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Whole genome sequencing reveals resemblance between ESBL-producing and carbapenem resistant *Klebsiella pneumoniae* isolates from Austrian rivers and clinical isolates from hospitals

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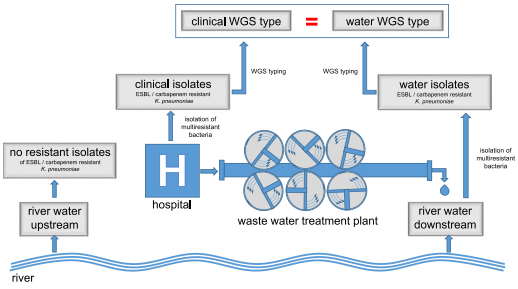
HIGHLIGHTS

- Detection of ESBL-, carbapenemase-producing *K. pneumoniae* in Austrian river water
- Relatedness of clinical and water isolates identified using WGS.
- Accordance of hospital wastewater effluent and water sampling location
- Evidence of anthropogenic pollution of river water in urban areas in Austria

GRAPHICAL ABSTRACT



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ABSTRACT

In 2016, the Austrian Agency for Health and Food Safety started a pilot project to investigate antimicrobial resistance in surface water. Here we report on the characterisation of carbapenem resistant and ESBL-producing *K. pneumoniae* isolates from Austrian river water samples compared to 95 clinical isolates recently obtained in Austrian hospitals.

Ten water samples were taken from four main rivers, collected upstream and downstream of major cities in 2016. For subtyping and comparison, public core genome multi locus sequence typing (cgMLST) schemes were used. The presence of AMR genes, virulence genes and plasmids was extracted from whole genome sequence (WGS) data.

In total three ESBL-producing strains and two carbapenem resistant strains were isolated. WGS based comparison of these five water isolates to 95 clinical isolates identified three clusters. Cluster 1 (ST11) and cluster 2 (ST985) consisted of doublets of carbapenem resistant strains (one water and one clinical isolate each). Cluster 3 (ST405) consisted of three ESBL-producing strains isolated from one water sample and two clinical specimens. The cities, in which patient isolates of cluster 2 and 3 were collected, were in concordance with the water sampling locations downstream from these cities. The genetic concordance

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between isolates from river water samples and patient isolates raises concerns regarding the release of wastewater treatment plant effluents into surface water. From a public health perspective these findings demand attention and strategies are required to minimize the spread of multiresistant strains to the environment.

1. Introduction

The Gram negative bacterium *Klebsiella pneumoniae* (*K. pneumoniae*) is a leading cause of human nosocomial infections, but can also be acquired in the community (Podschun and Ullmann, 1998; Shon and Russo, 2012). It can either be carried asymptomatically or can cause a wide spectrum of infections, for instance pneumonia; wound, soft tissue, urinary tract and bloodstream infections (Holt et al., 2015; Maatallah et al., 2014; Podschun and Ullmann, 1998).

The evolution, spread and emergence of bacterial antibiotic resistance represent one of the most important health care problems worldwide (Hawkey, 2008). Since the 1950s, infections caused by *Enterobacteriaceae* are treated with beta-lactam antibiotics. Following the introduction of broad-spectrum beta-lactam antibiotics, new extended spectrum beta-lactamases (ESBL) emerged (Grundmann et al., 2010). In 1996 the first carbapenemase, encoded by the bla_{KPC} gene, was detected in *K. pneumoniae* (Yigit et al., 2001). Subsequently, other carbapenemase-genes, such as bla_{NDM}, bla_{OXA-48}, bla_{VIM} and bla_{IMP-1} emerged (Fukigai et al., 2007; Kumarasamy et al., 2010; Miriagou et al., 2003; Wesselink et al., 2012). The global dissemination of carbapenemase-producing strains has been shown in rivers in different regions of the world (Khan et al., 2018; Mahon et al., 2017; Zarfel et al., 2017; Zurfluh et al., 2013).

Cumulating reports indicate that animals, food products and the environment may also constitute reservoirs for carbapenemase producing bacteria (Wyres and Holt, 2018; Zurfluh et al., 2013).

The discharge of hospital effluents into sewers are hotspots for antimicrobial resistant bacteria and antimicrobials (Duarte et al., 2018; Hocquet et al., 2016; Marti et al., 2014). The intermixture of bacteria from different anthropogenic sources (urban, industrial and agricultural waste) with environmental species may result in the transfer of antibiotic resistance genes. Subsequently this watery soup provides perfect conditions for the evolution of novel combinations of resistance genes (Amos et al., 2014) leading to the evolution and selection of new resistant species due to the presence of antibiotic residues in water (Chen et al., 2018; Gekenidis et al., 2018; Lupo et al., 2012; Rodriguez-Mozaz et al., 2015).

