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Antibiotic Sensitivity Screening of *Klebsiella* spp. and *Raoultella* spp. Isolated from Marine Bivalve Molluscs Reveal Presence of CTX-M-Producing *K. pneumoniae*

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Received: 17 October 2020; Accepted: 28 November 2020; Published: 30 November 2020

Abstract: *Klebsiella* spp. are a major cause of both nosocomial and community acquired infections, with *K. pneumoniae* being responsible for most human infections. Although *Klebsiella* spp. are present in a variety of environments, their distribution in the sea and the associated antibiotic resistance is largely unknown. In order to examine prevalence of *K. pneumoniae* and related species in the marine environment, we sampled 476 batches of marine bivalve molluscs collected along the Norwegian coast. From these samples, *K. pneumoniae* ($n = 78$), *K. oxytoca* ($n = 41$), *K. variicola* ($n = 33$), *K. aerogenes* ($n = 1$), *Raoultella ornithinolytica* ($n = 38$) and *R. planticola* ($n = 13$) were isolated. The number of positive samples increased with higher levels of faecal contamination. We found low prevalence of acquired resistance in all isolates, with seven *K. pneumoniae* isolates showing resistance to more than one antibiotic class. The complete genome sequence of cefotaxime-resistant *K. pneumoniae sensu stricto* isolate 2016-1400 was obtained using Oxford Nanopore and Illumina MiSeq based sequencing. The 2016-1400 genome had two contigs, one chromosome of 5,088,943 bp and one plasmid of 191,744 bp and belonged to ST1035. The β -lactamase genes *bla*_{CTX-M-3} and *bla*_{TEM-1}, as well as the heavy metal resistance genes *pco*, *ars* and *sil* were carried on a plasmid highly similar to one found in *K. pneumoniae* strain C17KP0055 from South-Korea recovered from a blood stream infection. The present study demonstrates that *K. pneumoniae* are prevalent in the coastal marine environment and that bivalve molluscs may act as a potential reservoir of extended spectrum β -lactamase (ESBL)-producing *K. pneumoniae* that may be transmitted through the food chain.

Keywords: *Klebsiella*; bivalve molluscs; antimicrobial resistance; CTX-M

1. Introduction

The genus *Klebsiella* contains several species known to cause nosocomial infections [1,2] and some that cause community acquired infections [3,4]. *Klebsiella* spp. are widely distributed outside the clinical environment, including environments like soil, plants, surface waters and other mammals [5]. In 2001, *K. terrigena*, *K. ornithinolytica*, *K. planticola* and *K. trevisanii* were assigned to the new genus *Raoultella* [6]. Although considered environmental species, *Raoultella* spp. have gained increased attention as opportunistic pathogens [7].

Within the genus, *K. pneumoniae sensu stricto* is responsible for the majority of human infections [5]. *K. pneumoniae* is closely related to *K. variicola*, *K. quasipneumoniae* subspecies *quasipneumoniae*, *K. quasipneumoniae* subsp. *similipneumoniae*, *K. variicola* subsp. *tropicalensis*, *K. africaensis* and *K. quasivariicola* and together these species constitute the *K. pneumoniae* species complex [8–11]. Although *Klebsiella* spp. are found in different environments, the opportunistic pathogen *K. pneumoniae* is often present in the human and animal gut [12].

K. pneumoniae is considered one of the most important opportunistic pathogens involved in the dissemination of antimicrobial resistance (AMR), as well as one of the most common causes of infections in health care settings [12]. In the EU/EAA countries, 37% of all *K. pneumoniae* isolates reported to the European Antimicrobial Resistance Surveillance Network (EARS-Net) in 2018, had acquired resistance to at least one class of antibiotics [13]. Of special concern is the emergence of carbapenem-resistant *K. pneumoniae* (CR-KP), often also co-resistant to multiple other classes of antibiotics [13]. Resistance to third-generation cephalosporins is a common type of resistance observed in clinical isolates of *K. pneumoniae* in the European countries [13]. This is largely caused by CTX-M-type extended spectrum β -lactamases (ESBLs). CTX-M encoding genes (*bla*_{CTX-M}) are often plasmid-borne and spread rapidly among the *Enterobacterales*. Cephalosporin-resistant *Enterobacterales* represents a public health concern as they severely limit the available treatment options [14]. Compared to many other countries, Norway has a low prevalence of AMR and the use of antibacterial agents in both humans and food-producing animals is low [13,15]. However, the prevalence of reported ESBL-producing *K. pneumoniae* in blood stream infections in Norway has increased from 1.9% in 2010 [16] to 8.5% in 2018 [15].

K. pneumoniae is genetically a diverse species and the majority of genes are part of the accessory genome [12] which is important in the acquisition of resistance genes [17]. The core genome includes a chromosomal *bla*_{SHV} conferring resistance to aminopenicillins, as well as *oqxAB* and *fosA* mediating reduced susceptibility to quinolones and fosfomycin, respectively [10]. Most of the acquired AMR genes in *K. pneumoniae* are located on plasmids [18], which can be spread among the members of microbial communities in different environments [19]. It has been suggested that *K. pneumoniae* is a particularly good acceptor of plasmids in diverse ecological niches, with the acquisition of these plasmids having a lower fitness cost in *K. pneumoniae* compared to *Escherichia coli* [12]. Many antibiotic resistance genes (ARGs) originate from the natural environment [20]. The environments affected by anthropogenic activities, for example, wastewater systems and animal manure, are considered hotspots for the development and spread of AMR [21]. However, the role of the marine environment in the development and dissemination of AMR is far from understood. There are multiple transmission routes of ARGs and antibiotic resistant bacteria to the marine environment, for example, sewage and runoff from land [21]. Although the literature is scarce on AMR in opportunistic pathogens in the Norwegian marine environment, previous studies have reported the occurrence of ESBL positive *E. coli* [22,23]. Bivalve molluscs have previously been shown as a good tool for monitoring AMR in the marine environment [22]. As filter feeders, they filter large volumes of water, retain and concentrate particles. As a result, they accumulate high numbers of microorganisms, including bacteria of both aquatic and anthropogenic origin [24]. Bivalve molluscs are therefore good indicators of faecal as well as chemical contamination status in a given marine environment [22,25].

K. pneumoniae is extensively studied in clinical settings and some studies have highlighted similarities between clinical and environmental isolates [26–28]. However, there is a lack of knowledge on the prevalence of *K. pneumoniae* and related species in the marine environment. The aim of our study was to examine the prevalence of *K. pneumoniae* and related species in the

Norwegian marine environment using bivalve molluscs as indicators and study their antibiotic sensitivity patterns.

2. Materials and Methods

2.1. Sampling

A total of 204 batch samples of bivalve molluscs were collected along the Norwegian coast from September 2019 to March 2020. An additional 272 samples collected in 2016 were included in the study. The samples comprised 384 blue mussels (*Mytilus edulis*), 48 oysters (*Crassostrea gigas*), 24 scallops (*Pecten maximus*), five horse mussels (*Modiolus modiolus*), three ocean quahogs (*Arctica islandica*), two cockles (*Cerastoderma edule*), two carpet shells (*Politapes rhomboides*) and one sand gaper (*Mya arenaria*). Even though not a bivalve mollusc, seven sea urchins (*Strongylocentrotus droebachiensis*) were also included. In total, 476 samples covering 77 different production areas and five non-rearing locations along the Norwegian coast was included in the study. Samples from production areas were collected through the surveillance programme on bivalves conducted by the Norwegian Food Safety Authority (NFSA). A detailed overview of samples and sampling locations is provided in supplementary Table S1.

2.2. Sample Preparation

Each batch sample comprised 10–20 individual bivalves. Live and closed bivalves were cleaned under cold tap water before they were opened using a sterile knife. Approximately 80–100 g soft tissue and intra-valvular fluid was weighed into sterile plastic bags (VWR, Radnor, PA, USA) and homogenised for 2.5 min using a stomacher (Seward, UK).

