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Klebsiella pneumoniae in the marine environment

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Acknowledgments

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Abbreviations

AMP – Ampicillin

ARGs – Antibiotic resistance genes

BPW – Buffered peptone water

ESBL – Extended spectrum β-lactamase

EUCAST – European Committee on Antimicrobial Susceptibility Testing

GFP – Green fluorescent protein

GI – Gastrointestinal

HGT – Horizontal gene transfer

HMRGs – Heavy metal resistance genes

hvKp – Hypervirulent *Klebsiella pneumoniae*

ICE – Integrative conjugative element

KAN – Kanamycin

KpSC – *Klebsiella pneumoniae* species complex

LPS – lipopolysaccharide

MALDI-TOF MS – Matrix-assisted laser desorption ionisation-time of flight mass spectrometry

MDR – Multidrug resistant

MDR-Kp – Multidrug resistant *Klebsiella pneumoniae*

MGE – Mobile genetic element

MHB – Mueller-Hinton broth

MIC – Minimum inhibitory concentration

MLST – Multilocus sequence typing

MPN – Most probable number

PBS – Phosphate buffered saline

PCU – Population correction unit

RIF – Rifampicin

SCAI – Simmons citrate agar with 1 % myo-inositol

SNP – Singe nucleotide polymorphism

ST – Sequence type

WGS – Whole-genome sequencing

WHO – World Health Organization

List of publications

Paper I

Håkonsholm, F., Hetland, M. A. K., Svanevik, C. S., Sundsfjord, A., Lunestad, B. T. & Marathe, N. P. (2020). Antibiotic Sensitivity Screening of *Klebsiella* spp. and *Raoultella* spp. Isolated from Marine Bivalve Molluscs Reveal Presence of CTX-M-Producing *K. pneumoniae*. *Microorganisms,* 8**,** 1909, doi:https://doi.org/10.3390/microorganisms8121909.

Paper II

Håkonsholm, F., Hetland, M. A. K., Svanevik, C. S., Lunestad, B. T., Löhr, I. H. & Marathe, N. P. (2022). Insights into the genetic diversity, antibiotic resistance and pathogenic potential of *Klebsiella pneumoniae* from the Norwegian marine environment using whole-genome analysis. *International Journal of Hygiene and Environmental Health,* 242**,** 113967, doi:https://doi.org/10.1016/j.ijheh.2022.113967.

Paper III

Håkonsholm, F., Hetland, M. A. K., Löhr, I. H., Lunestad, B. T. & Marathe, N. P. (202X). Complete genome sequences of *Klebsiella pneumoniae* isolates carrying antibiotic resistance genes and heavy metal resistance genes isolated from marine bivalves. (*Submitted to BMC Microbiology*).

Summary

Klebsiella pneumoniae is a common cause of infections in humans and is often associated with antibiotic resistance. *K. pneumoniae* is present in a wide range of environments. However, there is very limited knowledge about its prevalence, genetic diversity, associated antimicrobial resistance and pathogenic potential in the marine environment. The work presented in this thesis represents a comprehensive study on *K. pneumoniae* in the Norwegian marine environment.

Overall, 578 batch samples of bivalve molluscs, 53 fish, 24 sediment samples, 17 seawater samples and seven sea urchins were examined. Isolates were identified by MALDI-TOF MS and subjected to antibiotic susceptibility testing by disk diffusion. Whole genome sequencing was performed on 99 isolates identified as *K. pneumoniae sensu stricto (K. pneumoniae)*, while isolates carrying acquired antibiotic resistance genes (ARGs) were also long-read sequenced. The transferability of selected resistance plasmids was examined by filter mating experiments.

We found *K. pneumoniae* to be present in 14 % of examined bivalve samples and in 35 % of seawater samples. Eight isolates displayed acquired phenotypic resistance to one or more antibiotics included in the study. Acquired ARGs were present in six *K. pneumoniae* isolates, while ten carried the yersiniabactin siderophore. *K. pneumoniae* from bivalves carried all acquired ARGs on IncF family plasmids, and most of these plasmids also carry genes encoding resistance to heavy metals. One of these plasmids was transferable to an *Escherichia coli* recipient via conjugation. We observed high genetic diversity among *K. pneumoniae* from the marine environment and showed presence of globally disseminated sequence types in bivalves. Further analysis revealed close genetic relationship between a *K. pneumoniae* ST25 isolate from blue mussels and a clinical ST25 isolate from Germany.

This study shows that *K. pneumoniae* was present in marine bivalves and seawater collected from Norwegian coastal waters, including isolates carrying clinically relevant ARGs and virulence genes. This work illustrates the potential of the marine environment, especially coastal waters, and seafood organisms to serve as vectors for human exposure to opportunistic pathogens and antibiotic resistance. Furthermore, our results highlight the importance of surveillance of pathogens and antimicrobial resistance in the marine environment.

1 Introduction

1.1 Genus *Klebsiella*

The genus *Klebsiella* is named after the German microbiologist Edwin Klebs and comprise non-motile (except *K. aerogenes*), aerobic and facultative anaerobic, Gram-negative, rod shaped, often encapsulated bacteria, belonging to the family Enterobacteriaceae (Grimont and Grimont, 2015;Brisse et al., 2006). In 2001, three *Klebsiella* species, *K. ornithinolytica*, *K. planticola* and *K. terrigena*, were re-assigned to the novel genus *Raoultella* based on comparative analysis of 16S rRNA and *rpoB* sequences (Drancourt et al., 2001). However, more recent studies using whole-genome sequence data has revealed a monophyletic relationship between the *Klebsiella* genus and the *Raoultella* genus. Therefore it has been proposed that the genus *Raoultella* should be reunified with the *Klebsiella* genus, a reclassification of *R. electrica* as *K. electrica* has also been proposed (Ma et al., 2021).

The members of the genus can be isolated from a wide range of sources, such as the gastrointestinal (GI) tract of mammals, soil, surface waters and vegetation where they contribute to geochemical and biochemical processes (Brisse et al., 2006;Grimont and Grimont, 2015;Bagley, 1985;Wyres et al., 2020).

Several species in the genus can cause serious infections in humans, especially in health care settings, and also in animals (Brisse et al., 2006). Within the genus, *K. pneumoniae* is clinically the most relevant species with regards to infections in humans (Brisse et al., 2006).

1.2 *Klebsiella pneumoniae*

K. pneumoniae was first described in 1882 by Carl Friedlaender and initially given the name Friedlander's bacillus before it was assigned to the novel genus *Klebsiella* (Russo and Marr, 2019;Grimont and Grimont, 2015;Friedlaender, 1882). The major reservoirs of *K. pneumoniae* are not well known, but the human GI tract is recognised as an important habitat. The GI carriage has also been shown to be associated with development of clinical *K. pneumoniae* infections in hospitalised patients (Martin and Bachman, 2018;Paczosa and Mecsas, 2016;Gorrie et al., 2017). There are large geographical variations in GI carriage rates. It has been reported to be 6-19 % in intensive care patients in Australia (Gorrie et al., 2017), 16 % in community-based adults in Norway (Raffelsberger et al., 2021), and as high as 88 % in healthy carriers in Malaysia (Lin et al., 2012). However, it is important to note that some of the differences in the GI carriage rates in these studies could be caused by differences in the

isolation and identification methods, and study populations of the different studies (Lin et al., 2012;Raffelsberger et al., 2021). *K. pneumoniae* can also be found in the nasopharynx of humans, but with lower prevalence than in the GI tract (Grimont and Grimont, 2015). The GI tract of other mammals, like dogs, cattle, pigs and poultry can also be inhabited by *K. pneumoniae* (Wyres et al., 2020;Franklin-Alming et al., 2021;Leangapichart et al., 2021), and the bacterium has also been isolated from a range of environments, including soil, surface waters and plants, as well as food products (Podschun et al., 2001;Bagley, 1985;Thorpe et al., 2021;Davis and Price, 2016;Wyres et al., 2020).

Recently, whole-genome sequencing (WGS) has revealed that isolates often identified as *K. pneumoniae* using standard laboratory methods, actually belong to five closely related species, of which two contains subspecies. These species share ~95-96 % average nucleotide identity with *K. pneumoniae*, and ~90 % average nucleotide identity with other *Klebsiella* species (Wyres et al., 2020). Typically, strains belonging to the same species share ≥ 95 % average nucleotide identity, and subspecies can be identified based on this proposed threshold in combination with biochemical test (Richter and Rosselló-Móra, 2009;Rodrigues et al., 2019). Together these species are informally referred to as the *K. pneumoniae* species complex (KpSC). The KpSC consists of *K. pneumoniae sensu stricto*, *K. quasipneumoniae* subspecies *quasipneumoniae*, *K. variicola* subsp. *variicola*, *K. quasipneumoniae* subsp. *similipneumoniae*, *K. variicola* subsp. *tropica*, *K. quasivariicola*, and *K. africana* (**Figure 1**). Of the KpSC members, *K. pneumoniae sensu stricto* is responsible for the majority of human infections and is one of the most common causes of nosocomial infections globally (Wyres et al., 2020). However, *K. variicola* subsp. *variicola* is also considered an emerging human pathogen (Rodríguez-Medina et al., 2019), and has recently been found to be responsible for 25 % of KpSC blood stream infections in Norway (Fostervold et al., 2021). For the remainder of this doctoral thesis, *K. pneumoniae* refers to *K. pneumoniae sensu stricto*.

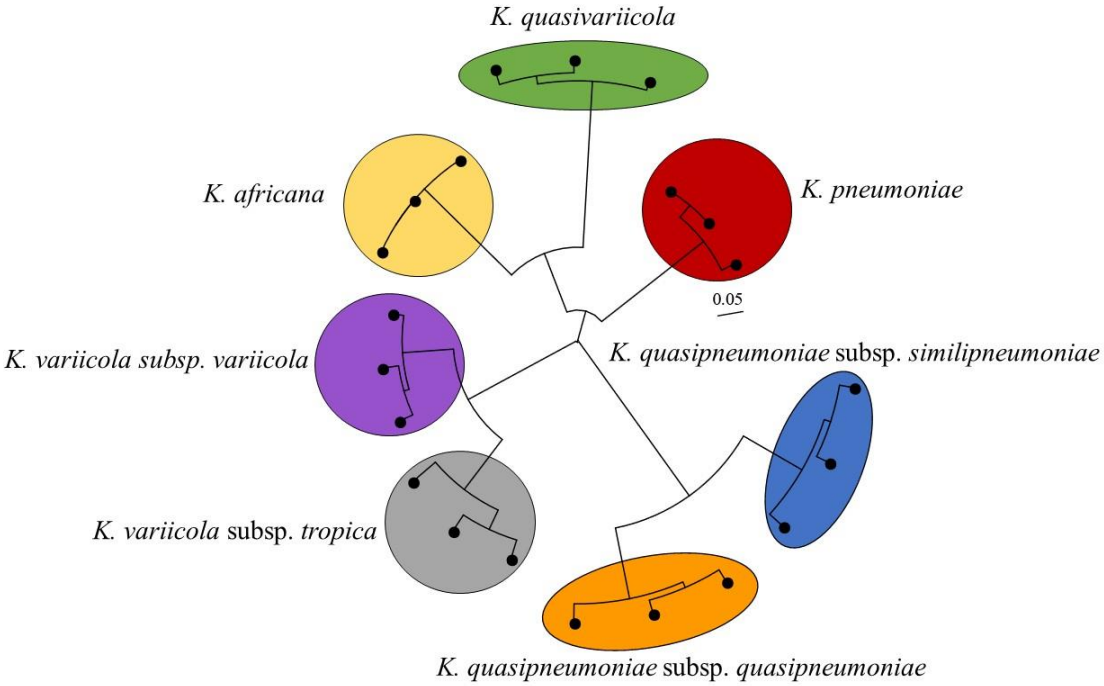

Figure 1. Core genome phylogeny of *Klebsiella pneumoniae* species complex members. The figure was created with genomes from own work and the following publicly available genomes: SRR13775017, SRR13775019, SRR12233581, SRR12233604, SRR12233579, SRR16202827, ERR2835897, ERR1217441, ERR3416001 and ERR3416096.

1.2.1 The *K. pneumoniae* genome

K. pneumoniae is a genetically diverse species (Wyres and Holt, 2018). The genome consists of core and accessory genes. The core genome is defined as the collection of genes present in ≥ 95 % of the members of a species, while genes that are present in individual members of a species are part of the accessory genome (Holt et al., 2015). Together, the core- and accessory genome constitutes the pangenome (Vernikos et al., 2015).