A mitigation of this critical situation can be achieved by consequent measurements leading to a reduction of antibiotic usage in hospitals and agriculture. This will reduce the risk of future emerging resistant pathogens, will also minimize the bacterial load in WWTPs subsequently increasing the efficiency of WWTPs in removing dangerous bacteria. In Austria with a total population of 8.75 million currently about 95% of the population are served by 1865 WWTPs (for a size >50 population equivalent (PE)) and 5% of the population are served by about 27,500 small WWTPs (<50 PE) (Langergraber et al., 2018). The most popular technologies for secondary treatment are conventional activated sludge (CAS), vertical flow (VF) wetlands as well as sequencing batch reactors (SBR), these processes can provide substantial but not complete removal of bacteria and this demands advanced treatment processes. However, 22.7% of small WWTPs use primary treatment only.

The aim of this study was to evaluate the diversity of ESBL and carbapenemase-harboring *K. pneumoniae* in water samples collected in four main Austrian rivers and to compare them with clinical isolates to identify possible sources of anthropogenic pollution.

2. Material and methods

2.1. Study design and strain isolation

Ten water samples were taken from main Austrian rivers, collected upstream (n = 5) and downstream (n = 5) of major cities in 2016, to screen for the presence of pathogenic, multiresistant bacterial organisms. Per sampling site, one 500 ml river water sample was collected upstream and one downstream (1 km to 3 km after waste water treatment plant effluent) from major Austrian cities: river Danube: cities Vienna and Linz; river Inn: city Innsbruck; river Glan: city Klagenfurt; river Traun: city Linz (Fig. 1).

A 100 ml sample aliquot was filtered and the filtrate incubated in BBL fluid thioglycollate medium (Becton Dickinson, NJ, USA) at 37 °C overnight. For extraction and detection of ESBL-producing and carbapenem resistant strains, 70 μl overnight cultures were plated on chromogenic media (chromID CARBA (bioMérieux, Marcy-l'Étoile, France), chromID ESBL (bioMérieux)). Per plate, morphologically different colonies were picked in triplicate and sub-cultured. Subcultivated single colonies were identified on species level by matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry.

2.2. Whole genome sequencing and sequence data analysis

High-molecular-weight (100–200 kb) DNA was isolated from isolates using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) and quantified fluorometrically with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using a target specific Qubit assay (dsDNA BR Assay Kit, Thermo Fisher Scientific).

To prepare ready-to-sequence libraries of bacterial genomes Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) was used according to the manufacturer's protocol and paired end sequenced (2 × 300 bp) on an Illumina Miseq instrument. Sequencing coverage calculator (www.illumina.com/CoverageCalculator) was used for calculating a desired mean coverage of at least 50-fold. *De novo* assembly of raw reads was performed using SPAdes (version 3.9.0) (Bankevich et al., 2012) and NGS data interpretation was carried out with the analysis software SeqSphere$^+$ (Ridom, Münster, Germany).

For phylogenetic analysis the MLST (multi-locus sequence type) (Diancourt et al., 2005) and the cgMLST (core genome multi-locus sequence type) were extracted from the whole genome sequence (WGS) data. Based on the defined *K. pneumoniae sensu lato* cgMLST in SeqSphere$^+$, comprising of 2358 target genes, a gene-by-gene approach was used to compare genomes. Isolates were visualised as minimum spanning trees (MST) and genotypically related isolates were identified with a Complex Type (CT) Distance of 15 alleles (https://www.cgmlst.org/ncs/schema/2187931/). The definition "good core genome targets" was according to the criteria described in detail in Ruppitsch et al. (2015).

PlasmidFinder 1.3 (Carattoli et al., 2014) available from the Center for Genomic Epidemiology web server (http://www.genomicepidemiology.org/) and the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017) were used to search for the presence of plasmids and genes conferring antibiotic resistance. The bla_{SHV} alleles were refined using the Institut Pasteur BIGSdb database. The existence of virulence genes was investigated by using the virulence allele library from the