2.3. Isolation and Identification of Presumptive *Klebsiella* spp.

Aliquots of 25 g were transferred to new sterile plastic bags and diluted 1:10 in Buffered Peptone Water (BPW) (VWR, USA), homogenised for 30 s and incubated aerobically at 37 °C for 18–24 h. After incubation, 10 µL of the enrichment cultures were streaked on Simmons Citrate Agar (Bio-Rad, Hercules, CA, USA) supplemented with 1% Myo-Inositol (Sigma-Aldrich, St. Louis, MI, USA) (SCAI), a highly selective media for the isolation of *Klebsiella* spp. and *Raoultella* spp. [29] and incubated aerobically at 37 °C for 48 h. Samples collected in 2016 had been enriched in BPW by the same protocol and stored at –80 °C in 20% glycerol. Before the samples were analysed, they were thawed in room temperature and approx. 1.5 mL transferred to 10 mL BPW and incubated at 37 °C over night. Yellow colonies representing presumptive *Klebsiella* spp. were sub-cultured to obtain pure cultures. The obtained isolates were cultured overnight on Plate Count Agar (PCA) (Oxoid, UK) at 37 °C. Colonies were transferred directly to disposable 96 spot targets (Bruker, Germany) and covered with 1 µL HCCA matrix (Bruker, Germany). The spots were air dried and the isolates were identified using Matrix Assisted Laser Desorption Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker, Germany).

2.4. Enumeration of *E. coli*

Enumeration of *E. coli* was done following ISO16649-3 [30]. The limit of quantification (LOQ) of the method is 18 *E. coli*/100 g samples, hereafter termed < LOQ. Based on the most probable number (MPN) *E. coli*/100 g sample, the sampling sites were categorised as A (<230), B (<4600), C (<46,000) or prohibited (>46,000) for cultivation of bivalves according to EU directive 854/2004 [31].

2.5. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was done by disk diffusion according to the European Committee on Antimicrobial Susceptibility testing (EUCAST) [32]. All isolates were tested against a panel of 17 antimicrobial agents belonging to 10 different classes using antibiotic disks (Oxoid, UK) on Mueller-Hinton agar (MH) (Oxoid, UK). The following agents were included: gentamicin (GEN,

10 µg), chloramphenicol (CHL, 30 µg), meropenem (MEM, 10 µg), ceftazidime (CAZ, 10 µg), cefotaxime (CTX, 5 µg), aztreonam (ATM, 30 µg), nitrofurantoin (NIT, 100 µg), amoxicillin-clavulanic acid (AMC, 20–10 µg), piperacillin-tazobactam (TZP, 30–6 µg), mecillinam (MEL, 10 µg), ampicillin (AMP, 10 µg), ciprofloxacin (CIP, 5 µg), trimethoprim-sulfamethoxazole (SXT, 1.25–23.75 µg), tetracycline (TET, 30 µg), tigecycline (TGC, 15 µg). *E. coli* CCUG17620 was included as quality control with each set up. The isolates were classified as sensitive (S), intermediate susceptible, increased exposure (I) or resistant (R) according to EUCAST breakpoints for *Enterobacterales* [33]. Breakpoints were unavailable for TET and no inhibition zone was the criterion used for classifying the isolates as resistant. Isolates falling within the area of technical uncertainty (ATU) for TZP were categorised as susceptible, increased exposure (I) to this agent. Isolates resistant to three or more antibiotic classes were defined as multidrug-resistant (MDR) [34].

2.6. Whole Genome Sequencing and Sequence Analysis

K. pneumoniae isolate 2016-1400 displayed phenotypic resistance to CTX and CXM and was analysed by whole genome sequencing using both short (Illumina) and long reads (Nanopore). For the short read sequencing, DNA was extracted using MagNA Pure 96 and Viral Small volume kit with the Pathogen Universal 200 4.0 purification protocol (Roche Applied Science, Penzberg, Germany). Genomic libraries were prepared using Illumina Nextera DNA Flex library prep and sequenced using the Illumina MiSeq system and the Illumina MiSeq Reagent Kit V3 (600 cycle) to obtain 2 × 300 bp paired end reads. For the long read sequencing, DNA was manually extracted using the Beckman Coulter Life science GenFind V3 kit (C34881) according to the supplemental protocol 'DNA extraction from Bacteria using GenFind v3' (Beckman Coulter, Brea, CA, USA). The DNA library was prepared with the Ligation sequencing kit (SQK-LSK109) (Oxford Nanopore Technologies (ONT), Oxford, UK), then loaded onto a R9.4.1 Flongle flow cell (FLO-FLG001) and sequenced on the ONT MinION Mk1B device (MIN-101B). Basecalling was performed with Guppy v4.2.2 + effba84 (available to ONT customers at <https://community.nanoporetech.com>) and quality filtered using FiltLong v0.2.0 (<https://github.com/rrwick/Filtlong>). Hybrid *de novo* assembly of the short and long read sequences was performed with Unicycler v0.4.8 [35]. Assembly statistics are available in supplementary Table S2. The assembled genome was analysed using Kleborate v2.0.0, a tool designed to accurately identify members of the *K. pneumoniae* species complex and sequence types (STs), acquired virulence factors associated with hypervirulent *K. pneumoniae* (yersiniabactin (*ybt*), aerobactin (*iuc*), salmochelin (*iro*) and colibactin (*clb*) as well as the hypermucoidy genes *rmpA/ompA2* and acquired ARGs (<https://github.com/katholt/Kleborate>). Further bioinformatic analysis was done using NCBI Antimicrobial Resistance Gene Finder (AMRFinderPlus) v3.2.3 [36], ABRicate v0.9.8 (<https://github.com/tseemann/abricate>) using the virulence factors database (VFDB) [37] and PlasmidFinder [38]. The genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline [39]. BLAST Ring Image Generator (BRIG) v0.95 [40] was used for sequence comparison and visualisation. BLASTn v2.9.0 [41] was used to query the *K. pneumoniae* C17KP0055 genome against the *K. pneumoniae* 2016-1400 assembly.

2.7. PCR Amplification of *blas_{SHV}*

To confirm the absence of *blas_{SHV}* *K. pneumoniae* isolate 2016-1400 was subjected to PCR amplification of the *blas_{SHV}* gene. DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Each 20 µL reaction contained 4 µL 5X Phusion HF buffer, 0.4 µL 10 mM dNTP mix, 0.2 µL of each 50 µM SHV specific primer (SHVF: 5'-ATGCGTTATATTCGCCTGTG-3', SHVR: 5'-TGCTTTGTTATTCGGGCCAA-3') [42], 0.2 µL Phusion DNA polymerase, 1 µL template DNA and 14 µL nuclease free water. PCR amplification was performed using the GeneAmp PCR system 9700 Thermal Cycler (Applied Biosystems, Waltham, MA, USA) and the following conditions: initial denaturation at 98 °C for 30 s, 30 cycles of 98 °C for 5 s, 62 °C for 5 s and 72 °C for 10 s, with a final extension at 72 °C for 3 min. *K. pneumoniae* CCUG 10,785 was included as a positive control and a *blas_{SHV}*-negative *E. coli* as a negative control. The PCR products were resolved on a 1% agarose gel

stained with Gel Red Nucleic Acid Stain (Biotium, Fremont, CA, USA) and visualised on a Bio-Rad ChemiDoc system (Bio-Rad, USA).