The genome of *K. pneumoniae* comprises ~5000 genes, and of these, only ~2000 are core genes, meaning that the majority of genes in *K. pneumoniae* are part of the accessory genome (Martin and Bachman, 2018;Holt et al., 2015). The core genes have been suggested to enable *K. pneumoniae* survival in different environmental niches by providing diverse metabolic and other capabilities. It has also been predicted that a large part of the pangenome encodes proteins with metabolic functions, which potentially could provide individual *K. pneumoniae* strains with abilities to survive in additional ecological niches (Wyres and Holt, 2018).

K. pneumoniae has a recognised ability to acquire mobile genetic elements (MGEs) through horizontal gene transfer (HGT), and can act as recipient of plasmids from a wide range of donors (Wyres and Holt, 2018). The accessory genome may include antibiotic resistance genes (ARGs) and virulence genes (Martin and Bachman, 2018), and the acquisition of MGEs have contributed to the development of two *K. pneumoniae* groups, multidrug resistant *K. pneumoniae* (MDR-Kp) and hypervirulent *K. pneumoniae* (hvKp) (Russo and Marr, 2019).

1.2.2 *K. pneumoniae* virulence

Numerous virulence factors are described in *K. pneumoniae*. The core genome includes type 1 and type 3 fimbriae involved in adhesion to surfaces and biofilm formation, the enterobactin siderophore involved in iron acquisition, as well a capsule and lipopolysaccharide (LPS) (Russo and Marr, 2019;Paczosa and Mecsas, 2016;Wyres et al., 2020).

Virulence factors associated with hvKp are carried on plasmids and integrative conjugative elements (ICEs). These include the additional salmochelin, yersiniabactin and aerobactin siderophores, the colibactin toxin and the *rmpA* and *rmpA2* genes involved in increased production of capsule polysaccharide and a hypermucoid phenotype (Russo and Marr, 2019;Paczosa and Mecsas, 2016;Wyres et al., 2020;Walker and Miller, 2020). Yersiniabactin is normally chromosomally encoded and mobilised by ICEs, while the remaining virulence factors usually are carried on large virulence plasmids, like the pLVPK plasmid (Russo and Marr, 2019;Wyres et al., 2020;Walker and Miller, 2020). In addition to *rmpA* and *rmpA2*, increased capsule production can be caused by the chromosomal *magA* gene (Paczosa and Mecsas, 2016). In contrast to the enterobactin siderophore, the acquired siderophores are not bound and neutralised by lipocalin-2, which is released by several cell types during infection (Wyres et al., 2020;Russo and Marr, 2019;Paczosa and Mecsas, 2016). Some capsule types are frequently associated with hvKp, and the most common of these are K1, K2, K5, K20, K54 and K57. Of these, the K1 and K2 capsule types account for the majority of hvKp isolates (Russo and Marr, 2019;Wyres et al., 2020). Several of the described virulence factors, *e.g.* those involved in biofilm formation, capsule production, iron acquisition as well as modifications of LPS, may be involved in protection of *K. pneumoniae* from the hosts immune response (Tiria and Musila, 2021;Paczosa and Mecsas, 2016;Martin and Bachman, 2018).

Although many virulence factors are associated with hvKp, the combination of these acquired virulence genes required for hypervirulence is largely unknown (Russo and Marr, 2019;Lan et al., 2021). In a previous study, hvKp has been defined as isolates carrying *rmpA* and/or *rmpA2*,

and/or at least one complete gene cluster among the aerobactin and salmochelin siderophores (Huynh et al., 2020).

1.2.3 *K. pneumoniae* infections

K. pneumoniae is an opportunistic pathogen causing nosocomial infections in patients with underlying disease and is among the most common causes of health care associated infections. However, hvKp can also cause infections in otherwise healthy individuals. Overall, *K. pneumoniae* accounts for around one third of all Gram-negative infections in hospital settings (Navon-Venezia et al., 2017). The most common infections caused by *K. pneumoniae* are urinary tract infections, bloodstream infections, and pneumonia (Wyres et al., 2020;Podschun and Ullmann, 1998;Russo and Marr, 2019;Bengoechea and Sa Pessoa, 2018). The hvKp strains can cause infections in multiple sites in the human body, especially liver abscess, but are able to spread and can cause infections like meningitis, necrotising fasciitis and non-hepatic abscess (Russo and Marr, 2019). Previously, hvKp has mainly been reported from Asia, while MDR-Kp has been dominating in Western countries (Russo and Marr, 2019). However, more recent reports are indicating an increased geographical spread of hvKp (ECDC, 2021). GI carriage of *K. pneumoniae* has been identified as a risk factor for subsequent development of infections also for hvKp infections (Paczosa and Mecsas, 2016).

Even though a link between colonisation and subsequent infection has been established, there are knowledge gaps in how colonisation develops into infection (Martin and Bachman, 2018). Some risk factors, such as density of colonising strains (colonisation pressure) has been suggested to play a role in progression to infection, and procedures, like endoscopy, can be potential sources of infections caused by colonising strains. In general, underlying conditions affecting the hosts immune status, like cancer and diabetes mellitus are associated with *K. pneumoniae* infection. However, it is not known if these also are associated with progression from colonisation to infection (Martin and Bachman, 2018).

1.2.4 *K. pneumoniae* sequence types

Multilocus sequence typing (MLST) has been the most common method for molecular typing of *K. pneumoniae* isolates (Chen et al., 2014). The method has made it possible to study the evolutionary origin and dissemination of bacterial strains (Diancourt et al., 2005). *K. pneumoniae* MLST is based on sequencing of seven housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*). From variations in the sequences of these genes, the strains are given a genetic profile, a sequence type (ST) (Chen et al., 2014). Currently, more

than 6 000 STs are registered in the *K. pneumoniae* MLST database (https://bigsdb.pasteur.fr/klebsiella, accessed 16.08.22). *K. pneumoniae* isolates can also be categorised into clonal groups based on core genome phylogeny or core genome MLST, where isolates sharing at least 594 of 694 core genome MLST alleles are assigned to the same group. The seven gene MLST can also be used to group isolates into clonal groups, but can potentially fail to distinguish groups due to frequent recombination events (Wyres et al., 2020).

Some STs are more frequently associated with human infections, some with hvKp and some with MDR-Kp, and are widely distributed geographically. MDR-Kp and hvKp frequently belong to specific STs. For example, the most common ST associated with hvKp is ST23, while ST258 is often associated with multidrug resistance (Lan et al., 2021;Wyres et al., 2020). Such challenging STs have also been isolated from animals and the environment (Wyres et al., 2020;Jelić et al., 2019;Silva-Sanchez et al., 2021). Even though specific STs are associated with MDR-Kp and hvKp, there are reports of convergence between MDR-Kp and hvKp strains, causing serious, difficult-to-treat infections (Bengoechea and Sa Pessoa, 2018;Lan et al., 2021;Lam et al., 2019).

1.3 Antibiotics

Antibiotics can be defined as substances that are able to kill or inhibit growth of bacterial cells (Nicolaou and Rigol, 2018). Most antibiotic agents used today are natural products produced by fungi and bacteria, or synthetical derivatives of these (Holmes et al., 2016;Wright and Poinar, 2012). It was the accidental discovery of the inhibitory effects of *Penicillium notatum* on *Staphylococcus aureus* by Alexander Fleming in 1928 which led to the identification of penicillin and subsequent commercial production as an antibiotic in the 1940`s. This marks the start of the modern era of antibiotics (Durand et al., 2019;Ventola, 2015). Few discoveries in the history of medicine has been more successful and important (Aminov, 2010;Marti et al., 2014). From the 1940`s – 1970`s, an era termed the golden age of antibiotics, more than 20 classes of antibiotics were discovered, mainly from the bacterial genus *Streptomyces*. However, very few new classes have been discovered since the 1980`s and most agents under development today are derived from existing antibiotics (Durand et al., 2019;Hutchings et al., 2019;Cook and Wright, 2022).

Antibiotics can affect bacteria by killing them (bactericidal) or they can inhibit bacterial growth (bacteriostatic) (Kohanski et al., 2010). However, the effect of antibiotics on bacteria is dependent on the concentration of the antibiotic agent (Wald-Dickler et al., 2017). Based on

their chemical structure or mechanism of action, antibiotics can be classified in groups (Mubeen et al., 2021). These groups can have several targets and mechanisms of action against bacterial cells, *e.g.* the β-lactam antibiotics targets and inhibits the cell wall synthesis. They can inhibit the DNA replication, like the quinolones or inhibit RNA synthesis, like rifamycins. Some antibiotics, *e.g.* trimethoprim-sulfamethoxazole, inhibit essential metabolic steps, like folate synthesis. Tetracyclines inhibits the protein synthesis, while polymyxins target the cytoplasmic or outer membrane (Madigan et al., 2015;Kohanski et al., 2010) (**Figure 2**).

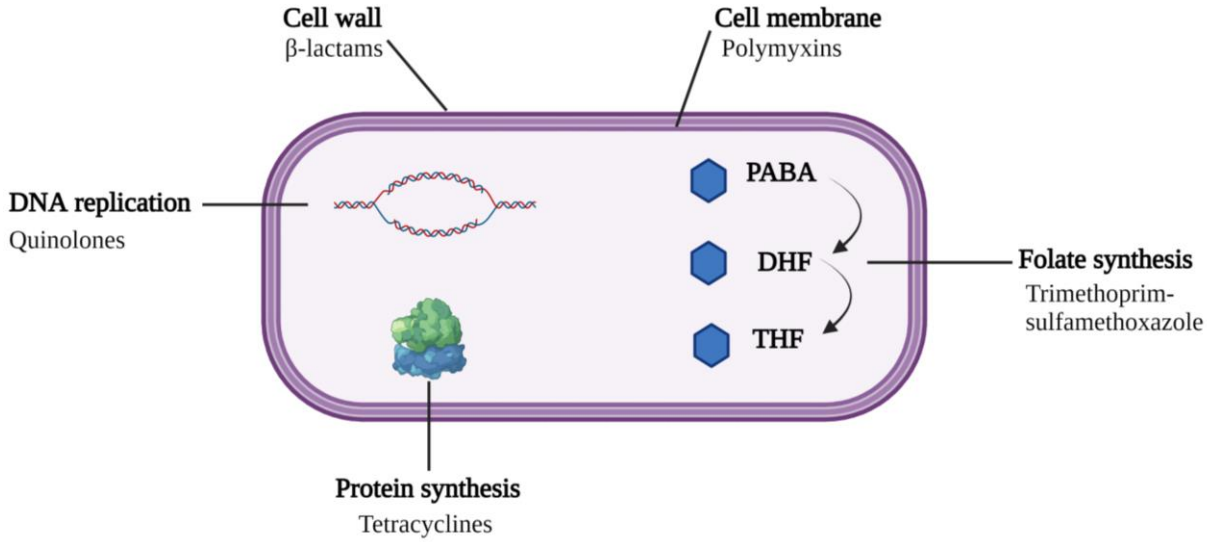

Figure 2. Molecular targets for antibiotic agents in the bacterial cell. PABA: para-aminobenzoic acid, DHF: dihydrofolate, THF: tetrahydrofolate. The figure was created with BioRender.com.

1.4 Antibiotic resistance

Antibiotic resistance can be defined as the ability of a bacterium to resist the inhibitory or toxic effect of an antibiotic agent (Davison et al., 2000). Thus, antibiotics will kill or suppress growth of sensitive bacteria, while resistant ones will survive and reproduce (Aslam et al., 2018). Dependent on the number of antibiotic classes a bacterial isolate is resistant to, it can be defined as multidrug resistant (MDR), extensively drug-resistant or pandrug-resistant (Magiorakos et al., 2012).

Resistance to antibiotics is an ancient phenomenon, it has been proposed that β-lactamases originated billions of years ago and that some of the genes encoding these enzymes have been present on plasmids for millions of years (Hall and Barlow, 2004;Allen et al., 2010). Since most antibiotics are natural products produced by bacteria and fungi, antibiotic producing bacteria

have developed mechanisms to protect themselves from the bactericidal and/or bacteriostatic effects of these products (Holmes et al., 2016;Wright and Poinar, 2012). However, since the introduction of antibiotics into human and veterinary medicine, the development of antibiotic resistance has accelerated, and the use of antibiotics in medical practice and food-producing animals is considered a major driving force for the development and selection of antibiotic resistant bacteria (Giubilini et al., 2017;Holmes et al., 2016). Currently, some antibiotics are considered to be critically important to human medicine by the World Health Organization (WHO). These are agents which are "the sole, or one of limited available therapies, to treat serious human infections", and that are used "to treat infections in people caused by either bacteria that may be transmitted to humans from non-human sources or bacteria that may acquire resistance genes from non-human sources". Among these critically important antibiotics are agents belonging to the class cephalosporins, carbapenems, polymyxins and aminoglycosides (WHO, 2019).