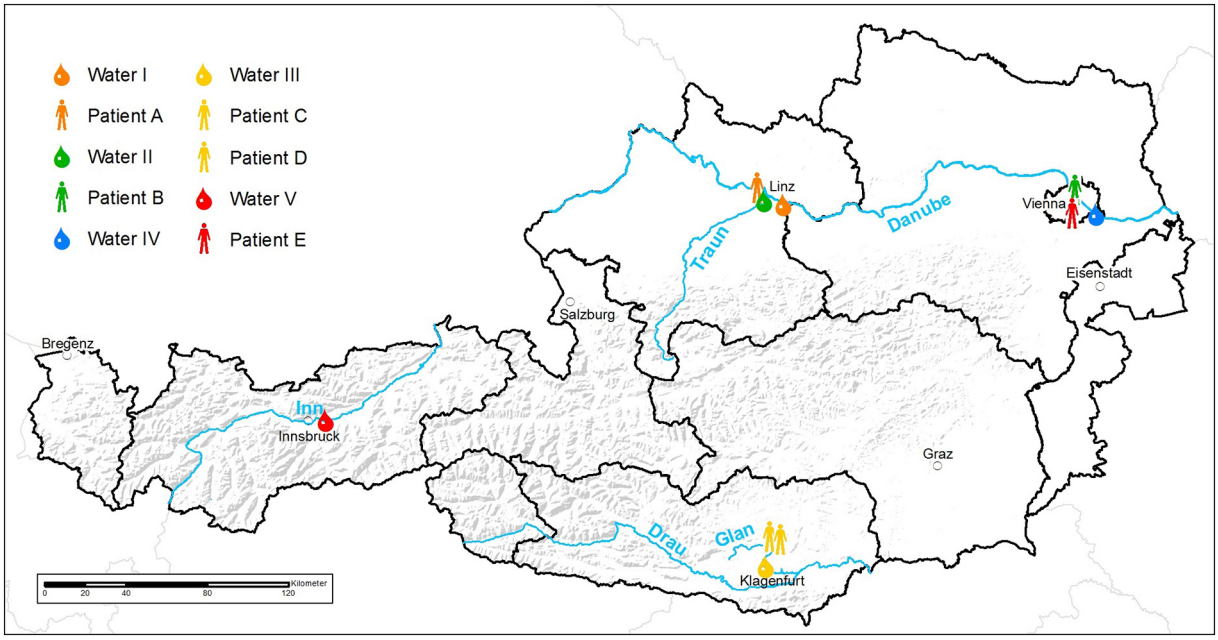


Fig. 1. Geographical map of Austria showing the sampling points for river-water samples yielding *K. pneumoniae* isolates and for the hospitals providing indistinguishable patient isolates. For water and clinical isolates belonging to the same MLST the same colour was assigned.

Institut Pasteur BIGSdb database for *K. pneumoniae* (http://bigsdb.pasteur.fr/klebsiella).

2.3. Collection of clinical isolates

To assess relationship of the river water isolates to clinical isolates, 95 isolates voluntarily provided by Austrian hospitals, available in the AGES "in-house" *K. pneumoniae* sequence database - comprising 95 isolates to date from 2011 (n = 4), 2012 (n = 1), 2015 (n = 8), 2016 (n = 46), 2017 (n = 24), 2018 (n = 12) were used for comparison.

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was determined for five water and four related clinical isolates with SensititreTM EUVSEC and EUVSEC2 plates (ThermoFisher Scientific, Waltham, USA) by microbroth dilution according to CLSI (Clinical and Laboratory Standards Institute) guidelines (CLSI standard M07). MIC values were interpreted according to EUCAST criteria (European Committee on Antimicrobial Susceptibility Testing, EUCAST Clinical Breakpoint Tables v.8.1, valid from 2018 to 05-15) for the following antibiotics: ampicillin, cefotaxime, ceftazidime, cefepime, ertapenem, imipenem, meropenem, gentamicin, ciprofloxacin and colistin.

Phenotypic detection of AmpC β-lactamase was carried out with Etest AmpC CN/CNI (bioMérieux).

2.5. Nucleotide sequence accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank including the following accession numbers: QWWA00000000 (water I), QWVW00000000 (water II), QWVY00000000 (water III), QWVZ00000000 (water IV), QWVX00000000 (water V), QWWF00000000 (patient A), QWWE00000000 (patient B), QWWD00000000 (patient C), QWWC00000000 (patient D), QWWB00000000 (patient E). The version described in this paper is the first version.

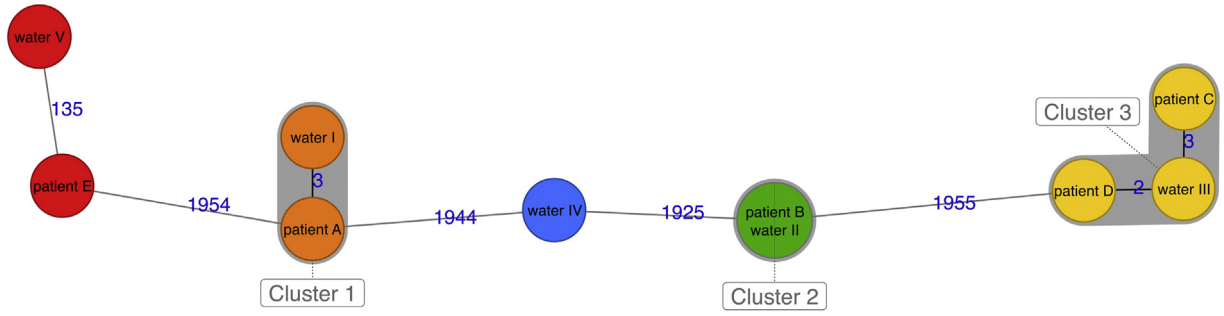
3. Results

3.1. Whole genome sequencing analysis

The five water samples collected upstream from cities Vienna (river Danube), Linz (n = 2, river Danube and Traun), Klagenfurt (river Glan) and Innsbruck (river Inn) yielded neither ESBL-producing nor carbapenem resistant *K. pneumoniae* isolates. All five samples taken downstream from the cities contained multidrug-resistant *K. pneumoniae* isolates: two isolates were from the Danube (one obtained downstream from Linz (isolate ID: water I), one downstream from Vienna (water IV)), and one each from the remaining river samples of Traun (water II), Glan (water III) and Inn (water V).