2.8. Filter Conjugation

Conjugation was carried out according to the method described by Jutkina et al., 2016 [43]. Briefly, kanamycin (KAN) and rifampicin (RIF) resistant *gfp* marked *E. coli* recipient was grown in Mueller Hinton broth (MHB) (Oxoid, UK) with 50 µg/mL KAN (Sigma-Aldrich, USA) at 30 °C with shaking overnight. The CTX and AMP resistant donor was grown over night in MHB supplemented with 2 µg/mL CTX (Sigma-Aldrich, USA) under the same incubation conditions. The donor and recipient were centrifuged at 2755× g for 15 min. and washed twice in phosphate buffered saline (PBS) (Sigma-Aldrich, USA) before final resuspension in PBS. The conjugation mixtures were prepared by mixing equal aliquots of donor and recipients (1:1 ratio). The conjugation mixture was pipetted on to 0.45 µm filters (Merck Millipore, Burlington, MA, USA) and placed on Mueller Hinton (MH) (Oxoid, UK) agar plates and incubated at 37 °C overnight. After incubation, the filter was removed and placed in a falcon tube with 10 mL PBS and sterile glass beads and the cells were removed from the filter by vortexing at maximum speed for 90 s. Serial dilutions up to 10⁻⁶ was prepared in PBS and 100 µL spread in duplicates on CHROMagar orientation (CHROMagar, Paris, France) plates supplemented with 50 µg/mL KAN, 50 g/mL RIF and 100 µg/mL AMP (Sigma-Aldrich, USA) and 50 µg/mL KAN, 50 µg/mL RIF and 2 µg/mL CTX. The plates were incubated at 37 °C for 24–30 h.

3. Results

3.1. Distribution of *Klebsiella* spp. and *Raoultella* spp. in Marine Bivalves

From the 476 samples, presumptive *Klebsiella* spp. were detected in 41% (*n* = 194) of the samples, with some samples positive for several morphotypes. A total of 204 isolates were obtained and identified as members of the genera *Klebsiella* and *Raoultella* using MALDI-TOF MS. In total, 78 isolates were identified as *K. pneumoniae*, 41 as *K. oxytoca*, 33 *K. variicola*, one *K. aerogenes*, 38 as *R. ornithinolytica* and 13 isolates were identified as *R. planticola* (Table 1).

Table 1. *Klebsiella* spp. and *Raoultella* spp. isolated from different bivalve mollusc species, *n* in brackets refer to the number of examined samples for each bivalve species

Species	Bivalve Species				Total no. Isolates
	<i>M. edulis</i> (<i>n</i> = 384)	<i>P. maximus</i> (<i>n</i> = 24)	<i>C. gigas</i> (<i>n</i> = 48)	<i>P. rhomboides</i> (<i>n</i> = 2)	
<i>K. pneumoniae</i>	70	2	5	1	78
<i>K. oxytoca</i>	40	0	1	0	41
<i>K. variicola</i>	29	1	3	0	33
<i>K. aerogenes</i>	1	0	0	0	1
<i>R. ornithinolytica</i>	25	5	8	0	38
<i>R. planticola</i>	12	0	1	0	13

The frequency of samples positive for *Klebsiella* spp. and/or *Raoultella* spp. increased with higher levels of faecal contamination as expressed by the number of *E. coli* detected, with a total of 24% (*n* = 51), 48% (*n* = 96) and 81% (*n* = 56) of the samples positive from < LOQ (*n* = 213) areas, class A (*n* = 194) areas and class B (*n* = 69) areas, respectively. The most frequently isolated species from class A and B areas was *K. pneumoniae* and *K. oxytoca* from locations where *E. coli* MPN/100 g was < LOQ (Figure 1). Detailed overview of isolates is provided in supplementary Table S3.

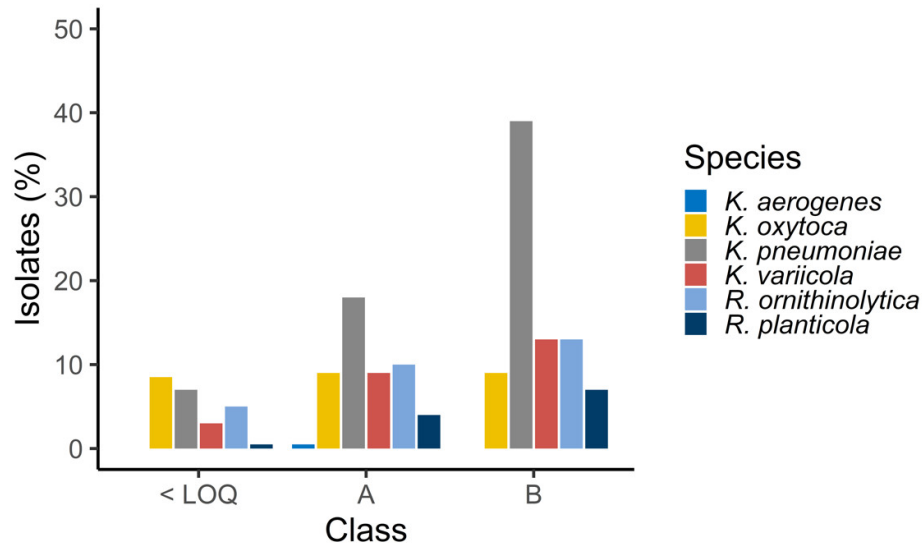


Figure 1. Distribution (%) of *Klebsiella* spp. and *Raoultella* spp. isolates recovered from areas of increasing *E. coli* load according to the EU classification A, B and C. < LOQ: < 18 MPN *E. coli*/100 g, A: 18–230 MPN *E. coli*/100 g, B: 230–4600 MPN *E. coli*/100 g. No results corresponded to *E. coli* counts above B classification.

3.2. Antimicrobial Susceptibility Patterns of *Klebsiella* spp. and *Raoultella* spp.

Among the *K. pneumoniae* isolates, resistance to more than one agent was seen in only eight isolates. Three MDR isolates were detected (Supplementary Table S4), while one isolate displayed phenotypic resistance to cefuroxime, cefotaxime and ampicillin, as well as intermediate susceptibility to aztreonam.

Phenotypic ampicillin susceptibility was observed in *K. pneumoniae* (5%, $n = 4$), *K. oxytoca* (5%, $n = 2$), *K. variicola* (21%, $n = 7$) and *R. ornithinolytica* (8%, $n = 3$) in repeated experiments. (Table 2). Measured antibiotic inhibition zones of all isolates are included in Supplementary Table S4.

Table 2. Prevalence of antibiotic resistance among *Klebsiella* spp. and *Raoultella* spp. isolated from marine bivalves.

Species	Antibacterial Agent																
	AMP	MEL	AMC	TZP	CHL	GEN	CIP	NIT	SXT	TET	TGC	CTX	CAZ	FOX	CXM	ATM	MEM
<i>K. pneumoniae</i> (n = 78)	74	0	3	3 *	2	0	1	2	2	3	0	1	0	0	1	1 *	0
<i>K. oxytoca</i> (n = 41)	39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>K. variicola</i> (n = 33)	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>K. aerogenes</i> (n = 1)	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
<i>R. ornithinolytica</i> (n = 38)	35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>R. planticola</i> (n = 13)	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Isolates categorised as intermediate susceptible, increased exposure. Abbreviations: AMP: Ampicillin, MEL: Mecillinam, AMC: Amoxicillin-clavulanic acid, TZP: Piperacillin-Tazobactam, CHL: Chloramphenicol, GEN: Gentamicin, CIP: Ciprofloxacin, NIT: Nitrofurantoin, SXT: Trimethoprim-sulfamethoxazole, TET: Tetracycline, TGC: Tigecycline, CTX: Cefotaxime, CAZ: Ceftazidime, FOX: Cefoxitin, CXM: Cefuroxime, ATM: Aztreonam, MEM: Meropenem.