1.4.1 Mechanisms of antibiotic resistance

To deal with the toxic effects of substances like antibiotics, bacteria have developed numerous resistance mechanisms (Holmes et al., 2016). These can be placed in three main categories (Blair et al., 2015) (**Figure 3**). **i)** Mechanisms that reduce the intracellular concentration of antibiotics by preventing entry into the bacterial cell or by active efflux of the agent. This category includes efflux pumps which actively remove the antibiotic from the cell, preventing it from reaching its intracellular target molecule. Some efflux pumps have a narrow specificity while other efflux pumps may have a broad substrate specificity (MDR efflux pumps) (Tenover, 2006;Blair et al., 2015). **ii)** Mechanisms that alter the target molecule either by mutation, protection of the target or acquisition of resistant target enzyme. Mutations in the target molecule of the antibiotic agent may prevent binding of the antibiotic to its target, *e.g.* mutations in the *gyrA* gene conferring resistance to quinolones (Blair et al., 2015;Hawkey and Jones, 2009). Protection of the target molecule may cause reduced susceptibility and/or resistance to antibiotics, *e.g.* the *qnr* genes that cause low-level resistance to quinolones by binding to DNA gyrase and thus protects the cell against the effects of quinolone antibiotics (Robicsek et al., 2006;Blair et al., 2015;Hawkey and Jones, 2009). Acquisition of genes encoding a resistant target enzyme can also cause antibiotic resistance, *e.g.* acquisition of *dfrA* genes encoding a trimethoprim resistant dihydrofolate reductase (Ambrose and Hall, 2021). **iii)** Enzymatic inactivation of antibiotics either by hydrolysis or modification of the antibiotic. Inactivation of antibiotics by enzymatic hydrolysis is a major resistance mechanism for β-lactam antibiotics.

Among such enzymes are the world-wide disseminated CTX-M extended spectrum β-lactamase (ESBL) enzymes and the carbapenemases responsible for resistance to carbapenem antibiotics and most other β-lactam antibiotics (Blair et al., 2015;Cantón and Coque, 2006).

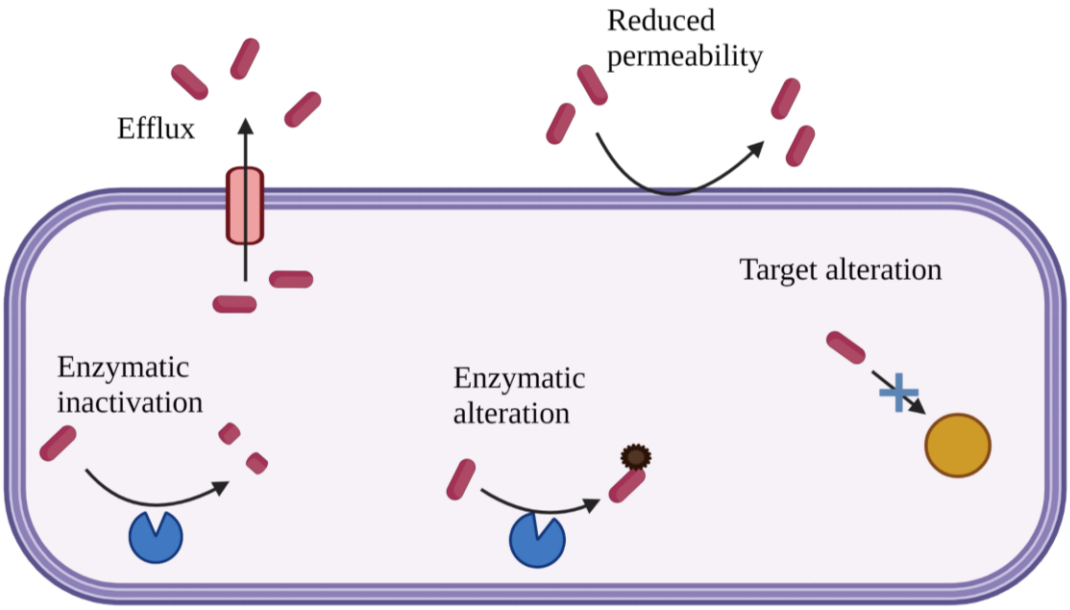

Figure 3. The main categories of antibiotic resistance mechanisms in bacteria. These include those that reduce the intercellular concentration of antibiotics (efflux and reduced permeability), altercation of the target molecule and enzymatic inactivation of antibiotics by hydrolysis or altercation of the antibiotic. The figure was created with BioRender.com.

1.4.2 Extended spectrum β-lactamases

β-lactam antibiotics, like penicillins, cephalosporins, monobactams and carbapenems are among the most commonly used antibiotics globally (Peirano and Pitout, 2019;Bush, 2018). The β-lactamases are able to hydrolyse β-lactam antibiotics and are therefore considered the most important resistance mechanism in Gram-negative bacteria (Cantón et al., 2012).

Based on their amino acid sequences, β-lactamases are divided into Ambler classes; class A, B, C and D. Class A, C and D use serine for hydrolysis of their target, while class B β-lactamases are metalloenzymes which needs zinc ions for hydrolysis of the substrate (Bush and Jacoby, 2010). These enzymes can also be grouped according to their functions, *e.g.* Group 1 which contains enzymes active against cephalosporins, Group 2 which amongst other includes ESBLs, and Group 3 containing metallo β-lactamases that can hydrolyse most β-lactams (Bush and Jacoby, 2010).

In contrast to more narrow spectrum β-lactamases, the ESBLs can hydrolyse penicillins, extended spectrum cephalosporins and monobactams (Bush, 2018;Peirano and Pitout, 2019;Cantón et al., 2012;Gniadkowski, 2001). Thus the ESBLs represent a major public health concern protecting bacteria against our most useful antibiotics (Castanheira et al., 2021).

The first ESBL, SHV-2, was identified in a clinical *Klebsiella* strain from Germany in the 1980`s, and shortly after, hospital outbreaks caused by ESBL-producing Gram-negative bacteria were reported in Europe and in the USA (Gniadkowski, 2001;Bush, 2018;Castanheira et al., 2021). Nowadays, ESBL-producing pathogens are common in both the clinics and in the community (Castanheira et al., 2021). Most ESBLs belong to Ambler class A and include the SHV, TEM and CTX-M enzymes. Initially, the TEM and SHV ESBL types were dominating, but since the early 2000`s the CTX-M type ESBLs have become the most prevalent ESBLs globally (Bush, 2018;Cantón and Coque, 2006;Cantón et al., 2012;Castanheira et al., 2021). Even though many different CTX-M variants exist, CTX-M-14 and CTX-M-15 are considered the most prevalent, present in humans, animals and environments worldwide (Cantón et al., 2012). Additionally, the high rates of ESBL-producing Enterobacteriaceae has caused an increase in the use of carbapenems, and this has again caused an increase in prevalence of carbapenemase-producing Enterobacteriaceae (Peirano and Pitout, 2019;Castanheira et al., 2021). Today, ESBL- and carbapenemase-producing Enterobacteriaceae are categorised by the WHO as bacteria for which new antibiotics are urgently needed (WHO, 2017).

1.4.3 Dissemination of antibiotic resistance

Bacteria can be intrinsically resistant to certain antibiotics, or they can acquire resistance through mutations and/or HGT (Blair et al., 2015;Cook and Wright, 2022).

Mutations can cause antibiotic resistance by several mechanisms, *e.g.* alterations in the target molecule of the antibiotic, they can affect the permeability of the bacterial cell or they can lead to increased efflux of the agent from the cell. Such mutations are normally transferred vertically (Martinez and Baquero, 2000).

HGT has been identified as the primary driver in the dissemination of antibiotic resistance (Vrancianu et al., 2020). There are at least three main mechanisms for HGT, transduction, transformation and conjugation, with conjugation being the most widespread mechanism among bacteria (Aminov, 2011;Pal et al., 2017). Transduction is the introduction of genetic material via bacteriophages infecting and injecting foreign DNA into a new host (Soucy et al.,

2015). When bacteriophages replicate, they can incorporate bacterial host DNA into their genome and form transducing particles. When the bacteriophage infects a new host, their genome, with the bacterial DNA, is injected into the new bacterial host. The foreign DNA can then be recombined into the host genome (**Figure 4A**). There are two types of transduction, generalised and specialised transduction. In generalised transduction, any bacterial DNA can be packaged into the phage and transferred to a new host, whereas specialised transduction is limited to the transfer of specific sets of genes linked to the prophage DNA after excision from a chromosomal integration site (Chiang et al., 2019). Transformation involves the uptake of foreign DNA from the environment by a recipient cell. The DNA can then be integrated into the recipient genome by homologues recombination. For a bacterium to acquire genetic material by this mechanism, it needs to express a state called competence which is genetically encoded (Johnston et al., 2014;Dubnau and Blokesch, 2019) (**Figure 4B**). Bacterial conjugation is a process which require physical cell to cell contact, mediated by pili. A cascade of steps then results in the formation of a mating bridge through which MGEs, such as conjugative plasmids, transposons and ICEs can be transferred to other strains, species or genera (**Figure 4C**). The transferred DNA is re-circularised, replicated and established in the recipient (Arutyunov and Frost, 2013;Virolle et al., 2020). The acquisition of antibiotic resistance is often associated with reduced fitness in the host and therefore, strains carrying ARGs will be outcompeted by susceptible strains in the absence of selection pressure (Andersson and Hughes, 2010;Martinez, 2012). However, plasmids carrying ARGs may be maintained by plasmid-host adaptation or mechanisms like toxin-antitoxin systems, responsible for killing plasmid-free progeny cells, even in environments free from selection pressure (Yang and Walsh, 2017;Martinez, 2009;Wein and Dagan, 2020). In addition to transduction, transformation and conjugation, other mechanisms, like the transfer of DNA by membrane vesicles and nanotubes, have more recently been suggested to be involved in HGT (Arnold et al., 2022;García-Aljaro et al., 2017;Abe et al., 2020).

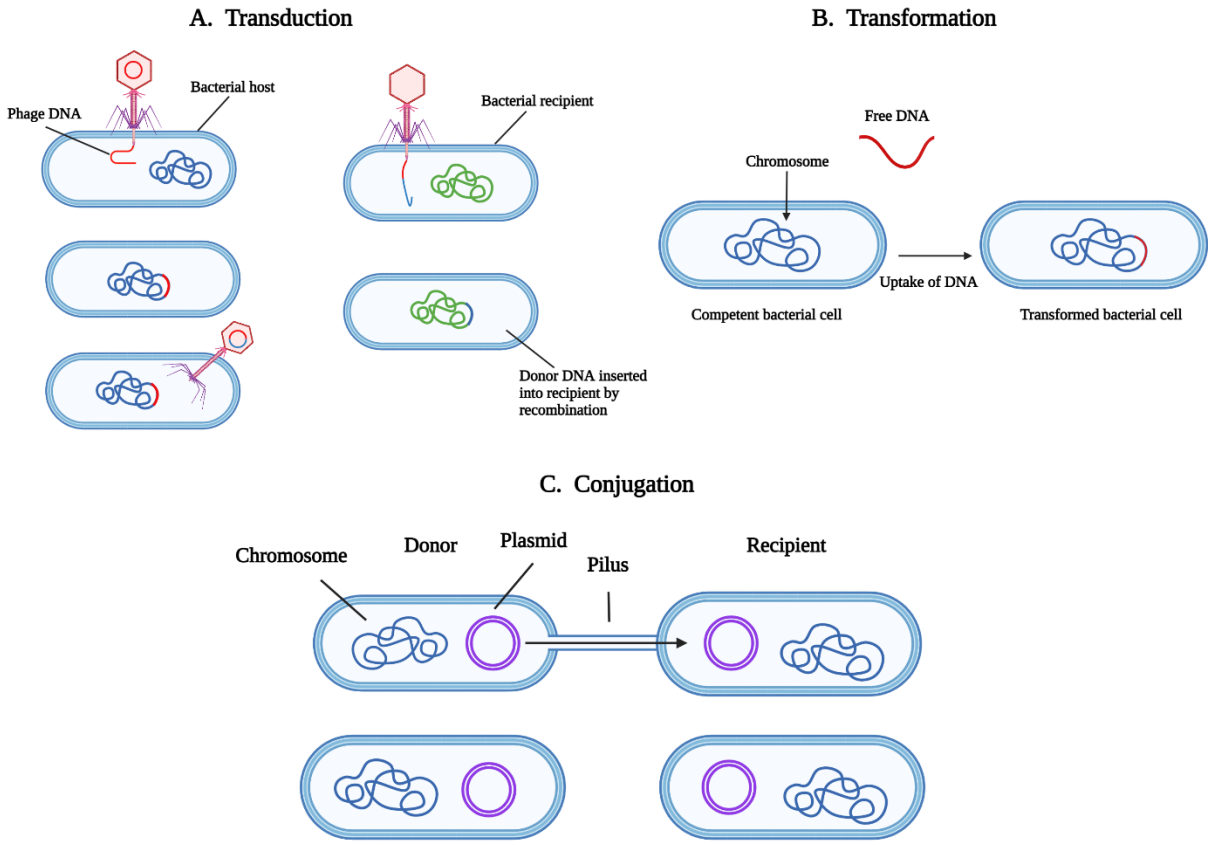

Figure 4. Mechanisms for horizontal gene transfer in bacteria. A: Transduction, B: Transformation, C: Conjugation. The figure was created with BioRender.com.