For five *K. pneumoniae* isolates from water the MLST type was extracted from the assembly data and identified five different STs and five respective CTs: ST11/CT1302 (water I), ST323/CT266 (water V), ST405/CT1363 (water III), ST985/CT1362 (water II) and a previously not described ST3400/CT2200 (water IV). The five *K. pneumoniae* isolates were sequenced with an average coverage of 77-fold (53 to 100-fold) and comprised on average 99.4% (99.2 to 99.7%) called cgMLST alleles.

A cgMLST based comparison of these five water isolates to 95 clinical *K. pneumoniae* isolates from the AGES *K. pneumoniae* sequence database identified three clusters (Fig. A.1). Cluster 1 (ST11) consisted of isolates water I and patient A with three allelic differences in their cgMLST profiles (CT1302). Cluster 2 (ST985) consisted of isolates water II and patient B which shared the same cgMLST profile (CT1362). Cluster 3 (ST405) consisted of isolates water III, patient C and patient D. Isolates from patient C and patient D showed three respectively two allelic differences from the water III isolate in their cgMLST profiles (CT1363) (Figs. 1, 2). We further applied the previously published Pasteur cgMLST scheme (Bialek-Davenet et al., 2014) and found 0 (cluster 1 and 2) or 1 (cluster 3) intra-cluster allelic mismatches out of 634 loci. In contrast, there were 31 mismatches between the two ST323 isolates. All genotypically related clinical isolates in clusters 1–3 (Table 1) were collected in 2016 and were from hospitals in Linz (cluster 1), Vienna (cluster 2) and Klagenfurt (cluster 3).



Sample ID	River	City	Good cgMLST targets	ST	Complex Type	Cluster
Water I	Danube	-	99.8 %	11	1302	Cluster 1
Patient A	-	Linz	99.8 %	11	1302	
Water II	Traun	-	99.2 %	985	1362	Cluster 2
Patient B	-	Vienna	99.2 %	985	1362	
Water III	Glan	-	99.7 %	405	1363	Cluster 3
Patient C	-	Klagenfurt	99.7 %	405	1363	
Patient D	-	Klagenfurt	99.7 %	405	1363	
Water IV	Danube	-	98.5 %	3400	2200	-
Water V	Inn	-	99.7 %	323	266	-
Patient E	-	Vienna	99.6 %	323	1577	-

Fig. 2. Minimum spanning tree including *K. pneumoniae* isolates collected from Austrian rivers and closely related clinical isolates collected from hospitals in Linz, Vienna and Klagenfurt in 2016. Each circle represents isolates with an allelic profile based on the cgMLST which consists out of 2358 alleles. Blue numbers correspond to the allelic differences between isolates; isolates with closely related genotypes are shaded in grey and marked as clusters. Isolates were coloured according to classical MLST.

Two river isolates, one from the Inn (water V) in Innsbruck and one from the Danube (water IV) in Vienna were singletons and showed no close relatedness to clinical isolates from Austria with 135 allelic differences (patient E collected in Vienna in 2016) and 1925 allelic differences to the closest related patient sample (patient B) (Fig. 2).

3.2. Phenotypic and genotypic antimicrobial resistance determination

In vitro susceptibility testing results of nine *K. pneumoniae* isolates are shown in Table A.1. All tested isolates were resistant to ampicillin, cefotaxime, ceftazidime, and cefepime; they were sensitive to colistin. Isolates from clusters 1 and 2, and from patients C and D were resistant to ertapenem too. Isolate water II was resistant to meropenem too. Isolates in cluster 3 and isolate water V were resistant to gentamicin too. All isolates except the isolates dubbed patient B and water IV were resistant to ciprofloxacin too. Out of all tested isolates only isolates of cluster 1 were confirmed as positive AmpC β-lactamase producer.

The analysis of antimicrobial resistance genes *via* the Comprehensive Antibiotic Resistance Database (CARD) identified 64 genes in total (Table 2) in the investigated *K. pneumoniae* isolates (n = 9), revealed resistances genes to beta-lactams, quinolones, aminoglycosides, phenicol, sulphonamides, macrolide, rifampicin, trimethoprim, fosfomycin and fluoroquinolones. Both isolates of cluster 1 shared the same set of resistance genes (n = 35), isolates of cluster 2 shared 26 resistance genes in total, and isolates of cluster 3 shared a total of 28 resistance genes. Isolate water IV carried 20 antimicrobial resistance genes in total, including narrow-spectrum (native) bla_{SHV-1} and $bla_{CTX-M-15}$. Isolate water V carried a total of 30 antimicrobial resistance genes, including narrow-spectrum (native) bla_{SHV-1}, $bla_{CTX-M-15}$, bla_{TEM-1} and bla_{OXA-1} (Table 2).