3.3. Genome Sequencing

The sequenced genome of *K. pneumoniae sensu stricto* isolate 2016-1400 was *de novo* assembled into two contigs, one 5,088,943 bp chromosome and one 191,744 bp plasmid. The isolate belonged to ST1035 and had the *wzi* allele 116, corresponding to capsule locus (KL) type 57. Further analysis of the sequenced genome revealed that the isolate carried *bla*_{CTX-M-3}, *bla*_{TEM-1}, *oqx**A*, *oqx**B*, *fos**A* and *erm*(*D*) conferring resistance to erythromycin but lacked the *bla*_{SHV} gene. This was confirmed by PCR analysis. A comparison of 2016-1400 with SHV-1-harboring *K. pneumoniae* ST1035 genomes (ENA run accession number ERR4859177 and ERR3416161) showed that there had likely been a deletion of a 10.1 Kbp region, which included *bla*_{SHV-1}, due to the insertion of an IS5 family transposase between a hypothetical protein and diguanylate phosphodiesterase in our isolate.

Several heavy metal resistance genes, including the plasmid borne copper resistance system(*pco*) gene cluster, the arsenic resistance genes (*ars*) and the *sil* operon genes conferring resistance to silver were identified. Virulence genes involved in iron acquisition (*ent* and *fep*), adherence (*ecp*), magnesium uptake (*mgt*) and immune evasion (*ompA*) were also detected in 2016-1400 (Table 3).

Table 3. Genes for antibiotic resistance, heavy metal resistance and virulence identified in *K. pneumoniae* isolate 2016-1400.

Isolate	ARGs	HRGs	VGs	Accession nos.
2016-1400	<i>bla</i> _{CTX-M-3} ,	<i>pcoA</i> , <i>pcoB</i> , <i>pcoC</i> , <i>pcoD</i> , <i>pcoR</i> ,	<i>ent</i> , <i>fep</i> , <i>ecp</i> , <i>mgt</i> , <i>ompA</i>	CP065034, CP065035
	<i>bla</i> _{TEM-1} , <i>oqx</i> <i>A</i> ,	<i>pcoS</i> , <i>pcoE</i> , <i>arsB</i> , <i>arsA</i> , <i>arsD</i> ,		
	<i>oqx</i> <i>B</i> , <i>fos</i> <i>A</i> ,	<i>arsR</i> , <i>arsC</i> , <i>arsH</i> , <i>SilE</i> , <i>SilS</i> , <i>SilR</i> ,		
	<i>erm</i> (<i>D</i>)	<i>SilC</i> , <i>SilF</i> , <i>SilB</i> , <i>SilA</i> , <i>SilP</i>		

Abbreviations: ARGs: Antibiotic resistance genes, HRGs: Heavy metal resistance genes, VGs: Virulence genes.

*bla*_{CTX-M-3} and *bla*_{TEM-1}, as well as the heavy metal resistance genes were carried on a IncFIB(K)/IncFII plasmid highly similar to the 190 582 bp non-conjugative CP052387.1 IncFIB(K)/IncFII plasmid (100% sequence coverage and 99.96% nucleotide identity) from a clinical *K. pneumoniae* strain (Figure 2). The plasmid was not transferable by filter conjugation and carried few conjugal transfer genes. These plasmids carried all of the same genes, except an IS630-like element ISSpu2 family transposase, which was carried by 2016-1400 only. Compared to CP052387.1, this insertion occurred between two base pairs in a pseudogene, an incomplete hypothetical protein. The 5 083 236 bp chromosome of the same published genome (CP052386.1) was also similar to 2016-1400, with 99.62% sequence coverage and 99.43% nucleotide identity.

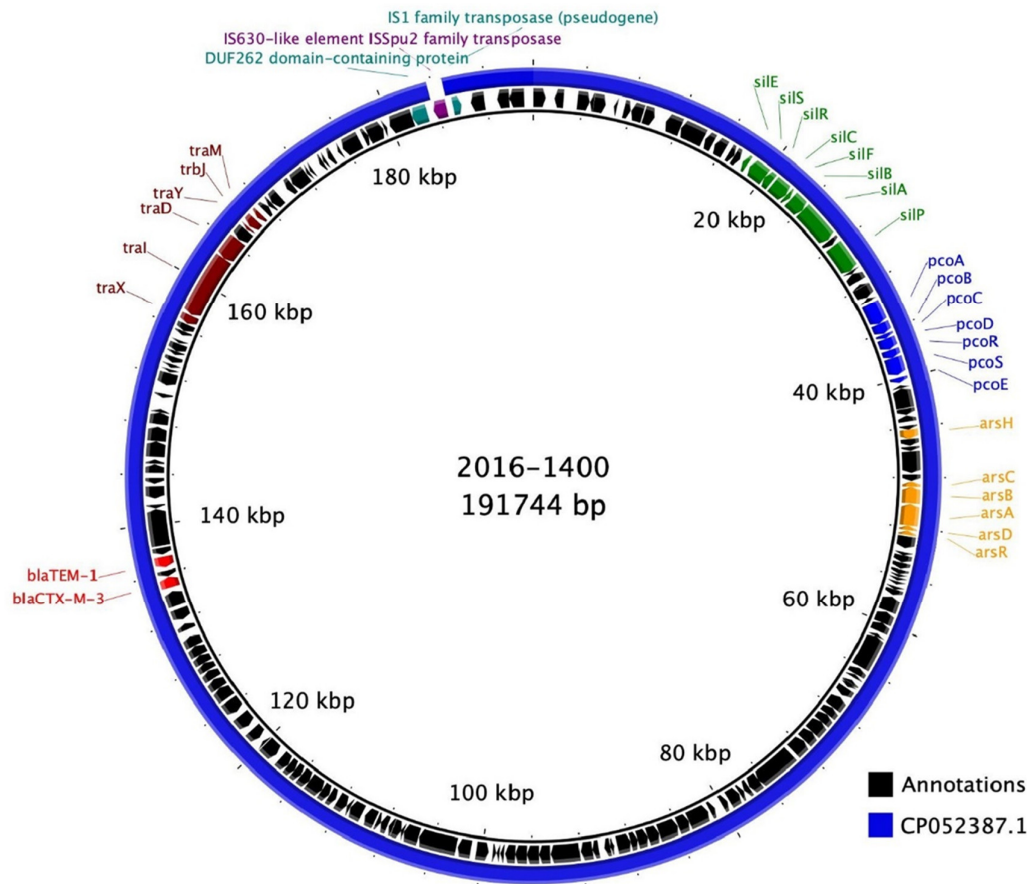


Figure 2. BLAST Ring Image Generator (BRIG) comparison of the *K. pneumoniae sensu stricto* 2016-1400 plasmid (accession number CP065035) and the plasmid from *K. pneumoniae* strain C17KP0055 (accession number CP052387.1). Ring 1 (innermost) shows the positions in the 2016-1400 plasmid, ring 2 shows its gene annotations and ring 3 shows the BLASTn result of CP052387.1 against the 2016-1400 plasmid. The locations of *bla*_{CTX-M-3}, *bla*_{TEM-1}, the *sil*, *pco*, *ars* operons and *tra* genes are indicated. One gene, an IS630-like element ISSpu2 family transposase, was not present in CP052387.1.

4. Discussion

Here we present a comprehensive study on the prevalence of *K. pneumoniae* and related species in marine bivalves collected from both areas used for commercial production of bivalve molluscs for human consumption along the Norwegian coast as well as non-rearing locations in Western and Southern Norway. We further show low prevalence of acquired antibiotic resistance in these isolates. However, one *K. pneumoniae* isolate carried a clinically important and potentially mobile ESBL gene.

K. pneumoniae sensu stricto isolate 2016-1400 recovered from *M. edulis* at a production area in Middle-Norway displayed phenotypic resistance to cefotaxime and carried a plasmid encoding the *bla*_{CTX-M-3} gene, first described in clinical *E. coli* and *Citrobacter freundii* isolates in Poland in 1996 [44]. The CTX-M genes originated from *Kluyvera* spp. and were spread through mobilisation from their chromosomal position [14,45] and since they have disseminated worldwide [14]. Cephalosporins are among the most commonly used classes of antibiotics worldwide [46] and ESBL-producing *K. pneumoniae* represents a threat to the public health as it limits the available therapeutic options [47]. The plasmid also carried a *bla*_{TEM-1} gene, a common plasmid-borne resistance gene among clinical Gram-negative bacteria, primarily conferring resistance to penicillins and first-generation cephalosporins [48]. No gene encoding the chromosomal *bla*_{SHV} was identified in *K. pneumoniae* 2016-1400. Interestingly, no chromosomal *bla*_{SHV} was detected in the chromosome (CP052386.1) of *K.*

pneumoniae strain C17KP0055 from South-Korea, which is highly similar to the strain in our study. This is rare but absence of SHV has previously been reported in some *K. pneumoniae* strains [49,50].