1.4.4 Antibiotic resistance in the environment

The environment is gaining attention as an important source and dissemination route of antimicrobial resistance (Bengtsson-Palme et al., 2017). Antibiotic resistant bacteria, antibiotic residues and ARGs can be spread to adjacent environments, including the marine environment, by many routes, *e.g.* through sewage, wastewater, run-off from land, including agricultural land, discarding of unused antibiotics and from production of pharmaceutical ingredients (Larsson, 2014;Fletcher, 2015;Allen et al., 2010;Zheng et al., 2021;Leonard et al., 2022;Marathe et al., 2018) (**Figure 5**). A large portion of antibiotics used by humans and in animals are released in a biological active form through faeces and urine (Leonard et al., 2022;Osunla and Okoh, 2017). Wastewater treatment plants are considered hotspots for the dissemination of antimicrobial resistance, since both ARGs and antibiotic selection pressure are present in wastewater. The antibiotic compounds, antibiotic resistant bacteria and ARGs can be poorly removed in the wastewater treatment plants and can therefore spread further to the environment through discharge of processed wastewater (von Wintersdorff et al., 2016;Grevskott et al., 2021;Fletcher, 2015;Zheng et al., 2021;Marathe et al., 2013;Marathe et al., 2017).

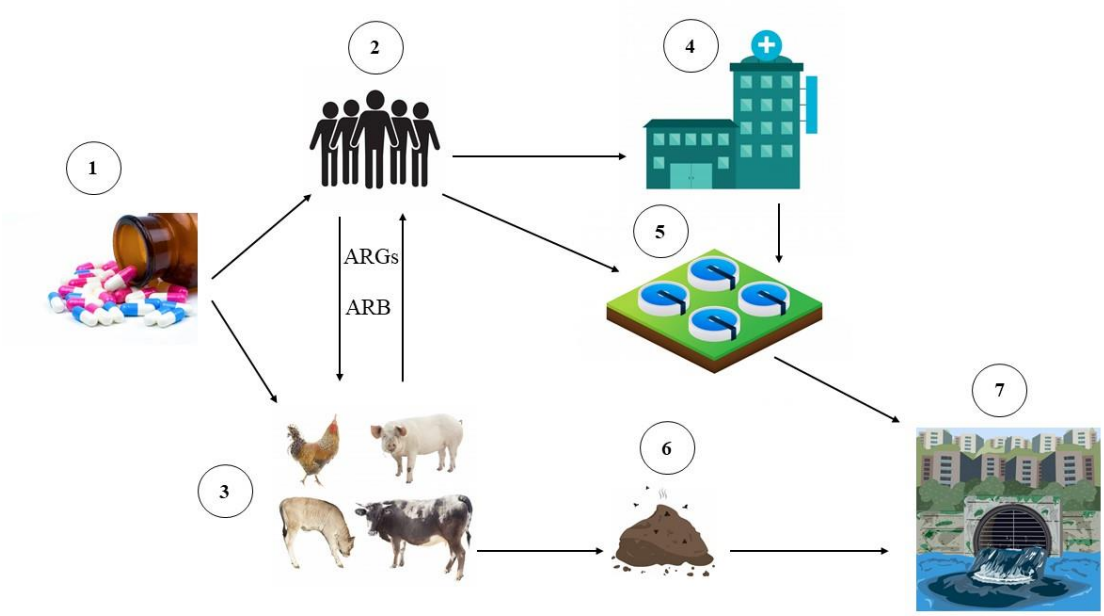

Figure 5. Dissemination routes of antibiotic residues, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) to the environment. 1: Antibiotics, 2: Human population, 3: Food-producing animals, 4: Clinics, 5: Wastewater treatment plants, 6: Manure, 7: Sewage outlet to the environment. The figure was created with images available from https://mostphotos.com and modified after "Review of antibiotic resistance in China and its environment". *Environ. Int.* 110**,** 160-172. doi:https://doi.org/10.1016/j.envint.2017.10.016.

The number of bacterial and archaeal cells on earth has been estimated to be 10^{30}, with large uncertainties, and the external environment may therefore represent a large reservoir for ARGs that may not yet have been seen in human pathogenic bacteria (Larsson, 2014;Flemming and Wuertz, 2019). In fact, many of the ARGs present in human pathogenic bacteria today originated in the environmental microbiota (Hernando-Amado et al., 2019). For example, the $bla_{\text{CTX-M}}$ genes originated in the environmental bacterium *Kluyvera*, and the mobile quinolone resistance gene *qnrA* and the OXA-48 carbapenemase have been suggested to originate in *Shewanella*, a marine genus (Poirel et al., 2005;Tacão et al., 2018;Poirel et al., 2012;Cantón et al., 2012;Bevan et al., 2017).

The antibiotic concentrations in non-clinical settings are generally low, however, the selection of antibiotic resistant bacteria can occur at low antibiotic concentrations, like the concentrations that can be found in natural environments, including aquatic environments (Marti et al.,

2014;Kraupner et al., 2018;Lundström et al., 2016;Bengtsson-Palme and Larsson, 2016). Even though the use of antibiotics is the most important driving force of antibiotic resistance, other compounds, such as heavy metals and biocides, may co-select for antibiotic resistance. Co-selection can occur if genes encoding resistance to antibiotics, heavy metals and/or biocides are present on the same MGE (co-resistance), or if different antimicrobial agents have the same molecular target in the bacterial cell (cross-resistance) (Baker-Austin et al., 2006). Heavy metals are the most abundant pollutants in industrialised and developing countries (Hernando-Amado et al., 2019). Furthermore, heavy metals are commonly used in antifouling agents in aquaculture and are also present in fish feed, and can thus be spread through faecal material, spilled feed and leakage from copper impregnated fish farm nets (Grefsrud et al., 2021). Metal compounds can also be present in livestock feed and are used in agriculture as pesticides, fertilisers and antimicrobials (Pal et al., 2017;Seiler and Berendonk, 2012;Hernando-Amado et al., 2019). In contrast to antibiotics, heavy metals are not degraded in the environment and can therefore represent long-term selection pressure (Baker-Austin et al., 2006). Additionally, biocides, such as quaternary ammonium compounds, are frequently used in cosmetic products, hygiene products and in the industry as disinfectants, and can thus reach the environment through treated wastewater (Hegstad et al., 2010;Zhang et al., 2015).

1.4.5 The One Health approach to antibiotic resistance

The One Health approach considers human, animal and environmental health as one instead of separate parts, thus, acknowledging that these niches are connected in the spread of antibiotic resistance (Hernando-Amado et al., 2019;Timme et al., 2020). This concept includes the idea that the increasing human population, climate change and increased pollution requires a multidisciplinary approach to ensure the future health and well-being of humans, animals as well as the environment (McEwen and Collignon, 2018). The major regulatory, economic and political bodies recognise that antimicrobial resistance cannot be addressed by studying antibiotic resistance in health care settings alone, it is also necessary to focus on other ecosystems as these contribute to the emergence, acquisition and dissemination of such resistance (Hernando-Amado et al., 2019). For example, soil naturally contains a large resistome, but also receives antibiotic resistant bacteria and ARGs from humans and animals which again can be transferred to humans through *e.g.* food and direct contact (Tiedje et al., 2019). The use of antibiotics in animals is an important contributor to antibiotic resistance (Allen et al., 2010), also among species that can cause infections in humans, like *Salmonella* spp. and *E. coli*, as well as in zoonotic bacteria, like *S. aureus* (McEwen and Collignon, 2018).

Thus, the use of antibiotics in the human, animal and/or environmental sector can subsequently lead to selection of antibiotic resistance in different niches, which again can have negative consequences for human and animal health (McEwen and Collignon, 2018). Therefore, increased surveillance of antimicrobial resistance, not only in clinical settings but also in animals and the environment, is an important element in addressing the challenges such resistance represents (Lammie and Hughes, 2016).

1.4.6 Consequences of antibiotic resistance

Antimicrobial resistance is today considered one of the biggest threats to global health and food security by the WHO and also represents a serious social and economic burden (ECDC et al., 2021;WHO, 2020). Antibiotic resistance can affect the public health by limiting the treatment options for bacterial infections, causing prolonged hospital stays, increased mortality and morbidity (MacGowan and Macnaughton, 2013).

Due to challenges in the treatment of infections caused by carbapenemase-producing Enterobacteriaceae, colistin has been used as a last resort antibiotic for the treatment of such infections. However, in 2015 the *mcr-1* gene encoding transferable resistance to colistin was reported in *Escherichia coli* in China and *mcr* genes have since been reported worldwide and in several species, mainly members of the Enterobacteriaceae family (Watkins et al., 2016;Quesada et al., 2016;Doumith et al., 2016;Caspar et al., 2017;Ling et al., 2020). Infections caused by bacteria resistant to carbapenems and colistin leave very few treatment options (Hasman et al., 2015). It has been estimated that antibiotic resistance is responsible for 33 000 deaths each year in Europe (Cassini et al., 2019) and more than 35 000 annual deaths in the USA (CDC, 2019). Recently, 1.27 million deaths were estimated globally as the direct consequence of antibiotic resistance in 2019 (Murray et al., 2022). Furthermore, antibiotic resistance can also reduce the efficacy of prophylactic use of antibiotics critical for successful surgical procedures, transplantations and cancer treatment, and can thus reverse improvements made in human medicine since the introduction of antibiotics (Martinez, 2014).

The global food production has increased the last decades, and it is estimated that 60 % more food will be needed by 2050 (FAO, 2017). Antibiotic agents are important in food-producing animals, both terrestrial and aquatic, for treatment of infections, and they are therefore essential for food security (FAO, 2016). Unfortunately, antimicrobials are not only used for health-related purposes in food production, they are also used as growth promoters in some countries (Allen et al., 2010;Boeckel et al., 2017). In fact, 73 % of all antimicrobial use are consumed in

animals worldwide. There are however large variations in the use of antimicrobials in food animals. In 2013, 8 mg / population correction unit (PCU) (one kg of animal product) was used in Norway, compared to 318 mg/PCU in China (Boeckel et al., 2017). The consequences of antibiotic resistance does not only affect human health, it also represents a serious threat to sustainable food production (FAO, 2016). Antibiotic resistant bacteria in the food production chain can represent a threat to public health, as food may serve as a transmission route of antibiotic resistant bacteria to the human population (Founou et al., 2016).

Even though regulations and control measures have been implemented to reduce the use of antibiotics and thereby slow the development of antimicrobial resistance, a major increase in the global use of antibiotics was reported from 2000-2015, and the use is predicted to further increase significantly by 2030. The increase is predicted to be the fastest in low- and middle-income countries, and much of the increase is expected to occur in the food animal production sector (Hernando-Amado et al., 2019).