3.3. Genotypic identification of virulence genes

Via the integrated *K. pneumoniae* virulence allele library (Bialek-Davenet et al., 2014; Lam et al., 2018) from Institut Pasteur database (http://bigsdb.pasteur.fr), 34 genes with attributes of virulence were detected (Table 3). Isolates belonging to the same ST shared the same set of virulence genes. Alleles of genes coding for yersiniabactin were identified and the observed combination was compared to the yersiniabactin sequence type (YbST) database from BIGSdb (Lam et al., 2018). Genes coding for yersiniabactin (*ybt*, n = 11) were identified in five isolates (cluster 1 and cluster 3) and the allelic profiles of YbST were assigned to sequence types YbST28 (ST11, n = 2) and YbST312

Table 1
Summarized data on patient isolates which were genotypically related to collected river samples.

Patient ID	Year of isolation	MLST	City of isolation	Age (years)	Sex	Specimen	Hospital ward
Patient A	2016	11	Linz	29	Male	Urine	Surgical ward
Patient B	2016	985	Vienna	2	Female	Urine	Paediatric ward
Patient C	2016	405	Klagenfurt	83	Female	Peritoneal fluid aspirate	Intensive care unit
Patient D	2016	405	Klagenfurt	81	Female	Urine	Urgent care center

MLST = multilocus sequence type.

(ST405, n = 3). Genes of type 3 fimbrial gene cluster (*mrk*), which is largely conserved within *K. pneumoniae*, and the ferric aerobactin receptor (*iutA*), which is part of the aerobactin gene cluster, were present in all nine isolates. Isolates from cluster 3 (water III, patient C, patient D; ST405), additionally carried genes which contribute to capsule formation (*kvgA*, *kvgS*), mediate uptake of ferric iron (*kfuA*, *kfuB*, *kfuC*) and genes belonging to the mammalian cell entry (*mce*) cluster coding for microcin E492, a channel-forming bacteriocin with activity against mammalian cells (Hetz et al., 2002).

3.4. Plasmid identification

The strains contained a total of nine plasmids (IncFIB(K), IncFII(K), IncR, IncL/M, IncFIA(HI1), Col440I, Col440II, IncX5, IncFIB(Mar)) which

Table 2
Genotypic antimicrobial resistance determination for five isolates from river water and four genotypically related human isolates. The presence of a gene is represented by a "red box (+)".

Resistance Mechanism	Drug Class	target	water I ST11	patient A ST11	water II ST985	patient B ST985	water III ST405	patient C ST405	patient D ST405	water IV ST3400	water V ST323
antibiotic inactivation	aminoglycosides	AAC(3)-IIc					+	+	+		+
		AAC(6')-Ib			+	+					
		APH(3')-Ia	+	+							
		APH(3")-Ib					+	+	+		+
		APH(6)-Id					+	+	+		+
		ANT(3")-Ii-AAC(6')-IId fusion protein			+						
		aadA			+	+					
		aadA2	+	+							
		aadA15			+	+					
		aadA21			+						
		aadA24				+					
	aminoglycosides; fluoroquinolones	AAC(6')-Ib-cr	+	+			+	+	+		+
antibiotic efflux	aminoglycosides; aminocoumarins	baeR	+	+	+	+	+	+	+	+	+
antibiotic inactivation	cephalosporin	CTX-M-15	+	+			+	+	+	+	+
	cephalosporin; cephamycin	DHA-1	+	+							
	cephalosporin; penam	OXA-1	+	+			+	+	+		+
		OXA-10			+	+					
		OXA-48	+	+							
	cephalosporin; carbapenem; penam	SHV-1								+	+
		SHV-11	+	+							
		SHV-76					+	+	+		
		SHV-83			+	+					
	cephalosporin; penem; penam; monobactam	TEM-1					+	+	+		+
	cephalosporin; carbapenem; penem; penam; cephamycin	VIM-1			+	+					
antibiotic target replacement	diaminopyrimidine	dfrA12	+	+							
		dfrA14			+	+	+	+	+		+
antibiotic target alteration	elfamycin	EF-Tu mutation (R234F)			+		+	+	+	+	+
antibiotic efflux	fluoroquinolones	emrB	+	+	+	+	+	+	+	+	+
		emrR	+	+	+	+	+	+	+	+	+
		qacH			+	+					
antibiotic target alteration		parC mutation (S80I)	+	+							
antibiotic target protection		QnrB1					+	+	+		+
		QnrB4	+	+							
		QnrS1								+	
		QnrVC4			+	+					
antibiotic target alteration	fosfomycin	UhpT mutation (E350Q)	+	+	+	+	+	+	+	+	+
antibiotic inactivation		FosA5								+	
		FosA6	+	+	+	+	+	+	+		+
antibiotic inactivation	macrolides	mphA	+	+							
		Mrx	+	+							
antibiotic inactivation	phenicols	catB3	+	+			+	+	+		+
		catI	+	+							
antibiotic efflux		cmlA1				+					
		cmlA5			+	+					