A recent study on antibiotic resistance in *E. coli* from marine bivalves collected in Norway identified *bla*_{CTX-M} genes (*bla*_{CTX-M-15} and *bla*_{CTX-M-14}) in only 1% of the isolates [22]. This is in line with our findings, indicating a low prevalence of CTX-M- producing *Enterobacterales* in the Norwegian marine environment. Although the occurrence of ESBL-producing *Enterobacterales* in Norway is low compared to many other countries, the prevalence of clinical ESBL-producing *Klebsiella* spp. isolates is increasing [15]. Hence, the presence of ESBL-producing *Enterobacterales* in reared bivalves intended for human consumption is concerning.

Furthermore, the *K. pneumoniae* 2016-1400 plasmid carried genes conferring resistance to silver, copper and arsenic. The plasmid was highly similar to the plasmid of a *K. pneumoniae* strain (C17KP0055) isolated from the blood of a South-Korean patient, encoding the indistinguishable ARGs and heavy metal resistance genes. Co-localisation of heavy metal resistance genes and ARGs on the same mobile genetic element (MGE) increases the chance of co-selection by heavy metals [51]. High copper and arsenic levels have been detected in some marine environments in Norway [52] and this may contribute to the co-selection of this plasmid/strain.

K. pneumoniae ST1035 has been associated with human infections [53–56]. Furthermore, capsule type (K) 57 has been identified in clinical isolates and associated with pyogenic liver abscess (PLA) [57,58]. However, isolate 2016-1400 did not harbour any of the genes associated with hypervirulent *K. pneumoniae* (hvKp) or the capsule types (K1 and K2) which are mainly associated with hvKP. Analysis of *K. pneumoniae* strain C17KP0055 showed that this strain belonged to the same ST and did not harbour any of the hvKP associated virulence genes.

One MDR *K. pneumoniae* isolate (2019-1764) was recovered from a location close to the city centre of Bergen. The location is likely to be heavily influenced by anthropogenic activities, indicating that this isolate is of human or animal origin. This could pose a threat to the public health as the sampling location is in close proximity to bathing areas [59].

The overall results from our study are in accordance with previous studies on AMR in bacteria from the Norwegian marine environment, where low prevalence of acquired resistance was found in *E. coli* and *Vibrio* spp. [22,60,61]. In the present study, acquired resistance was only found in few *K. pneumoniae* isolates and in none of the other isolated species. These results reflect the restrictive use of antibiotics in Norway, both in clinics, food production and companion animals. In Norwegian clinical *K. pneumoniae* isolates, resistance to amoxicillin-clavulanic acid, trimethoprim, trimethoprim-sulfamethoxazole, cefuroxime and mecillinam are the most commonly observed resistance phenotypes [15]. Resistance to these agents, except mecillinam, was also found among the marine *K. pneumoniae* isolates. No carbapenemase-producing isolates were recovered. This is in contrast to the increase of CR-KP in some European countries [13] as well as several reports on the occurrence of CR-KP in the environment [59,62–64]. Although all of the isolated species are considered to be intrinsically resistant to aminopenicillins due to the presence of chromosomal class A β -lactamases [5,65], we found some ampicillin-susceptible isolates among all species, except *K. aerogenes* and *R. planticola*. This has previously been described in *Klebsiella* spp. isolates [66–69] and is in *K. pneumoniae* likely caused by differential expression [70] or lack of the *bla*_{SHV} gene.

K. pneumoniae was the most common species isolated from our samples. This is in accordance with a study by Podschun et al. [28] on *Klebsiella* spp. in surface waters from fresh, brackish and seawater in temperate regions. The presence of *K. oxytoca* seems to be less dependent on faecal contamination than *K. pneumoniae*. As *K. oxytoca* is known to be present in terrestrial environments [71], there are several transmission routes for this species to the marine environment. The relative distribution of the isolated genera in our study is in contradiction to the current description of the genus *Raoultella* as an environmental genus known to be associated with aquatic environments [6,65,72]. Even from samples with *E. coli* MPN/100 g below the LOQ, *Klebsiella* was the more frequently isolated compared to *Raoultella*.

This study provides enhanced understanding about the prevalence and AMR of *K. pneumoniae* found in the Norwegian marine environment. Although low prevalence of acquired resistance was

detected, our study demonstrates presence of ESBL-producing *K. pneumoniae* in an area used for production of marine bivalves for human consumption. This indicates that bivalve molluscs may act as a potential reservoir of ESBL-producing *K. pneumoniae* for transmission to the community through the food chain. Our study also highlights the importance of the marine environment in dissemination of opportunistic human pathogens and ARGs.

Supplementary Materials: The following are available online at www.mdpi.com/2076-2607/8/12/1909/s1, Table S1: Sample origin, bivalve species and *E. coli*/MPN 100 g, Table S2: Assembly statistics for whole genome sequence of *K. pneumoniae* isolate 2016-1400, Table S3: Species identification by MALDI-TOF-MS, isolate origin and *E. coli*/MPN 100 g, Table S4: Measured inhibition zones (mm) from AST of *Klebsiella* spp. and *Raoultella* spp.

Author Contributions: F.H., N.P.M., B.T.L., A.S., and C.S.S. contributed to the design and conception of the study, F.H. performed the experiments with assistance from N.P.M., bioinformatic analyses were done by F.H. and M.A.K.H. F.H. prepared the first draft of the manuscript, all authors reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was part of the KLEB-GAP project (project number TMS2019TMT03) funded by the Trond Mohn Foundation (<https://mohnfoundation.no/amr-prosjekter/>).

Acknowledgments: We wish to thank the Norwegian Food Safety Authority (NFSA), Eli Gustad at the Institute of Marine Research (IMR) Flødevigen, Cathinka Krogness at IMR Austevoll and Peter Hovgaard for providing samples for this study. A thanks to Tone Galluzzi, Leikny Fjeldstad, Betty Irgens and Kateryna Selezska Natvik for help with processing of samples and identification of isolates. We also acknowledge Ragna-Johanne Bakksjø and Eva Bernhoff at Stavanger University Hospital (SUS) for help with genome sequencing.

Conflicts of Interest: The authors declare no conflict of interest.

Data Availability: The genome sequence included in the study has been submitted to GenBank with the accession numbers CP065034 (chromosome) and CP065035 (plasmid). The short reads (ERR4570363) and long reads (ERR4859178) are available under BioProject PRJEB40149 in the European Nucleotide Archive.