1.4.7 Usage of antibiotics and antibiotic resistance in Norway

Norway is a low prevalence country with regards to antibiotic resistance, and the use of antibiotics in human and veterinary medicine is low. In 2021, the total sales of antibacterial agents for terrestrial animals were 4 875 kg measured in active substance, of which 4 500 kg was for use in terrestrial food-producing animals. In aquaculture, the total antibiotic use was 953 kg, and only florfenicol (896 kg), oxolinic acid (57 kg) and enrofloxacin (0.25 kg) were applied. This represents a reduction of more than 99 % since 1987 (NORM/NORM-VET, 2022). Even though a 43 % reduction in the sale of antibiotics for animals was seen among 25 European countries reporting data to the European Medicines Agency between 2011-2020, the use in Norway is still low compared to most other countries (EMA, 2021) (**Figure 6**).

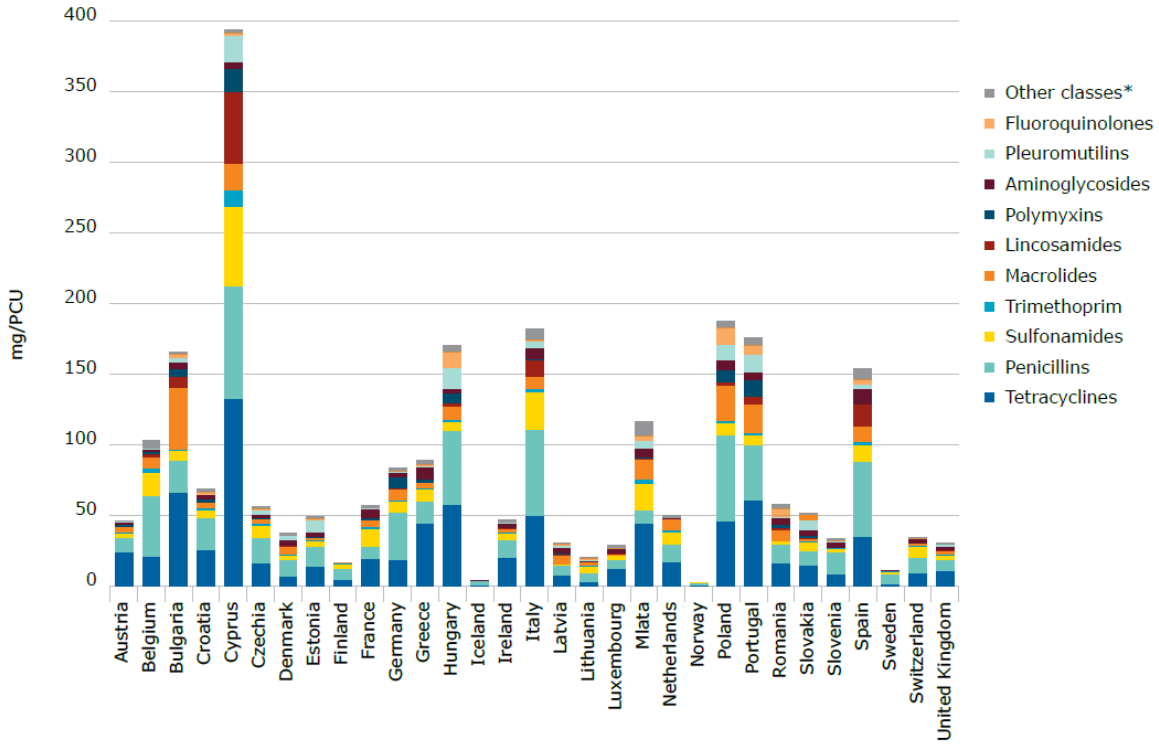

Figure 6. Antibiotic sales for food-producing animals in 2020 in 31 European countries represented by milligrams (mg) of active substance sold per population correction unit (PCU). Reprinted from "Sales of veterinary antimicrobial agents in 31 European countries in 2019 and 2020", https://www.ema.europa.eu/en (EMA, 2021).

Also, for human medicine the usage of antibiotics is low in Norway, with a total of 11.2 defined daily doses/1000 inhabitants/day sold in 2021. In 2015, the Norwegian government launched a national strategy to reduce the use of antibiotics in humans by 30 % and by 10 % in food-producing animals within 2020. In 2021, a 25 % reduction in the sale of antibiotics for food-producing terrestrial animals was reported, while a reduction of 33 % in the sale of antibiotics for human use was observed compared to 2012. Even though there has been a marked reduction in the usage of antibiotics in humans in Norway since 2012, a significant reduction in prescription of antibiotics was seen during the COVID-19 pandemic, in particular in the community sector (NORM/NORM-VET, 2022).

Antibiotic resistance still represents a comparably limited problem in Norway (NORM/NORM-VET, 2022). In 2020, 14.9 % of the invasive *E. coli* isolates reported to the European Antimicrobial Resistance Surveillance Network and the Central Asian and European Surveillance of Antimicrobial Resistant were resistant to third generation cephalosporins, with the highest prevalence in east and south-east Europe, while the frequency of ESBL-producing

E. coli from blood cultures in Norway was 5.8 % in 2020 (WHO Regional Office for Europe/ECDC, 2022) (**Figure 7**).

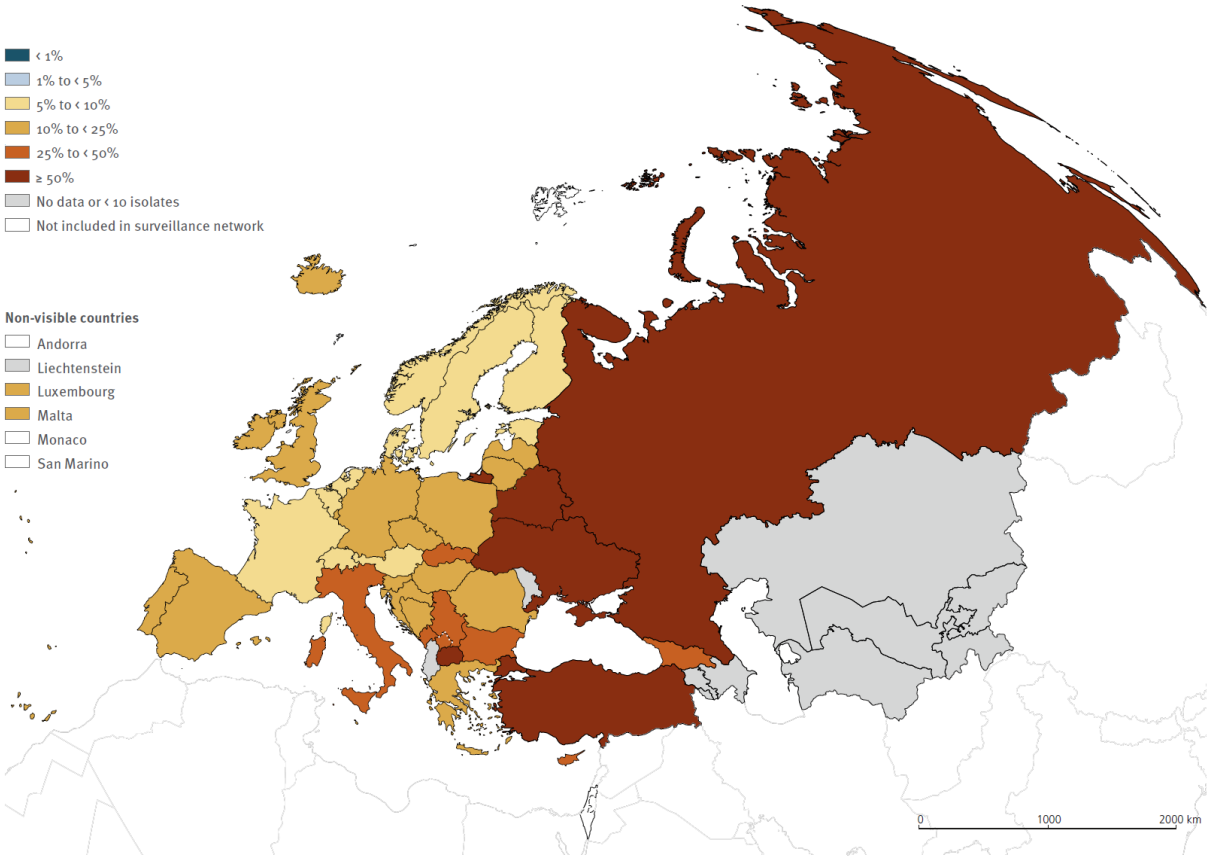

Figure 7. Percentage of invasive *Escherichia coli* isolates resistant to third generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime) in the WHO European Region, 2020. Reprinted from "Antimicrobial resistance surveillance in Europe 2022 – 2020 data", https://www.ecdc.europa.eu/en (WHO Regional Office for Europe/ECDC, 2022). The material is licensed under CC BY 3.0 IGO.

1.5 *K. pneumoniae* and antibiotic resistance

K. pneumoniae carries the bla_{SHV} gene on its chromosome and is therefore intrinsically resistant to penicillins, including aminopenicillins (Wyres and Holt, 2018). The core genome of *K. pneumoniae* also contains the *fosA* gene and the *oqxAB* efflux pump involved in reduced susceptibility to fosfomycin and quinolones, respectively. However, *fosA* and *oqxAB* does not confer clinical resistance to these classes of antibiotics (Wyres et al., 2020). The majority of ARGs in *K. pneumoniae* are acquired via plasmids through HGT, and several clinically relevant

ARGs were first detected in *K. pneumoniae*. These ARGs include the quinolone resistance genes *qnrA* and *qnrB*, the *K. pneumoniae* carbapenemase (KPC), the OXA-48 carbapenemase, and the New Delhi metallo-β-lactamase-1 (NDM-1). Since their detection, these ARGs have been spread to other species worldwide, especially within the Enterobacteriaceae family (Wyres and Holt, 2018).

K. pneumoniae is considered a major source of antibiotic resistance and is a member of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) (Pendleton et al., 2013;Wyres and Holt, 2018). These are opportunistic pathogens frequently associated with antibiotic resistant infections in health care settings (Pendleton et al., 2013;Navon-Venezia et al., 2017). *K. pneumoniae* is defined as a critical pathogen for which new treatment options are considered urgent by the WHO (WHO, 2017). Furthermore, carbapenem-resistant *K. pneumoniae* are considered as an urgent threat to human health (CDC, 2019). In 2020, 38 % of invasive *K. pneumoniae* isolates reported to the European Antimicrobial Resistance Surveillance Network and the Central Asian and European Surveillance of Antimicrobial Resistant were resistant to at least one group of antibiotics, with resistance to third generation cephalosporins being most common (WHO Regional Office for Europe/ECDC, 2022) (**Figure 8**).