(continued on next page)

Table 2 (*continued*)

antibiotic inactivation	rifamycin	**arr-3**	+	+							
antibiotic target replacement	sulfone; sulfonamides	**sul1**	+	+	+	+					
		sul2					+	+	+		+
antibiotic efflux	tetracyclines	**tet(C)**									+
antibiotic target alteration	triclosan	**gyrA mutation (S83F)**							+		
antibiotic efflux; reduced permeability to antibiotic	monobactam; cephalosporin; cephamycin; triclosan; glycylcycline; penem; carbapenem; penam; rifamycin; tetracycline; phenicol; fluoroquinolone	**marA**	+	+	+	+	+	+	+	+	+
antibiotic efflux	cephalosporin; tetracycline; triclosan; glycylcycline; phenicol; penam; rifamycin; fluoroquinolone	**marR**	+	+	+	+	+	+	+	+	+
		acrA	+	+	+	+	+	+	+	+	+
	cephalosporin; cephamycin; tetracycline; macrolide; penam; fluoroquinolone	**H-NS**	+	+	+	+	+	+	+	+	+
	penam; fluoroquinolone; macrolide	**CRP**	+	+	+	+	+	+	+	+	+
	tetracycline; fluoroquinolone	**adeF**	+	+	+	+	+	+	+	+	+
	tetracycline; triclosan; macrolide	**MexK**									+
	nitrofuran; tetracycline; glycylcycline; diaminopyrimidine; fluoroquinolone	**oqxA**	+	+	+	+	+	+	+	+	+
		oqxB	+	+							
	nitroimidazole	**msbA**	+	+	+	+	+	+	+	+	+
	pleuromutilin; streptogramin	**vgaC**	+	+						+	
antibiotic target alteration	cephamycin; monobactam; carbapenem; penam; cephalosporin	**PBP3 mutation (S357N, D350N)**	+	+	+	+	+	+	+	+	+
	nybomycin; fluoroquinolone	**gyrA mutation (S83I)**	+	+							
reduced permeability to antibiotic	monobactam; penem; cephalosporin; cephamycin; carbapenem; penam	**OmpK37**	+	+	+	+	+	+	+	+	+

showed between 95.95% and 100% identity to query sequences (Table 4). Isolates (n = 2) belonging to ST11 carried IncFIB(K), IncFII (K), IncR, IncL/M (pOXA-48). Whereas resistance genes *aadA2*, *dfrA12*, *mphA* are carried by IncFIB(K) plasmids, the OXA-48 carbapenemase gene is carried by IncL/M plasmids. Isolates (n = 2) belonging to ST985 carried IncFIB(K), IncFII(K), IncFIA(HI1), Col440I and Col440II. Isolates (n = 3) belonging to ST405 carried IncFIB(K) and IncFII(K). One isolate (ST3400) carried IncFIB(K), IncFII(K), IncR, IncX5, IncFIB (Mar) and one isolate (ST323) carried IncFIB(K) and IncFII(K). Water isolates had the same plasmid content as their related human isolates sharing the same MLST.

4. Discussion

The increasing number of antimicrobial resistant microorganisms poses a major problem for public health (WHO, 2015). Consequently, the World Health Organization (WHO) defined a list of global priority pathogens for antibiotic resistant bacteria to support the definition of priorities in research and development of new and effective drugs (http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/). Results of the European antimicrobial resistance surveillance report 2016 revealed that in Austria 9.6% of tested invasive *K. pneumoniae* isolates were resistant to 3rd generation cephalosporins and 0.7% were resistant to carbapenems (ECDC, 2017).

In 2016, the Austrian Agency for Health and Food Safety started a pilot project to survey the prevalence of clinically relevant, antibiotic resistant human pathogens in surface water. A common outcome of our study was that all water samples from rivers taken before a city were negative for ESBL and carbapenemase-producing *K. pneumoniae* whereas all samples taken one to three kilometers downstream of main Austrian cities wastewater plant release points were positive, which shows the impact of wastewater effluents and anthropogenic pollution on the aquatic environment (Amos et al., 2014).

