and environmental), similarities in resistance of ESBL isolates have a bold R.

	Isolate	AMP	AMC	PIT	CLE	CXM	CXI	CTA	CTZ	CEP	IMI	MER	GEN	AMI	MOX	CIP	TET	TIG	CHL	TRS	COL	R_{sum}	ESBL
water upstream	TMU_07/21_8	R	S	S	R	R	S	R	R	R	S	S	R	S	R	R	R	S	R	R	S	12	
biofilm upstream	*TMU-BF_04/21_22*	***R***	***R***	*S*	***R***	***R***	*S*	***R***	***R***	*R*	*S*	*S*	*S*	*S*	*S*	*S*	*R*	*R*	*S*	*R*	*S*	*10*	*CTX-M-15*
biofilm downstream	TMD-BF_01/21_21	R	R	R	R	R	S	S	S	S	S	S	S	S	R	R	R	S	R	R	S	10	
biofilm downstream	TMD-BF_04/21_3	R	R	S	R	R	R	R	R	R	S	S	S	R	R	S	S	S	S	R	S	11	
biofilm downstream	*TMD-BF_08/21_1*	***R***	***R***	*S*	***R***	***R***	*R*	***R***	***R***	*R*	*S*	*S*	*S*	*S*	*S*	*S*	*S*	*S*	*S*	*R*	*S*	*9*	*CTX-M-15*
inpatient	*patient_14*	***R***	***R***	*S*	***R***	***R***	*S*	***R***	***R***	*S*	*S*	*S*	*S*	*S*	*R*	*S*	*S*	*S*	*S*	*R*	*S*	*8*	*CTX-M-15*
inpatient	*patient_21*	***R***	***R***	*S*	***R***	***R***	*S*	***R***	***R***	*R*	*S*	*S*	*R*	*S*	*R*	*R*	*S*	*S*	*S*	*S*	*S*	*10*	*CTX-M-15*
inpatient	*patient_179*	***R***	***R***	*S*	***R***	***R***	*S*	***R***	***R***	*R*	*S*	*S*	*S*	*S*	*R*	*R*	*S*	*S*	*S*	*S*	*S*	*9*	*CTX-M-15*
inpatient	patient_193	R	S	S	R	R	S	R	R	R	S	S	S	S	R	R	R	S	S	R	S	10	
inpatient	patient_208	R	R	S	R	R	S	R	R	R	S	S	R	S	R	R	R	S	S	R	S	12	
inpatient	patient_420	R	S	S	R	R	S	R	R	R	S	S	S	S	R	R	R	S	S	R	S	10	
inpatient	*patient_526*	***R***	***R***	*S*	***R***	***R***	*S*	***R***	***R***	*R*	*S*	*S*	*S*	*S*	*R*	*R*	*R*	*S*	*S*	*S*	*S*	*10*	*CTX-M-15*
inpatient	*patient_549*	***R***	***R***	*S*	***R***	***R***	*R*	***R***	***R***	*R*	*S*	*S*	*S*	*S*	*R*	*R*	*S*	*S*	*R*	*R*	*S*	*12*	*CTX-M-15*
inpatient	patient_567	R	S	S	R	R	S	R	R	R	S	S	S	S	R	R	R	S	R	R	S	11	
inpatient	*patient_618*	***R***	***R***	*S*	***R***	***R***	*S*	***R***	***R***	*R*	*S*	*S*	*R*	*S*	*R*	*R*	*R*	*S*	*S*	*S*	*S*	*11*	*CTX-M-15*
inpatient	patient_666	R	S	S	R	R	S	R	R	R	S	S	R	S	R	R	R	S	S	R	S	11	
outpatient	patient_131	R	S	S	R	S	S	R	R	R	S	R	R	S	R	S	R	S	S	R	S	10	
outpatient	patient_172	R	R	S	R	R	R	S	S	S	S	S	S	S	R	R	R	S	R	R	S	10	
outpatient	patient_263	R	R	S	R	R	S	R	R	R	S	R	R	S	R	S	R	S	S	R	S	12	
outpatient	patient_296	R	R	S	R	R	R	R	R	R	S	S	S	S	R	S	R	S	S	R	S	11	
outpatient	*patient_342*	***R***	***R***	*S*	***R***	***R***	*R*	***R***	***R***	*R*	*S*	*S*	*S*	*S*	*S*	*S*	*R*	*S*	*S*	*R*	*S*	*10*	*CTX-M-14*
outpatient	patient_385	R	R	S	R	R	R	R	R	S	S	S	S	S	R	R	S	S	S	R	S	10	
outpatient	patient_637	R	R	R	S	R	S	S	S	R	S	S	R	S	R	R	R	S	R	R	S	11	