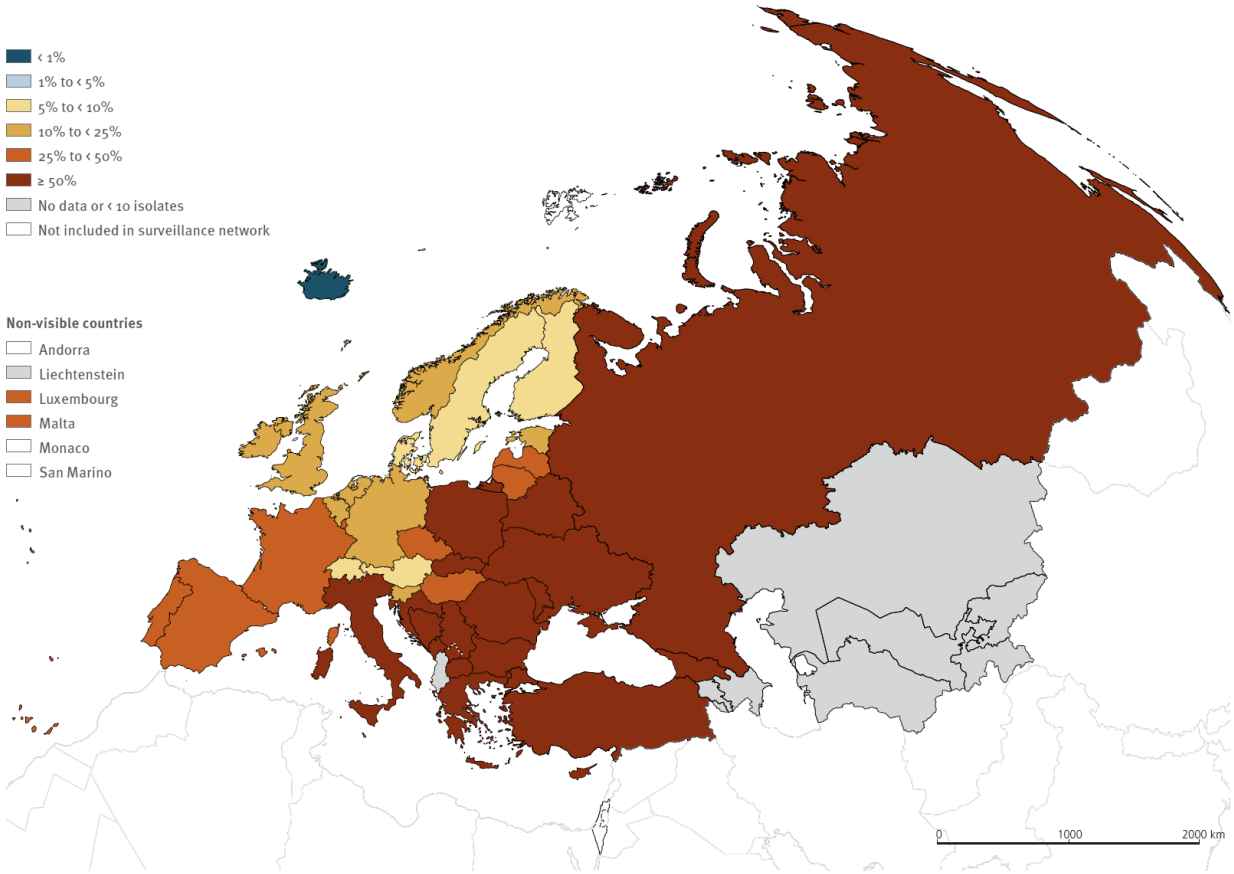

Figure 8. Percentage of invasive *Klebsiella pneumoniae* isolates resistant to third generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime) in the WHO European Region, 2020. Reprinted from "Antimicrobial resistance surveillance in Europe 2022 – 2020 data", https://www.ecdc.europa.eu/en (WHO Regional Office for Europe/ECDC, 2022). The material is licensed under CC BY 3.0 IGO.

Antibiotic resistant *K. pneumoniae* in European Union/European Economic Area countries is problematic, and the frequency of carbapenem-resistant strains is increasing in several European countries, with the highest frequency reported from southern, eastern and south-eastern countries (WHO Regional Office for Europe/ECDC, 2022) (**Figure 9**). In a study by the European Centre for Disease Prevention and Control, it was estimated that the number of deaths caused by carbapenem-resistant *K. pneumoniae* increased significantly between 2007 and 2015 (ECDC, 2019).

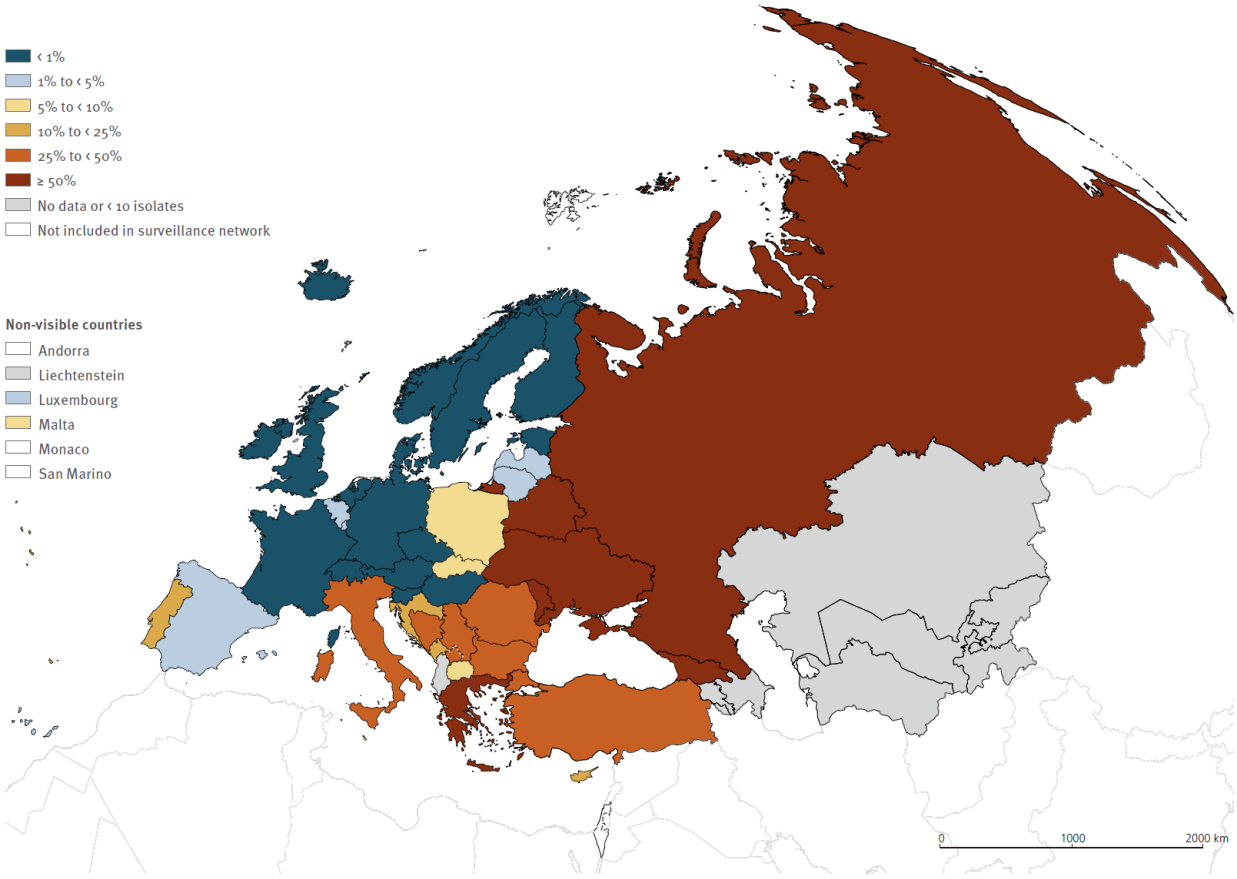

Figure 9. Percentage of invasive *Klebsiella pneumoniae* isolates resistant to carbapenems (meropenem, imipenem) in the WHO European Region, 2020. Reprinted from "Antimicrobial resistance surveillance in Europe 2022 – 2020 data", https://www.ecdc.europa.eu/en, (WHO Regional Office for Europe/ECDC, 2022). The material is licensed under CC BY 3.0 IGO.

The resistance rates among invasive *K. pneumoniae* isolates in Norway are lower than in many other countries. In 2021, 0.3 % of 759 reported blood culture isolates (including KpSC members) were phenotypically resistant to meropenem (NORM/NORM-VET, 2022). However, the frequency of *K. pneumoniae* isolates resistant to third generation cephalosporins is increasing in clinical settings also in Norway, with 10.1 % of *K. pneumoniae* isolates reported in 2020 resistant to these antibiotics compared to 5.8 % in 2016 (**Figure 10**) (WHO Regional Office for Europe/ECDC, 2022).

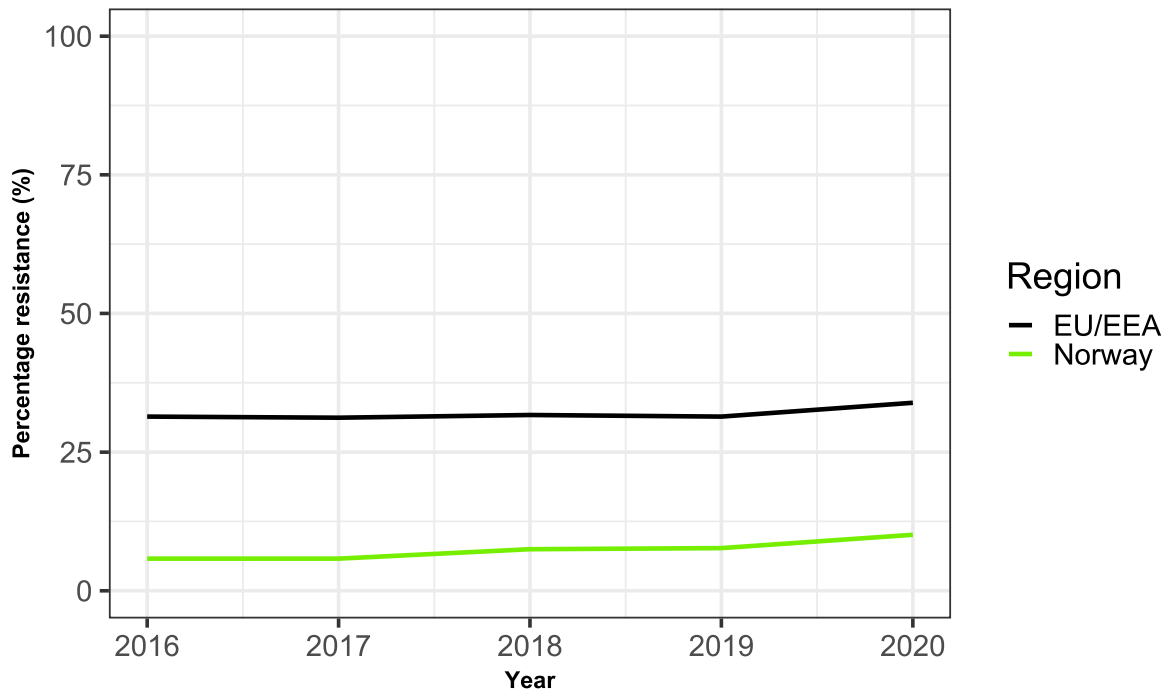

Figure 10. Percentage of invasive *Klebsiella pneumoniae* isolates resistant to third generation cephalosporins in Norway (green) compared to the EU/EEA population-weighted mean (black), 2016-2020. Data obtained from "Antimicrobial resistance surveillance in Europe 2022 – 2020 data", https://www.ecdc.europa.eu/en (WHO Regional Office for Europe/ECDC, 2022).

1.6 The marine microbiome

The oceans cover around 70 % of the earth's surface and along with marine sediments, the oceans represent one of the largest microbiomes on earth. In the oceans, the microorganisms play important roles in biogeochemical processes (Whitman et al., 1998). It has been estimated that one ml of seawater contains approximately 10^6 bacterial cells and an even higher abundance of viruses (Azam et al., 1983;Saha et al., 2018;Suttle, 2005;Bergh et al., 1989). Bacteria that are naturally occurring in the marine environment often have a requirement for NaCl, grow at relative low temperatures and are adapted to low concentrations of organic and nitrogenous compounds like those present in the oceans (Adams and Moss, 2008a).

Large-scale metagenome analyses of samples collected from most of the global oceans have shown a high diversity of species present. In total, > 35 000 different operational taxonomic units were identified in 238 samples from 68 sampling stations, with Proteobacteria representing the most common phylum (Sunagawa et al., 2015).

In coastal waters, which can be affected by anthropogenic and terrestrial activities, *e.g.* sewage discharge and other faecal pollution, the bacterial community can be different and may contain bacteria not indigenous to the marine environments, and pathogenic bacteria which may cause human and animal infections (Baquero et al., 2008).

1.7 *K. pneumoniae* in the environment and food

As previously mentioned, *K. pneumoniae* can be found in many environmental niches, both free-living and as a commensal (Wyres et al., 2020). It has also been found in diseased and stranded marine mammals (Roe et al., 2015;Jang et al., 2010), its presence in bivalve molluscs has been reported (Bueris et al., 2022) and it has been implicated in mass mortality of freshwater fish in Brazil (Vaneci-Silva et al., 2022). Although it has been recovered from numerous sources, there is limited knowledge on its prevalence, genetic diversity and features in the different environmental niches (Wyres et al., 2020), especially in the marine environment.

Although not a classical foodborne pathogen, food has been suggested as a risk factor for GI colonisation by *K. pneumoniae* in humans (Huynh et al., 2020;Lepuschitz et al., 2020). Its presence has been reported in different types of foods, including chicken, salad (Rodrigues et al., 2022) and seafood (Sanjit Singh et al., 2017). However, the association between *K. pneumoniae* in food and GI colonisation is unclear (Wareth and Neubauer, 2021). The sources of *K. pneumoniae* in food are not well known, but contamination during harvest, slaughter and/or processing has been suggested to be involved (Wyres et al., 2020).