A linkage of water and clinical isolates was demonstrated by WGS based comparison of isolates using two existing cgMLST schemes: https://www.cgmlst.org/ncs/schema/2187931/ and the Pasteur scheme (Bialek-Davenet et al., 2014). Three different clusters comprising water and clinical isolates were identified by both schemes. Isolates within the same cluster revealed similar antimicrobial resistance profiles, shared the same set of virulence genes and had the same plasmid content. Cluster 1 (ST11, CT1302) contained a water isolate collected downstream the waste water treatment plant (WWTP) of the city of Linz and a clinical isolate collected from a hospital in Linz in 2016, differing by three alleles in their cgMLST. ST11 is a common multidrug-resistant ST, mainly found in Asia and South America (Deleo et al., 2014; Dong et al., 2018; Munoz-Price et al., 2013). Although ST11 – OXA-48 outbreaks have been described in Europe previously (Jayol et al., 2016; Pérez-Blanco et al., 2018), the occurrence of this type in the clinic and in the environment in Austria may present an upcoming major public health threat. The capability of these strains to carry different classes of carbapenemases (OXA-48, VIM, NDM, KPC dramatically hampers medical treatment-options (Oteo et al., 2016; Pena et al., 2014; Voulgari et al., 2014). The ST11 isolates from our study were ESBL positive ($bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$) and showed reduced susceptibility to carbapenems, attributable to the presence of $bla_{\text{OXA-48}}$ located on a self-transferable IncL/M-type plasmid. Carbapenemase genes have been associated with multiple separate acquisition events mediated by plasmids of various sizes belonging to a huge range of incompatibility groups including broad and narrow host ranges, such as IncF, IncA/C, IncL/M, IncH, IncN and IncX3.6 (Voulgari et al., 2014). The occurrence of IncF-type, IncR and IncL/M plasmids in water as well as in patient isolates is highly alarming due to the possibility of acquiring further resistance genes and the occurrence of novel combinations of resistance genes (Amos et al., 2014). Additionally the detection of the yersiniabactin locus in these isolates reflects the virulence properties of these strains, since yersiniabactin is significantly associated with invasive infections in humans (Holt et al., 2015).

The second cluster (ST985, CT1362) contained a water isolate and a patient isolate (two-year-old baby girl with recurring urinary tract infection). These isolates were collected approximately 200 km away, which reveals for the first time the possible survival distance of multiresistant *K. pneumoniae* strains in river water. Both isolates had

Table 3
Identified virulence genes in five isolates from river water and four genotypically related patient isolates.

	water I ST11	patient A ST11	water II ST985	patient B ST985	water III ST405	patient C ST405	patient D ST405	water IV ST3400	water V ST323	
ybST	**28**	**28**			**312**	**312**	**312**			yersiniabactin
ybtS	16	16			6	6	6			
ybtX	12	12			62	62	62			
ybtQ	4	4			60	60	60			
ybtP	3	3			4	4	4			
ybtA	3	3			1	1	1			
irp2	35	35			145	145	145			
irp1	50	50			148	148	148			
ybtU	3	3			2	2	2			
ybtT	10	10			39	39	39			
ybtE	23	23			69	69	69			
fyuA	2	2			2	2	2			
mrkA	2	2	20	20	4	4	4		6	type 3 fimbrial gene cluster
mrkB	2	2	3	3	1	1	1	9	NAT	
mrkC	2	2	NAT	NAT	NAT	NAT	NAT		10	
mrkD	12	12	39	39	NAT	NAT	NAT		NAT	
mrkF			8	8	38	NAT	38		NAT	
mrkH	7	7	10	10	15	15	15	2	1	
mrkI	15	15	7	7	18	18	18	3	1	
mrkJ	12	12	6	6	1	1	1		6	
iutA	NAT	NAT	NAT	NAT	NAT	NAT	NAT	NAT	NAT	aerobactin transport
kvgA					2	2	2			contribute to capsule formation
kvgS					NAT	NAT	NAT			
kfuA					NAT	NAT	NAT			
kfuB					NAT	NAT	NAT			mediates uptake of ferric iron, intestinal colonization factor
kfuC					NAT	NAT	NAT			
mceA					1	1	1			mammalian cell entry (mce) gene cluster
mceB					2	2	2			
mceC					1	1	1			
mceD					3	3	3			
mceE					2	2	2			
mceG					NAT	NAT	NAT			
mceH					5	5	5			
mceI					NAT	NAT	NAT			
mceJ					NAT	NAT	NAT			

NAT = new allele type.

ESBL genes bla_{SHV-83} and bla_{OXA-10} and carbapenemase bla_{VIM-1}. Endemicity of VIM-producing *K. pneumoniae* isolates are mainly reported in Italy and Greece (Nordmann et al., 2011). First occurrence in Austria was reported the last decade and bla_{VIM} harboring *Enterobacter cloacae* isolates were recently found in Austrian surface water samples (Zarfel et al., 2017). To the best of our knowledge *K. pneumoniae* strains belonging to ST985 positive for bla_{VIM-1} have not been described before.

Table 4
Identified plasmids in five isolates from river water and four genotypically related patient isolates.