4.2. ESBL-harbouring E. coli from patients are dominated by $bla_{CTX\text{-}M\text{-}15}$ and do not carry resistances to last-line antibiotics

In total, seven patient isolates harboured ESBL, six of them carrying the $bla_{CTX\text{-}M\text{-}15}$ gene and one $bla_{CTX\text{-}M\text{-}14}$. With two exceptions, the patient ESBL isolates were also multi-resistant with ten or more resistances to single antibiotics, but none of them was resistant to the two carbapenems tested, piperacillin/tazobactam, nor to one of the last-line antibiotics amikacin, colistin or tigecycline. Overall, only 1% of the patient isolates carried an ESBL gene which is a very low proportion compared to other studies (Benaissa et al., 2021). The finding of $bla_{CTX\text{-}M\text{-}15}$ in all of the inpatient ESBL *E. coli* isolated from urine was not surprising, as this gene is the most often reported in UTIs worldwide (Naas et al., 2007; Peirano and Pitout, 2010). A single ESBL isolate from

an outpatient carried the $bla_{CTX\text{-}M\text{-}14}$ gene, which was also found less often in Germany and seemed to be associated with a long-term care facility stay (Rohde et al., 2020). This gene has only one substitution from $bla_{CTX\text{-}M\text{-}9}$ (Ma et al., 2002) and in medical context it was reported as one of dominant types in Asia (Song et al., 2009; Liu et al., 2018). A study evaluating the genetic background of ESBL in inpatients and outpatients in South-East Austria also found $bla_{CTX\text{-}M\text{-}1\text{-}group}$ being the most abundant gene (~72% in *E. coli*) and ~61% of the tested *E. coli* carried $bla_{CTX\text{-}M\text{-}15}$ (Paulitsch-Fuchs et al., 2022). Nearly 10% of the *E. coli* carried $bla_{CTX\text{-}M\text{-}14}$ and the $bla_{CTX\text{-}M\text{-}9\text{-}group}$ was the second most abundant reported group (Paulitsch-Fuchs et al., 2022). In Europe, $bla_{CTX\text{-}M\text{-}14}$ was recently found on plasmids in *E. coli* isolated from French rivers and some were closely related to plasmids derived from humans (Baron et al., 2020).

4.3. Isolates from biofilms displayed higher resistance levels than water isolates

In the present study, the resistance levels of the riverine biofilm isolates were significantly higher than those of the water isolates. Not many studies exist that have compared water and biofilm resistance levels in riverine *E. coli* populations. A study from Canada also showed that the resistance proportion in *E. coli* isolated from river biofilms (and bottom sediments) was higher than in water (Maal-Bared et al., 2013). Biofilms are known to have high genetic exchange rates within different bacterial species (Balcazar et al., 2015) and potentially promote the transfer of antibiotic resistance genes (Aubertheau et al., 2017; Matviichuk et al., 2023). The authors stated that biofilms are able to sorb pharmaceutical compounds from the surrounding water and may select for resistant bacteria (Matviichuk et al., 2023). On single antibiotic level we saw for three compounds, ampicillin, amoxicillin/clavulanic acid and cefoxitin, higher resistance levels in biofilm which might be partly explained with such selection pressure as ampicillin and amoxicillin/clavulanic acid are two antibiotics to which the highest resistance levels were detected. Therefore, a second explanation could be the picking of clones from the biofilms as cefoxitin resistance was obviously higher in July and August than in the other months (Supplementary Table S3). Specifically downstream of WWTPs, the levels of pharmaceuticals in biofilms may be higher resulting in an increased selective pressure for antibiotic resistance in the biofilm communities in comparison to water (Aubertheau et al., 2017; Matviichuk et al., 2023).