References

1. Podschun, R.; Ullmann, U. *Klebsiella* spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods and Pathogenicity Factors. *Clin. Microbiol. Rev.* **1998**, *11*, 589–603, doi:10.1128/cmr.11.4.589.
2. Rodríguez-Medina, N.; Barrios-Camacho, H.; Duran-Bedolla, J.; Garza-Ramos, U. *Klebsiella variicola*: An emerging pathogen in humans. *Emerg. Microbes Infect.* **2019**, *8*, 973–988, doi:10.1080/22221751.2019.1634981.
3. Breurec, S.; Melot, B.; Hoen, B.; Passet, V.; Schepers, K.; Bastian, S.; Brisse, S. Liver Abscess Caused by Infection with Community-Acquired *Klebsiella quasipneumoniae* subsp. *quasipneumoniae*. *Emerg. Infect. Dis.* **2016**, *22*, 529–531, doi:10.3201/eid2203.151466.
4. Shankar, C.; Veeraraghavan, B.; Nabarro, L.E.B.; Ravi, R.; Ragupathi, N.K.D.; Rupali, P. Whole genome analysis of hypervirulent *Klebsiella pneumoniae* isolates from community and hospital acquired bloodstream infection. *BMC Microbiol.* **2018**, *18*, 6, doi:10.1186/s12866-017-1148-6.
5. Brisse, S.; Grimont, F.; Grimont, P.A.D. The Genus *Klebsiella*. In *Proteobacteria: Gamma Subclass*; Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., Eds.; Springer: New York, NY, USA, 2006; Volume 6.
6. Drancourt, M.; Bollet, C.; Carta, A.; Rousselier, P. Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. *Int. J. Syst. Evol. Microbiol.* **2001**, *51*, 925–932, doi:10.1099/00207713-51-3-925.
7. Sękowska, A. *Raoultella* spp.—Clinical significance, infections and susceptibility to antibiotics. *Folia Microbiol.* **2017**, *62*, 221–227, doi:10.1007/s12223-016-0490-7.
8. Brisse, S.; Passet, V.; Grimont, P.A.D. Description of *Klebsiella quasipneumoniae* sp. nov., isolated from human infections, with two subspecies, *Klebsiella quasipneumoniae* subsp. *quasipneumoniae* subsp. nov. and *Klebsiella quasipneumoniae* subsp. *similipneumoniae* subsp. nov. and demonstration that *Klebsiella singaporensis* is a junior heterotypic synonym of *Klebsiella variicola*. *Int. J. Syst. Evol. Microbiol.* **2014**, *64*, 3146–3152, doi:10.1099/ijss.0.062737-0.

9. Rodrigues, C.; Passet, V.; Rakotondrasoa, A.; Diallo, T.A.; Criscuolo, A.; Brisse, S. Description of *Klebsiella africanensis* sp. nov., *Klebsiella variicola* subsp. tropicalensis subsp. nov. and *Klebsiella variicola* subsp. variicola subsp. nov. *Res. Microbiol.* **2019**, *170*, 165–170, doi:10.1016/j.resmic.2019.02.003.
10. Wyres, K.L.; Lam, M.M.C.; E Holt, K. Population genomics of *Klebsiella pneumoniae*. *Nat. Rev. Genet.* **2020**, *18*, 344–359, doi:10.1038/s41579-019-0315-1.
11. Long, S.W.; Linson, S.E.; Saavedra, M.O.; Cantu, C.; Davis, J.J.; Brettin, T.; Olsen, R.J. Whole-Genome Sequencing of a Human Clinical Isolate of the Novel Species *Klebsiella quasivariicola* sp. nov. *Genome Announc.* **2017**, *5*, e01057–e02017, doi:10.1128/genomea.01057-17.
12. E Holt, K.; Wyres, K.L. *Klebsiella pneumoniae* as a key trafficker of drug resistance genes from environmental to clinically important bacteria. *Curr. Opin. Microbiol.* **2018**, *45*, 131–139, doi:10.1016/j.mib.2018.04.004.
13. ECDC. Surveillance of Antimicrobial Resistance in Europe: Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2018; Stockholm, Sweden 2019 Available online: <https://www.ecdc.europa.eu/en/publications-data/surveillance-antimicrobial-resistance-europe-2018> (accessed on 17 October 2020).
14. Bevan, E.R.; Jones, A.M.; Hawkey, P.M. Global epidemiology of CTX-M β -lactamases: Temporal and geographical shifts in genotype. *J. Antimicrob. Chemother.* **2017**, *72*, 2145–2155, doi:10.1093/jac/dkx146.
15. NORM/NORM-VET. NORM/NORM-VET: Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway 2018; Norwegian Surveillance System for Antibiotic Resistance in Microbes/ Norwegian Veterinary Institute/Norwegian Institute of Public Health, Tromsø/Oslo, Norway, 2018. Available online: <https://www.vetinst.no/en/surveillance-programmes/norm-norm-vet-report> (accessed on 17 October 2020).
16. NORM/NORM-VET. NORM/NORM-VET: Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway 2010; Norwegian Surveillance System for Antibiotic Resistance in Microbes/ Norwegian Veterinary Institute/Norwegian Institute of Public Health, Tromsø/Oslo, Norway 2010. Available online: <https://www.vetinst.no/en/surveillance-programmes/norm-norm-vet-report> (accessed on 17 October 2020).
17. Martin, R.M.; Bachman, M. Colonization, Infection and the Accessory Genome of *Klebsiella pneumoniae*. *Front. Cell. Infect. Microbiol.* **2018**, *8*, 4, doi:10.3389/fcimb.2018.00004.
18. Wyres, K.L.; E Holt, K. *Klebsiella pneumoniae* Population Genomics and Antimicrobial-Resistant Clones. *Trends Microbiol.* **2016**, *24*, 944–956, doi:10.1016/j.tim.2016.09.007.
19. Von Wintersdorff, C.J.H.; Penders, J.; Van Niekerk, J.M.; Mills, N.D.; Majumder, S.; Van Alphen, L.B.; Savelkoul, P.H.M.; Wolffs, P.F.G. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Front. Microbiol.* **2016**, *7*, 173, doi:10.3389/fmicb.2016.00173.
20. Martínez, J. Antibiotics and Antibiotic Resistance Genes in Natural Environments. *Science* **2008**, *321*, 365–367, doi:10.1126/science.1159483.
21. Berendonk, T.U.; Manaia, C.M.; Merlin, C.; Fatta-Kassinos, D.; Cytryn, E.; Walsh, F.; Buergermann, H.; Sørum, H.; Norström, M.; Pons, M.-N.; et al. Tackling antibiotic resistance: The environmental framework. *Nat. Rev. Genet.* **2015**, *13*, 310–317, doi:10.1038/nrmicro3439.
22. Grevskott, D.H.; Svanevik, C.S.; Sunde, M.; Wester, A.L.; Lunestad, B.T. Marine Bivalve Mollusks As Possible Indicators of Multidrug-Resistant *Escherichia coli* and Other Species of the Enterobacteriaceae Family. *Front. Microbiol.* **2017**, *8*, 24, doi:10.3389/fmicb.2017.00024.
23. Jørgensen, S.; Sørås, A.V.; Arnesen, L.S.; Leegaard, T.M.; Sundsfjord, A.; Jenum, P.A. A comparison of extended spectrum β -lactamase producing *Escherichia coli* from clinical, recreational water and wastewater samples associated in time and location. *PLoS ONE* **2017**, *12*, e0186576, doi:10.1371/journal.pone.0186576.
24. Bernard, F.R. Uptake and Elimination of Coliform Bacteria by Four Marine Bivalve Mollusks. *Can. J. Fish. Aquat. Sci.* **1989**, *46*, 1592–1599, doi:10.1139/f89-203.
25. Kibria, G.; Hossain, M.; Mallick, D.; Lau, T.; Wu, R. Monitoring of metal pollution in waterways across Bangladesh and ecological and public health implications of pollution. *Chemosphere* **2016**, *165*, 1–9, doi:10.1016/j.chemosphere.2016.08.121.
26. Runcharoen, C.; Moradigaravand, D.; Blane, B.; Paksanont, S.; Thammachote, J.; Anun, S.; Parkhill, J.; Chantratita, N.; Peacock, S.J. Whole genome sequencing reveals high-resolution epidemiological links between clinical and environmental *Klebsiella pneumoniae*. *Genome Med.* **2017**, *9*, 1–10, doi:10.1186/s13073-017-0397-1.