Accession no.	Plasmid	water I ST11	patient A ST11	water II ST985	patient B ST985	water III ST405	patient C ST405	patient D ST405	water IV ST3400	water V ST323
JN233704	**IncFIB(K)**	100.00 %	100.00 %	98.93 %	98.93 %	98.93 %	98.93 %	98.93 %	98.93 %	98.93 %
CP000648	**IncFII(K)**	100.00 %	100.00 %	98.65 %	98.65 %	95.95 %	95.95 %	95.95 %	97.97 %	95.95 %
DQ449578	**IncR**	100.00 %	100.00 %						99.20 %	
JN626286	**IncL/M (pOXA-48)**	100.00 %	100.00 %							
AF250878	**IncFIA(HI1)**			96.91 %	96.91 %					
CP023920.1	**Col440I**			100.00 %	100.00 %					
CP023921.1	**Col440II**			97.52 %	97.52 %					
MF062700.1	**IncX5**								99.65 %	
JN420336	**IncFIB(Mar)**								99.54 %	

The third cluster (ST405, CT1363) comprised one water isolate and two patient isolates differing in their cgMLST by two and three alleles. All isolates harbored ESBL genes bla_{SHV-76}, $bla_{CTX-M-15}$ and bla_{OXA-1}. ST405 is among the predominant clones in Spanish hospitals and carries the bla_{OXA-48} gene on IncL/M-type plasmids (Pérez-Vázquez et al., 2016). In contrast to the Spanish ST405 strains, bla_{OXA-48} was not present in our ST405 isolates. However, the Austrian ST405 isolates revealed by far the highest content of virulence genes. Genes belonging to yersiniabactin, genes of the type 3 fimbrial gene cluster, the ferric aerobactin receptor, which is part of the aerobactin gene cluster, genes which contribute to capsule formation, mediate uptake of ferric iron and genes belonging to the mammalian cell entry cluster (responsible for microcin E492 production) were present. The presence of these virulence genes enhances colonization and adherence to the host, invasive infections, and biofilm formation. The increased virulence potential of this clone subsequently might lead to an increase of community-acquired infections in young and healthy individuals (Clegg and Murphy, 2016).

Two ESBL positive water isolates, one with the new sequence type ST3400 and the other with ST323 were collected in the river Danube downstream from Vienna and in the river Inn downstream from Innsbruck; both lacked matching clinical *K. pneumoniae* isolates. Missing links from water to patient isolates were expected, since *K. pneumoniae* isolates are not routinely sent to the Austrian reference laboratory and comparison was therefore carried out on a limited number of sequenced clinical isolates available in the AGES *K. pneumoniae* sequence database. Both water-isolates carried IncF plasmids, among others, which have been termed “epidemic resistance plasmids” due to their ability to acquire resistance determinants and propensity to rapid dissemination (Mathers et al., 2015). They are specifically linked with certain beta-lactamase genes such as CTX-M-15, which was present in both water samples.

The screening of Austrian surface water revealed two carbapenem resistant and three ESBL-producing *K. pneumoniae* isolates in total, in five river samples taken downstream from WWTP effluents. The fact that we found these clinically important clones, of which three were indistinguishable from contemporarily collected patient isolates, indicates that pathogens find their way from hospitals into rivers, as described elsewhere recently (Amos et al., 2014; Hocquet et al., 2016; Khan et al., 2018; Mahon et al., 2017). Contaminated rivers provide a milieu for antibiotic-resistant bacteria to persist, disseminate, evolve and exchange antibiotic resistance determinants. This implicates the hazard of spreading to animals, humans and clinically relevant settings. However, the fact that patient isolates had corresponding genotypes to river isolates does not necessarily implicate them as direct contamination source, but rather, reflect the presence of such genotypes in the human population. We not only can confirm the previous finding, that WWTPs pose a worrying reservoir of highly resistant enteric bacteria in the environment (Amos et al., 2014), our results also show that hospital patients could be a source of multiresistant Gram negative organisms spilling into rivers when hospital effluents are not properly treated.

5. Conclusions

The results of this pilot study on the release of antimicrobial-resistant *K. pneumoniae* strains into the environment and the detection of clinically relevant strains in the environment is alarming and appears an emerging future public health problem, which demands increased attention. Based on our findings future projects should cover rivers from all over the country with repeated sampling to obtain a better picture of the situation. Immediate actions as a consequence of recent publications (Amos et al., 2014; Mahon et al., 2017; Zarfel et al., 2017; Zurfluh et al., 2013) and our study results include proper treatment of hospital effluents and operation of WWTPs with state-of-the-art techniques (Kistemann et al., 2008). We recommend the development of new strategies for treating WWTP effluents, and the establishment of a surveillance system - at least downstream the major cities - to monitor for multiresistant clinically relevant bacterial species in surface water, especially such used for recreational activities.

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CRediT authorship contribution statement

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Conflict of interest

None declared.

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