4.4. Resistance patterns in environmental E. coli populations in Austria compared to other parts of the world

During Joint Danube Survey 2013, the resistance pattern of *E. coli* was analysed along the whole Danube River (Kittinger et al., 2016a). Our sampling sites are located in the upper section of the Danube where resistance levels of 45% and multi-resistance levels of approximately 10% with the same set of antibiotics tested as in this study were reported (Kittinger et al., 2016a). Our resistance levels were lower with about 30% and multi-resistance levels around 7%, indicating that the selected sampling site does not represent a hotspot of resistance despite the inflow of a WWTP. Comparable resistance proportions in Europe were found by Blaak et al. (2015) who isolated *E. coli* from different Dutch surface waters and reported resistance levels of 26% and 11% multi-resistance, while in the Rhine River, coliform isolates showed resistance levels up to 54% (Stange et al., 2016). However, in surface waters of Eastern European countries such as Serbia (Veljovic et al., 2015) and in Asia (India, China) (Gomi et al., 2017; Diwan et al., 2018; Peng et al., 2020) higher resistance levels have been recorded. For example, in an Indian river, *E. coli* reached multi-drug resistance levels of 24% and 23% in autumn and winter (Diwan et al., 2018) and a calculated annual average, based on the published data, of roughly 18% which is clearly more than the annual average of approximately 7% in our study.

Isolates resistant to last-line antibiotics such as colistin or tigecycline (Zurfuh et al., 2016; Hladicz et al., 2017; Tuo et al., 2018; Cho et al., 2020) and clinically relevant serotypes carrying resistance genes (Dhanji et al., 2011; Yang et al., 2017; Zarfel et al., 2017; Dantas Palmeira et al., 2022) were found in aquatic environments all over the world. Luckily, resistances to such antibiotics are still rare but they are often combined with multiple other resistances or ESBL production. For example, in a Chinese river, 1.6% of the analysed isolates were colistin-resistant from which most were multi-resistant, harbouring additional ARGs (Tuo et al., 2018). A similar colistin resistance level than in China was reported in Switzerland (Zurfuh et al., 2016). Concerning tigecycline, a study conducted in Georgia found resistance levels of 6.4% in enterococci isolated from surface waters. The enterococci originated from different species without any primary species contribution (Cho et al., 2020). In Austria, two tigecycline-resistant *Klebsiella pneumoniae* were isolated from the Mur River (Hladicz et al., 2017) which were genetically different but both shared susceptibility to all tested antibiotics except tetracycline and tigecycline. We only found one tigecycline resistant isolate in one biofilm sample upstream of the WWTP effluent which harboured resistances to the beta-lactam class and was ESBL-positive, showing a very low level of contamination with isolates resistant to last line antibiotics.