1.8 Accumulation of bacteria in bivalve molluscs

Bivalve molluscs belong to the Mollusca phylum and the Bivalvia class, and includes commercially important bivalves like mussels, clams, scallops, and oysters (Gosling, 2003a;Gosling, 2003b). Most bivalve molluscs are filter feeders that retain and concentrate particles present in their surroundings (Strand and Ferreira, 2019). As bivalves filter large volumes of water on a daily basis, they typically contain marine bacteria, for example *Vibrio* spp., *Aeromonas* spp. and *Shewanella* spp. (Odeyemi et al., 2018). However, they can also accumulate human pathogenic bacteria and viruses of anthropogenic or terrestrial origin if present in their surroundings (Gosling, 2003c). Therefore, bivalve molluscs can act as good indicators of the faecal contamination status in a given marine environment at a given time (Grevskott et al., 2017).

1.9 Microbiota of fish

The microbiota in the marine environment is largely dependent on the ambient temperature, and therefore, the temperature also affects the microbiota of freshly caught fish (Huss, 1997). The microbiota of fish caught in cold and temperate waters will be dominated by psychrotrophic Gram-negative bacteria like *Aeromonas* spp., *Shewanella* spp., *Pseudomonas* spp., *Flavobacterium* spp. and members of the Vibrionaceae family. Fish caught in tropical waters often contain higher loads of Gram-positive and enteric bacteria compared to those caught in temperate waters (Gram and Huss, 1996;Rathod et al., 2022;Visciano et al., 2012). However, fish caught in coastal waters may contain pathogenic bacteria as result of contamination from terrestrial sources (Huss, 1997;Parlapani, 2021;Rathod et al., 2022).

Normally, the muscle and the internal organs of healthy fresh fish are sterile. However, high bacterial loads can be found in the gills (10^3-10^9 cfu/g), the gut (10^3-10^9 cfu/g) and on the skin (10^2-10^7 cfu/cm^2) (Adams and Moss, 2008b;Rathod et al., 2022).

2 Objectives

The main aim of this work was to examine the role of the Norwegian marine environment and seafood as a potential dissemination route for *K. pneumoniae* to humans. This was achieved by investigating the prevalence, genetic diversity, associated antibiotic resistance and pathogenic potential of *K. pneumoniae* in the marine environment. Six specific objectives were defined:

I. Examine the prevalence of *K. pneumoniae* in different niches in the Norwegian marine environment by sampling of bivalve molluscs, fish, seawater and sediments.

II. Investigate antibiotic susceptibility by disk diffusion in obtained isolates.

III. Explore the genetic diversity, presence of acquired ARGs and the pathogenic potential of *K. pneumoniae* isolated from the marine environment using short-read sequencing.

IV. Examine if specific *K. pneumoniae* STs from the marine environment are related to isolates from other sources belonging to the same STs by performing phylogenetic analysis.

V. Combine short- and long-read sequencing to obtain complete genome sequences of antibiotic resistant *K. pneumoniae* from marine sources for in depth characterisation of such isolates.

VI. Examine the ability of antibiotic resistant *K. pneumoniae* isolates to transfer acquired ARGs to other bacteria by conjugation.

3 Materials and methods

3.1 Sampling

To examine the prevalence of *K. pneumoniae* in the Norwegian marine environment (**Objective I)**, a total of 578 bivalve batch samples, seven batch samples of sea urchins, 53 fish, 17 water samples and 24 sediment samples were collected.

Batch samples of bivalve molluscs were collected in 2016 (n=271), 2019 (n=144) and 2020 (n=163). The majority of bivalve samples (n=563) were collected from locations used for commercial production and monitoring stations, covering 79 different locations, of which 75 were sampled more than once. Samples covering production locations and monitoring stations were obtained through the annual surveillance programme on bivalves conducted by the Institute of Marine Research on behalf of the Norwegian Food Safety Authority. The samples also included products cleared for the market (n=30). Batch samples of bivalves collected from six locations not covered by the program were also included (n=15). The total collection of batch samples consisted of blue mussels (*Mytilus edulis*) (n=476), oysters (*Crassostrea gigas*) (n=58), scallops (*Pecten maximus*) (n=31), horse mussels (*Modiolus modiolus*) (n=5), ocean quahogs (*Arctica islandica*) (n=3), carpet shells (*Politapes rhomboides*) (n=2), cockles (*Cerastoderma edule*) (n=2) and one single batch sample of sand gappers (*Mya arenaria*). Even though not classified as a bivalve mollusc, seven sea urchins (*Strongylocentrotus droebachiensis*) were collected through the annual surveillance programme and included in the study. All samples were transported to the laboratory in Styrofoam boxes with cooling elements to keep the temperature at ~4 ºC. The samples were analysed within 24 h after collection.

Fish from the North and Norwegian Sea were collected by commercial fishing vessels during the harvest seasons in 2019 and 2020 (**Figure 11A**) and comprised herring (*Clupea harrengus*) (n=40) and mackerel (*Scomber scombrus*) (n=5). Fish from coastal waters outside the Sotra island (**Figure 11A**) were caught by recreational fishermen and comprised pollack (*Pollachius pollachius*) (n=2), tusk (*Brosme brosme*) (n=2), ling (*Molva molva*) (n=2) and hake (*Merluccius merluccius*) (n=1). All fish samples were transferred to sterile plastic bags (VWR, USA) at time of capture and stored at chilled temperature (~4 ºC) under transportation. Each fish was analysed individually shortly after arrival.

Seawater samples (n=17) were collected from four stations in the North Sea (n=5) were one station was sampled twice, as well as from coastal waters (n=12) (**Figure 11B**). Water samples

were collected at approximately 3-10 m depth using a Van Dorn water sampler (KC Denmark, Denmark) and each sample consisted of a minimum of 1 l seawater. The water samples were transferred to sterile plastic or glass bottles and stored at chilled conditions until analysis.

Sediment samples (n=24) were collected from the North Sea (n=2) and from coastal areas (n=22), covering in total nine different locations (**Figure 11C**). Sediments were collected with an Van Veen sediment grab (KC Denmark, Denmark) and transferred to sterile plastic bags (VWR, USA) using sterile spoons. The samples were stored at ~4 ºC until processing.

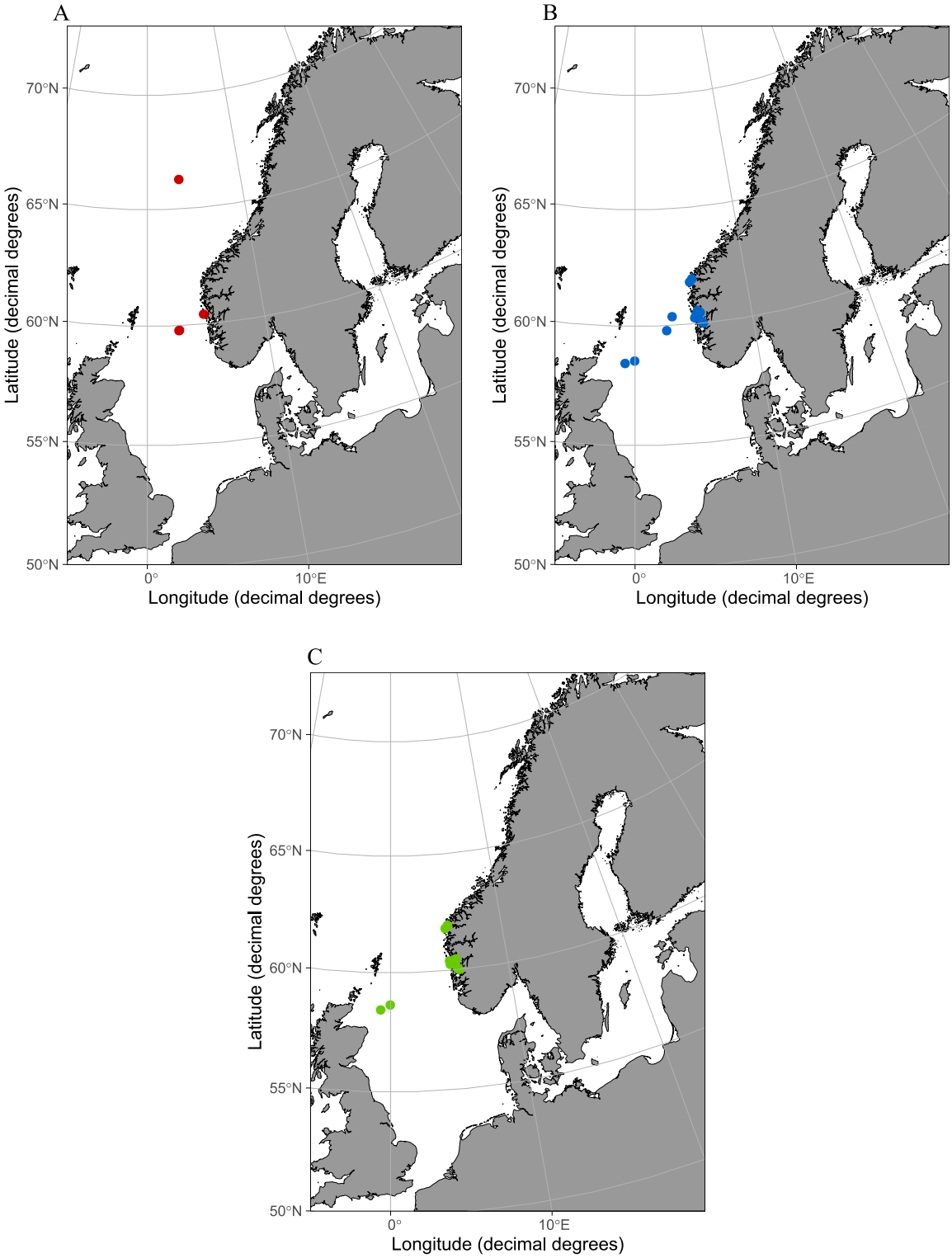

Figure 11. Maps showing sampling locations for; A: fish, B: seawater, C: sediments.

3.2 Processing of samples and isolation of *K. pneumoniae*

3.2.1 Bivalves

Bivalves were cleansed under cold tap water, and only living organisms, *i.e.* individuals closing their valves when exposed to water, were included. The bivalves were opened using a sterile knife. Each batch sample consisted of at least 10 individual bivalves, or more to obtain at least 75 g of soft tissue and intra-valvular fluid. The samples were homogenised in a stomacher (Seward, UK) for 2.5 min at 185 rpm before 25 g were transferred to new sterile plastic bags. Each sample was diluted 1:10 in buffered peptone water (BPW) (Sigma-Aldrich, USA), homogenised for 30 sec at 185 rpm and incubated aerobically at 37 ºC for 24 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 at room temperature and approximately 1.5 ml transferred to 10 mL BPW and incubated at 37 °C over night. Following enrichment, 10 µl of the culture was streaked on Simmons citrate agar (Sigma-Aldrich, USA) supplemented with 1 % Myo-inositol (Sigma-Aldrich, USA) (SCAI) and incubated aerobically at 37 ºC for 48 h. SCAI agar is a selective media originally designed for isolation of *K. pneumoniae* and *K. oxytoca* from stool samples (Van Kregten et al., 1984). Colonies corresponding to characteristics described by Van Kregten et al. (1984) were picked and re-streaked to obtain pure cultures. No standard protocols for isolation of *K. pneumoniae* from environmental sources and food samples exist. However, enrichment in BPW and subsequent streaking of 10 µl enrichment culture on SCAI has recently been shown to be effective for isolation of *K. pneumoniae* from food samples (Rodrigues et al., 2022). It has also been shown that incubation of SCAI plates at higher temperature (44 ºC) may yield slightly higher recovery rates of *K. pneumoniae* from food products (Rodrigues et al., 2022). All bivalve samples were routinely examined for *E. coli* most probable number (MPN)/100 g, following the ISO16649-3 method (ISO, 2019) with conformation on the chromogenic tryptone bile x-glucuronide agar. The limit of quantification of the *E. coli* MPN method is 18 MPN/100 g.

3.2.2 Fish

To ensure that *K. pneumoniae,* if detected in fish, was not due to post harvest contamination, the intestinal contents of the fish was sampled. The fish were opened using sterile scalpels before the intestine was carefully removed using sterile forceps. To access the intestinal contents, a small incision was made with scalpels and the intestine was thereafter transferred to sterile plastic bags. The intestinal content was diluted 1:10 in BPW and homogenised for 30

sec at 185 rpm. Enrichment, incubation and isolation followed the steps described for bivalve molluscs.