27. Struve, C.; Krogfelt, K.A. Pathogenic potential of environmental *Klebsiella pneumoniae* isolates. *Environ. Microbiol.* **2004**, *6*, 584–590, doi:10.1111/j.1462-2920.2004.00590.x.
28. Podschun, R.; Pietsch, S.; Höller, C.; Ullmann, U. Incidence of *Klebsiella* Species in Surface Waters and Their Expression of Virulence Factors. *Appl. Environ. Microbiol.* **2001**, *67*, 3325–3327, doi:10.1128/aem.67.7.3325-3327.2001.
29. Van Kregten, E.; A Westerdaal, N.; Willers, J.M. New, simple medium for selective recovery of *Klebsiella pneumoniae* and *Klebsiella oxytoca* from human feces. *J. Clin. Microbiol.* **1984**, *20*, 936–941, doi:10.1128/jcm.20.5.936-941.1984.
30. ISO. ISO16649-3. Microbiology of the Food Chain—Horizontal Method for the Enumeration of Beta-Glucuronidase-Positive *Escherichia Coli*—Part 3: Detection and Most Probable Number Technique Using 5-bromo-4-chloro-3-indolyl- β -D-Glucuronide; International Organization for Standardization (ISO): Geneva, Switzerland, 2015.
31. EU. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Off. J. Eur. Communities* **2004**, *24*, 83–127.
32. EUCAST. Antimicrobial Susceptibility Testing-EUCAST Disk Diffusion Method. Available online: <https://www.eucast.org> (accessed on 9 January 2020).
33. EUCAST. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Available online: <https://www.eucast.org/> (accessed on 10 July 2020).
34. Magiorakos, A.-P.; Srinivasan, A.; Carey, R.; Carmeli, Y.; Falagas, M.; Giske, C.; Harbarth, S.J.; Hindler, J.; Kahlmeter, G.; Olsson-Liljequist, B.; et al. Multidrug-Resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* **2012**, *18*, 268–281, doi:10.1111/j.1469-0691.2011.03570.x.
35. Wick, R.R.; Judd, L.M.; Gorrie, C.L.; E Holt, K. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* **2017**, *13*, e1005595, doi:10.1371/journal.pcbi.1005595.
36. Feldgarden, M.; Brover, V.; Haft, D.H.; Prasad, A.B.; Slotta, D.J.; Tolstoy, I.; Tyson, G.H.; Zhao, S.; Hsu, C.-H.; McDermott, P.F.; et al. Validating the AMRFinder Tool and Resistance Gene Database by Using Antimicrobial Resistance Genotype-Phenotype Correlations in a Collection of Isolates. *Antimicrob. Agents Chemother.* **2019**, *63*, doi:10.1128/aac.00483-19.
37. Chen, L.; Zheng, D.; Liu, B.; Yang, J.; Jin, Q. VFDB 2016: Hierarchical and refined dataset for big data analysis—10 years on. *Nucleic Acids Res.* **2016**, *44*, D694–D697, doi:10.1093/nar/gkv1239.
38. Carattoli, A.; Zankari, E.; García-Fernández, A.; Larsen, M.V.; Lund, O.; Villa, L.; Aarestrup, F.M.; Hasman, H. In Silico Detection and Typing of Plasmids using PlasmidFinder and Plasmid Multilocus Sequence Typing. *Antimicrob. Agents Chemother.* **2014**, *58*, 3895–3903, doi:10.1128/aac.02412-14.
39. Tatusova, T.; DiCuccio, M.; Badretdin, A.; Chetvernin, V.; Nawrocki, E.P.; Zaslavsky, L.; Lomsadze, A.; Pruitt, K.D.; Borodovsky, M.; Ostell, J. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res.* **2016**, *44*, 6614–6624, doi:10.1093/nar/gkw569.
40. Alikhan, N.-F.; Petty, N.K.; Ben Zakour, N.L.; Beatson, S.A. BLAST Ring Image Generator (BRIG): Simple prokaryote genome comparisons. *BMC Genom.* **2011**, *12*, 402, doi:10.1186/1471-2164-12-402.
41. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.S.; Bealer, K.; Madden, T.L. BLAST+: Architecture and applications. *BMC Bioinform.* **2009**, *10*, 421, doi:10.1186/1471-2105-10-421.
42. Paterson, D.L.; Hujer, K.M.; Hujer, A.M.; Yeiser, B.; Bonomo, M.D.; Rice, L.B.; Bonomo, R.A. Extended-Spectrum β -Lactamases in *Klebsiella pneumoniae* Bloodstream Isolates from Seven Countries: Dominance and Widespread Prevalence of SHV- and CTX-M-Type β -Lactamases. *Antimicrob. Agents Chemother.* **2003**, *47*, 3554–3560, doi:10.1128/aac.47.11.3554-3560.2003.
43. Jutkina, J.; Rutgersson, C.; Flach, C.-F.; Larsson, D.G.J. An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. *Sci. Total. Environ.* **2016**, 131–138, doi:10.1016/j.scitotenv.2016.01.044.
44. Gniadkowski, M.; Schneider, I.; Palucha, A.; Jungwirth, R.; Mikiewicz, B.; Bauernfeind, A. Cefotaxime-Resistant Enterobacteriaceae Isolates from a Hospital in Warsaw, Poland: Identification of a New CTX-M-3 Cefotaxime-Hydrolyzing β -Lactamase That Is Closely Related to the CTX-M-1/MEN-1 Enzyme. *Antimicrob. Agents Chemother.* **1998**, *42*, 827–832, doi:10.1128/aac.42.4.827.
45. Cantón, R.; Egonzalez-Alba, J.M.; Galan, J.-C. CTX-M Enzymes: Origin and Diffusion. *Front. Microbiol.* **2012**, *3*, 110, doi:10.3389/fmicb.2012.00110.