4.5. ESBL in environmental isolates and potential links to humans

Overall, we found two ESBL-producing isolates in the biofilm sample set both carrying the $bla_{CTX\text{-}M\text{-}15}$ gene. This gene was already reported as the most abundant in Danube River ESBL isolates from water samples collected within the frame of a whole river survey (Kittinger et al., 2016a). In total, Kittinger and colleagues isolated 17 ESBL-carrying *E. coli* among 629 isolates over the whole stretch of the Danube River (Kittinger et al., 2016a). This general low frequency may partly explain why we found only two ESBL-harbouring isolates as we sampled in the upper stretch of the Danube River, where less resistant *E. coli* were reported. In the Mur, another Austrian river, Zarfel et al. (2017) evaluated the diversity of ESBL-carrying *Enterobacteriaceae* and found $bla_{CTX\text{-}M\text{-}15}$ as most abundant ESBL gene. Similar results, concerning $bla_{CTX\text{-}M\text{-}15}$, were found in other European surface waters (Zurfluh et al., 2013; Baron et al., 2020; Tacao et al., 2022). In India, the percentage of ESBL-positive *E. coli* has been reported to be higher with up to 12% carrying an ESBL-gene dependent on the season (Diwan et al., 2018). However, a large study conducted in different Chinese surface waters had with 2.8% comparable levels of ESBL-carrying *E. coli* (Liu et al., 2018) as the 2.7% of isolates of the Danube River (Kittinger et al., 2016a). The most abundant ESBL-gene isolated from the positive *E. coli* in China was $bla_{CTX\text{-}M\text{-}14}$ which was found in 46% of the isolates, followed by $bla_{CTX\text{-}M\text{-}9}$ which was carried by 22% (Liu et al., 2018) and Gomi et al. (2017) reported $bla_{CTX\text{-}M\text{-}14}$ as the most common ESBL gene found in *E. coli* isolated from a Japanese river. In general, among ESBL the $bla_{CTX\text{-}M}$-type is the most common worldwide (Castanheira et al., 2021).

Fagerström et al. reported the occurrence of similar lineages of ESBL *E. coli* in wastewater and humans with $bla_{CTX\text{-}M\text{-}15}$ as the most abundant gene (Fagerstrom et al., 2019). The Danube River receives treated wastewater at many sites and it is therefore highly likely that the two ESBL *E. coli* (and other resistant isolates) were from WWTPs further upstream. A direct connection between patient and riverine samples could not be confirmed with our results, although, in the literature such connections have quite often been reported. For example, Lepuschitz et al. found three genetic clusters of ESBL producing *Klebsiella pneumoniae* from river water and isolates from patients, from which one water isolate linked to a patient had a distance from 200 km, indicating a long survival of ARB in the water environment (Lepuschitz et al., 2019). In Germany, genetic analysis of ESBL/carbapenemase-producing bacteria isolated from river water and sediment showed that nearly 90% of them are originating from clinically relevant species (Falgenhauer et al., 2019). Recently, Davidova-Gerzova et al. characterized

ESBL/carbapenemase-producing *E. coli* from UTI patients, wastewaters and the receiving river with a whole-genome sequencing approach (Davidova-Gerzova et al., 2023). Although, they specialized on ESBL-harbouring isolates, they got similar results as our cultivation-based approach with patient isolates being more resistant than isolates obtained from water samples. The finding of rare resistance types suggests a transmission from hospital (wastewater) to river water (Davidova-Gerzova et al., 2023). For a deeper understanding on resistance gene fluxes from patients to the environment, a representative and sufficiently high number of isolates (a few hundred) should be sequenced by whole genome sequencing, but this was beyond the scope and capabilities of our study.

5. Conclusions

The *E. coli* isolates from in- and outpatients investigated in this study showed significantly higher resistance levels than the water and biofilm isolates from the Danube River. No resistance to imipenem and the last-line antibiotics tigecycline and colistin were found in the patient isolates indicating a generally moderate resistance situation in Austria. Even though we found a low number of multi-resistant *E. coli* and some resistances to last-line antibiotics (amikacin, tigecycline and imipenem) in the environmental sample set, we conclude that the investigated sampling sites in the Danube are less polluted when compared to other rivers in the world. Although we did not see any significant differences between water and biofilm isolates concerning last-line antibiotics, both ESBL-positive isolates, one tigecycline-resistant isolate and slightly higher imipenem resistance levels were found in the riverine biofilm. Potentially, the biofilm can be a better indicator of clinical influence in rivers than the water.

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CRediT authorship contribution statement

Melanie Leopold: Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Angelika Kabicher:** Investigation, Methodology, Writing – review & editing. **Ildiko-Julia Pap:** Conceptualization, Investigation, Writing – review & editing. **Barbara Ströbele:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Gernot Zarfel:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Andreas H. Farnleitner:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Alexander K.T. Kirschner:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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