46. Klein, E.Y.; Van Boeckel, T.P.; Martinez, E.M.; Pant, S.; Gandra, S.; Levin, S.A.; Goossens, H.; Laxminarayan, R. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E3463–E3470.
47. Rawat, D.; Nair, D. Extended-spectrum β -lactamases in gram negative bacteria. *J. Glob. Infect. Dis.* **2010**, *2*, 263–274, doi:10.4103/0974-777x.68531.
48. Bush, K.; Jacoby, G.A. Updated Functional Classification of β -Lactamases. *Antimicrob. Agents Chemother.* **2009**, *54*, 969–976, doi:10.1128/aac.01009-09.
49. Bialek-Davenet, S.; Criscuolo, A.; Ailloud, F.; Passet, V.; Jones, L.; Delannoy-Vieillard, A.-S.; Garin, B.; Le Hello, S.; Arlet, G.; Nicolas-Chanoine, M.-H.; et al. Genomic Definition of Hypervirulent and Multidrug-Resistant *Klebsiella pneumoniae* Clonal Groups. *Emerg. Infect. Dis.* **2014**, *20*, 1812–1820, doi:10.3201/eid2011.140206.
50. Rodrigues, C.; D’Humières, C.; Papin, G.; Passet, V.; Ruppé, E.; Brisse, S. Community-acquired infection caused by the uncommon hypervirulent *Klebsiella pneumoniae* ST66-K2 lineage. *Microb. Genom.* **2020**, *6*, e000419, doi:10.1099/mgen.0.000419.
51. Baker-Austin, C.; Wright, M.S.; Stepanauskas, R.; McArthur, J. Co-selection of antibiotic and metal resistance. *Trends Microbiol.* **2006**, *14*, 176–182, doi:10.1016/j.tim.2006.02.006.
52. Green, N.W.; Schøyen, M.; Hjermann, D.Ø.; Øxnevad, S.; Ruus, A.; Lusher, A.; Beylich, B.; Lund, E.; Tveiten, L.A.; Håvardstun, J.; et al. Contaminants in Coastal Waters of Norway 2017. Available online: <https://www.miljodirektoratet.no/globalassets/publikasjoner/m1120/m1120.pdf> (accessed on 17 October 2020).
53. Cubero, M.; Grau, I.; Tubau, F.; Pallarés, R.; Dominguez, M.; Liñares, J.; Ardanuy, C. Hypervirulent *Klebsiella pneumoniae* clones causing bacteraemia in adults in a teaching hospital in Barcelona, Spain (2007–2013). *Clin. Microbiol. Infect.* **2016**, *22*, 154–160, doi:10.1016/j.cmi.2015.09.025.
54. Poirel, L.; Aires-De-Sousa, M.; Kudyba, P.; Kieffer, N.; Nordmann, P. Screening and Characterization of Multidrug-Resistant Gram-Negative Bacteria from a Remote African Area, São Tomé and Príncipe. *Antimicrob. Agents Chemother.* **2018**, *62*, 01021–01018, doi:10.1128/aac.01021-18.
55. Hansen, S.K.; Kaya, H.; Roer, L.; Hansen, F.; Skovgaard, S.; Justesen, U.S.; Hansen, D.S.; Andersen, L.P.; Knudsen, J.D.; Røder, B.L.; et al. Molecular characterization of Danish ESBL/AmpC-producing *Klebsiella pneumoniae* from bloodstream infections. *J. Glob. Antimicrob. Resist.* **2020**, doi:10.1016/j.jgar.2020.05.014.
56. Higashino, M.; Murata, M.; Morinaga, Y.; Akamatsu, N.; Matsuda, J.; Takeda, K.; Kaku, N.; Kosai, K.; Uno, N.; Hasegawa, H.; et al. Fluoroquinolone resistance in extended-spectrum β -lactamase-producing *Klebsiella pneumoniae* in a Japanese tertiary hospital: Silent shifting to CTX-M-15-producing *K. pneumoniae*. *J. Med. Microbiol.* **2017**, *66*, 1476–1482, doi:10.1099/jmm.0.000577.
57. Pan, Y.-J.; Fang, H.-C.; Lin, T.-L.; Hsieh, P.-F.; Tsai, F.-C.; Keynan, Y.; Wang, J.-T. Capsular Polysaccharide Synthesis Regions in *Klebsiella pneumoniae* Serotype K57 and a New Capsular Serotype. *J. Clin. Microbiol.* **2008**, *46*, 2231–2240, doi:10.1128/jcm.01716-07.
58. Hsu, C.-R.; Liao, C.-H.; Lin, T.-L.; Yang, H.-R.; Yang, F.-L.; Hsieh, P.-F.; Wu, S.-H.; Wang, J.-T. Identification of a capsular variant and characterization of capsular acetylation in *Klebsiella pneumoniae* PLA-associated type K. *Sci. Rep.* **2016**, *6*, 31946, doi:10.1038/srep31946.
59. Lepuschitz, S.; Schill, S.; Stoeger, A.; Pekard-Amenitsch, S.; Huhulescu, S.; Inreiter, N.; Hartl, R.; Kerschner, H.; Sorschag, S.; Springer, B.; et al. Whole genome sequencing reveals resemblance between ESBL-producing and carbapenem resistant *Klebsiella pneumoniae* isolates from Austrian rivers and clinical isolates from hospitals. *Sci. Total. Environ.* **2019**, *662*, 227–235, doi:10.1016/j.scitotenv.2019.01.179.
60. Håkonsholm, F.; Lunestad, B.T.; Sánchez, J.R.A.; Martinez-Urtaza, J.; Marathe, N.P.; Svanevik, C.S. Vibrios from the Norwegian marine environment: Characterization of associated antibiotic resistance and virulence genes. *Microbiology* **2020**, *9*, e1093, doi:10.1002/mbo3.1093.
61. Grevskott, D.H.; Salvà-Serra, F.; Moore, E.R.B.; Marathe, N.P. Nanopore sequencing reveals genomic map of CTX-M-type extended-spectrum β -lactamases carried by *Escherichia coli* strains isolated from blue mussels (*Mytilus edulis*) in Norway. *BMC Microbiol.* **2020**, *20*, 10, doi:10.1186/s12866-020-01821-8.
62. Jelic, M.; Hrenović, J.; Dekić, S.; Goić-Barišić, I.; Andrašević, A.T. First evidence of KPC-producing ST258 *Klebsiella pneumoniae* in river water. *J. Hosp. Infect.* **2019**, *103*, 147–150, doi:10.1016/j.jhin.2019.04.001.
63. Nascimento, T.; Cantamessa, R.; Melo, L.; Fernandes, M.R.; Fraga, E.; Dropa, M.; Sato, M.I.; Cerdeira, L.; Lincopan, N. International high-risk clones of *Klebsiella pneumoniae* KPC-2/CC258 and *Escherichia coli* CTX-M-15/CC10 in urban lake waters. *Sci. Total. Environ.* **2017**, *598*, 910–915, doi:10.1016/j.scitotenv.2017.03.207.

64. Piedra-Carrasco, N.; Fàbrega, A.; Calero-Cáceres, W.; Cornejo-Sánchez, T.; Brown-Jaque, M.; Mir-Cros, A.; Muniesa, M.; González-López, J.J. Carbapenemase-producing enterobacteriaceae recovered from a Spanish river ecosystem. *PLoS ONE* **2017**, *12*, e0175246, doi:10.1371/journal.pone.0175246.
65. Ponce-Alonso, M.; Rodríguez-Rojas, L.; Del Campo, R.; Cantón, R.; Morosini, M.-I. Comparison of different methods for identification of species of the genus *Raoultella*: Report of 11 cases of *Raoultella* causing bacteraemia and literature review. *Clin. Microbiol. Infect.* **2016**, *22*, 252–257, doi:10.1016/j.cmi.2015.10.035.
66. Babini, G.S.; Livermore, D.M. Are SHV β -Lactamases Universal in *Klebsiella pneumoniae*? *Antimicrob. Agents Chemother.* **2000**, *44*, 2230, doi:10.1128/aac.44.8.2230-2230.2000.
67. Potter, R.F.; Lainhart, W.; Twentyman, J.; Wallace, M.A.; Wang, B.; Burnham, C.-A.D.; Rosen, D.A.; Dantas, G. Population Structure, Antibiotic Resistance and Uropathogenicity of *Klebsiella variicola*. *mBio* **2018**, *9*, e02481–e03018, doi:10.1128/mbio.02481-18.
68. Hartantyo, S.H.P.; Chau, M.L.; Koh, T.H.; Yap, M.; Yi, T.; Cao, D.Y.H.; GutiérrezR.A.; Ng, L.C. Foodborne *Klebsiella pneumoniae*: Virulence Potential, Antibiotic Resistance and Risks to Food Safety. *J. Food Prot.* **2020**, *83*, 1096–1103, doi:10.4315/jfp-19-520.
69. Matsen, J.M.; Spindler, J.A.; Blosser, R.O. Characterization of *Klebsiella* Isolates from Natural Receiving Waters and Comparison with Human Isolates. *Appl. Microbiol.* **1974**, *28*, 672–678, doi:10.1128/aem.28.4.672-678.1974.
70. Fu, Y.; Zhang, F.; Zhang, W.; Chen, X.; Zhao, Y.; Ma, J.; Bao, L.; Song, W.; Ohsugi, T.; Urano, T.; et al. Differential expression of blaSHV related to susceptibility to ampicillin in *Klebsiella pneumoniae*. *Int. J. Antimicrob. Agents* **2007**, *29*, 344–347, doi:10.1016/j.ijantimicag.2006.10.015.
71. Imhoff, J.F. “Enterobacteriales”. In *Bergey’s Manual® of Systematic Bacteriology: Volume Two the Proteobacteria Part B the Gammaproteobacteria*; Brenner, D.J., Krieg, N.R., Staley, J.T., Garrity, G.M., Boone, D.R., De Vos, P., Goodfellow, M., Rainey, F.A., Schleifer, K.-H., Eds.; Springer: Boston, MA, USA, 2005; pp. 587–850.
72. Bagley, S.T.; Seidler, R.J.; Brenner, D.J. *Klebsiella planticola* sp. nov.: A new species of enterobacteriaceae found primarily in nonclinical environments. *Curr. Microbiol.* **1981**, *6*, 105–109, doi:10.1007/bf01569013

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