3.2.3 Water samples

Depending on sample volume, between 1-5 l of seawater was analysed. To avoid clogging of the filters, the water samples were filtered through three separate 0.45 µm filers (Merck Millipore, Germany) using the using the EZ-fit Manifold 3-place system (Merck Millipore, Germany). The three filters were folded using sterile forceps and placed together in 100 ml BPW. Incubation conditions, cultivation and isolation of *Klebsiella* sp. followed the protocol described for bivalve samples.

3.2.4 Sediment samples

Using a sterile spoon, approximately 10 g from each sediment sample was transferred to new sterile plastic bags with filters (VWR, USA). The samples were diluted 1:10 in BPW and homogenised for 30 sec at 185 rpm. Incubation, cultivation and isolation followed the steps described for bivalve samples.

3.3 Identification of presumptive *K. pneumoniae*

Typical *K. pneumoniae* colonies were presumptively identified using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) (Bruker, Germany). MALDI-TOF MS is a simple and sensitive method for identification of bacteria to the genus and species level. Identification by MALDI-TOF MS is based on the molecular mass of ribosomal proteins (Angeletti and Ciccozzi, 2019;Sauget et al., 2017). The spectra generated during the analysis are compared to a database containing spectra from microbes responsible for the most important human infections. The more spectra for each species present in the database, the more reliable the identification will be (Kailasa et al., 2020;Angeletti and Ciccozzi, 2019). However, the standard MALDI-TOF MS library only contains spectra of two of the KpSC members, *K. pneumoniae* and *K. variicola* subsp. *variicola*. Thus MALDI-TOF MS is not able to identify the remaining species within the complex and has limitations to discriminate between the KpSC members (Long et al., 2017;Rodrigues et al., 2018).

3.4 Antibiotic susceptibility testing

To examine the prevalence of antibiotic resistance among *K. pneumoniae* and closely related species isolated from the marine environment (**Objective II**), all isolates identified as members of the *Klebsiella* or *Raoultella* genera were subjected to antibiotic susceptibility testing

applying the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk diffusion method (Matuschek et al., 2014). The disk diffusion method is based on the diffusion of the antibiotic agent into the agar and its inhibition of bacterial growth. The diameters of the inhibition zones are related to the susceptibility of the test isolate against the selected antibiotic agents and the diffusion rate of the antibiotic into the agar. The measured inhibition zones are compared to standardised zone diameter breakpoints (Jorgensen and Ferraro, 1998;Reller et al., 2009), like those provided by EUCAST (https://www.eucast.org/clinical_breakpoints). This comparison allows for the categorisation of the test isolate as sensitive (S), susceptible, increased exposure (I) or resistant (R) to the antibiotic agents applied to the agar.

In the present work, the isolates were tested against a panel of 17 antibiotic agents belonging to 10 different classes (**Table 1**). All antibiotic disks were obtained from Oxoid, UK.

Table 1. Antibiotic disks used in disk diffusion susceptibility testing of *K. pneumoniae* isolated from the marine environment.

Class	Antibiotic	Abbreviation	Disk potency (µg)
Penicillin	Ampicillin	AMP	10
	Amoxycillin/Clavulanic acid	AMC	20/10
	Piperacillin/Tazobactam	TZP	30/6
	Mecillinam	MEC	10
Cephalosporin	Cefoxitin	FOX	30
	Cefuroxime	CXM	30
	Ceftazidime	CAZ	10
	Cefotaxime	CTX	5
Monobactam	Aztreonam	ATM	30
Carbapenem	Meropenem	MEM	10
Aminoglycoside	Gentamicin	GEN	10
Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole	SXT	1.25/23.75
Quinolone	Ciprofloxacin	CIP	5
Tetracycline	Tetracycline	TET	30
	Tigecycline	TGC	15
Amphenicol	Chloramphenicol	CHL	30
Nitrofuran	Nitrofurantoin	NIT	100

Even tough disk diffusion is a well-established and much used method, it is not suitable for susceptibility testing for all antibiotics, like colistin. Due to the large size of the colistin molecule, this agent has a poor diffusion rate in agar, and it is therefore not possible to discriminate between susceptible and resistant isolates using this method. Minimum inhibitory concentration (MIC) determination using the broth microdilution method is therefore the recommended method for susceptibility testing of polymyxin antibiotics (Rodríguez-Santiago et al., 2021;Matuschek et al., 2018). The MIC can be defined as the lowest concentration of an antibiotic agent that inhibits the visible growth of a microbe (EUCAST, 2003).

To examine colistin susceptibility in strains isolated from the marine environment with mutations associated with colistin resistance, the sensititre^{TM} EUVSEC plates (Thermo Fisher Scientific, USA) were used and results interpreted according to EUCAST breakpoints. The EUVSEC panel contains 14 antibiotic agents belonging to 11 different classes of antibiotics, including colistin (1-16 mg/l).

3.5 Short-read sequencing, *de novo* assembly and genome annotation

To examine the diversity of *K. pneumoniae* isolated from marine sources (**Objective III**) and to determine the presence of acquired ARGs and virulence factors (**Objective III**), all isolates identified as *K. pneumoniae* using MALDI-TOF MS were subjected to short-read sequencing. Short-read sequencing platforms, such as the Illumina MiSeq platform, produces short, low-error, paired end reads. This approach enables the determination of phylogenetic relationships and detection of important loci, like ARGs and virulence genes (Besser et al., 2018;De Maio et al., 2019), and is a powerful tool for the discrimination and identification of the closely related KpSC members (Wyres et al., 2020).

Extraction of genomic DNA was performed with the MagNA Pure 96 and Viral small volume kit with the Pathogen universal 200 4.0 purification protocol (Roche Applied Science, Germany). Sequencing libraries were prepared using Illumina Nextera DNA Flex library prep, and the libraries were sequenced with the Illumina MiSeq system and Illumina MiSeq Reagent kit V33 (600 cycle) to obtain 2x300 bp paired end reads.

The raw short-reads were adapter and quality trimmed using TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore) assembled with Unicycler (Wick et al., 2017). Draft genome sequences were annotated through the prokaryotic annotation pipeline (Tatusova et al., 2016).

3.6 Long-read sequencing, hybrid assembly and genome annotation

Although short-read sequencing provides important insights into genome sequences, it rarely enables assembly of complete genomes. Plasmids often contain repeat regions, which also can be present on the chromosome, complicating assembly of complete genomes from short-read sequences (Arredondo-Alonso et al., 2017). This results in fragmented assemblies and makes plasmid reconstruction difficult (Ben Khedher et al., 2022;Smalla et al., 2015;de Toro et al., 2014). These challenges can be resolved by using long-read sequencing technologies, which enables the assembly of complete genomes. However, long-read sequencing methods, like Nanopore sequencing (Oxford Nanopore Technologies, UK), often produces higher error rates compared to short-read sequencing (Ben Khedher et al., 2022;Kono and Arakawa, 2019). By combining the short- and long-read sequences, high quality closed genomes can be obtained (Ben Khedher et al., 2022). For in depth characterisation of antibiotic resistant *K. pneumoniae* isolated from the marine environment (**Objective V**), isolates carrying ARGs were subjected to long-read sequencing.

DNA was extracted manually with the Beckman Coulter Life science GenFind V3 with the "DNA extraction from Bacteria using GenFind V3" protocol (Beckman Coulter, USA). Sequencing libraries were prepared with the Ligation sequencing kit (SQK-LSK-109) (Oxford Nanopore Technologies, UK), loaded onto a MINion flow cell (Oxford Nanopore Technologies, UK) and sequenced on a GridION device (Oxford Nanopore Technologies, UK). For one isolate, 2016-1400, long-read sequencing was performed with a MinIon device (Oxford Nanopore Technologies, UK). For basecalling, Guppy v4.22 + effbaf (https://nanoporetech.com) was used, and Filtlong v0.2.0 (https://github.com/rrwick/Filtlong) was used for quality filtering. Hybrid *de novo* assembly of short- and long-read sequences obtained from antibiotic resistant *K. pneumoniae* was done using Unicycler (Wick et al., 2017). The complete genomes were annotated using the prokaryotic annotation pipeline (Tatusova et al., 2016).

3.7 Whole-genome sequence-based characterisation of *K. pneumoniae*

To examine the diversity of *K. pneumoniae* isolated from the marine environment as well as the presence of ARGs and virulence genes (**Objective III**) several bioinformatic tools were used. To determine the ST of our isolates, as well as the presence of acquired ARGs and virulence genes, Kleborate was used (Lam et al., 2021). Capsule (K) and O antigen (LPS) prediction was performed with Kaptive (Wyres et al., 2016). Further identification of acquired

ARGs as well as ”plus” genes (stress, heavy metal resistance genes and some virulence factors) was done with AMRFinderPlus (Feldgarden et al., 2021). Further identification of heavy metal resistance genes (HMRGs) was done searching the BIGSdb-Kp (https://bigsdb.pasteur.fr/klebsiella) and by manually searching the annotated genomes. The virulence factor database (VFDB) (Chen et al., 2016) v2021-4-8 available through ABRicate (https://github.com/tseemann/abricate) was used for further identification of virulence factors. Plasmid replicons were identified with PlasmidFinder (Carattoli et al., 2014). In cases were intrinsic ARGs or virulence factors were not identified in assemblies or annotated files, the raw-reads were screened with SRST2 (Inouye et al., 2014). All bioinformatic tools were run with default settings and are summarised in **Table 3**.

Table 3. Bioinformatic tools used to predict virulence factors, antibiotic resistance genes, heavy metal resistance genes, plasmid replicons, capsule antigen, O-antigen and multi locus sequence type.

Tool	Version	Target	Reference
Kleborate	2.1.0	ARGs, MLST, virulence factors	Lam et al., 2021
Kaptive	0.7.3	Capsule (K) and O antigen (LPS) prediction	Wyres et al., 2016
AMRFinder Plus	3.9.8	ARGs, stress, HMRGs, virulence factors	Feldgarden et al., 2021
PlasmidFinder	2.1	Plasmid replicons	Carattoli et al., 2014
VFDB	2021-4-8*	Virulence factors	Chen et al., 2016
BIGSdb-Kp	1.31.0	HMRGs	https://bigsdb.pasteur.fr/klebsiella
SRST2	0.2.0	ARGs, virulence factors	Inouye et al., 2014

*Database version available via ABRicate. Abbreviations: ARGs: antibiotic resistance genes, HMRGs: heavy metal resistance genes, MLST: Multi locus sequence typing

3.8 Phylogenetic analysis

To examine if specific *K. pneumoniae* STs present in the marine environment resemble those recovered from other sources, including from human infections (**Objective IV**), an ST specific core genome single nucleotide polymorphism (SNP) analysis was performed using the RedDog pipeline v1.beta.11 (https://github.com/katholt/RedDog). To achieve high resolution, the analyses were run individually for each ST, and the trimmed raw reads were mapped to a reference genome belonging to the same ST. By using a reference genome closely related to the genomes to be analysed, the chances of mismapping are reduced, also the number of regions in the reference that the reads are mapped to will increase when using a closely related genome as reference (Schürch et al., 2018). To avoid variability due to recombination events, which may affect the phylogeny, Gubbins v2.4.1 (Croucher et al., 2014) was used to filter out SNPs in recombination sites. SNP-sites (Page et al., 2016) was used to extract the total number of SNPs in the aligned genomes and SNP matrices were created with SNP-dists (https://github.com/tseemann/snp-dists). Maximum likelihood phylogenies from the SNP alignments were created with RAxML v8.2.12 (Stamatakis, 2014) and phylogenetic trees were visualised with ggtree (Yu et al., 2017).

3.9 Conjugation experiments

The transferability of specific plasmids carrying ARGs (**Objective VI**) was examined by filter mating experiments following the method described by Jutkina et al. (2018). A green fluorescent protein (GFP)-marked, rifampicin (RIF) and kanamycin (KAN) resistant *E. coli* strain was used as recipient and selected antibiotic resistant *K. pneumoniae* isolates recovered from marine sources were used as donors. Prior to mating, the recipient was grown over night in Mueller-Hinton broth (Oxoid, UK) (MHB) supplemented with 50 µg/ml KAN, while the donor strains were grown in MHB supplemented with 100 µg/ml ampicillin (AMP) according to their resistance patterns and incubated in 30 ºC over night. After incubation, donor and recipient were centrifuged at 2755 x g for 15 min and washed twice in phosphate buffered saline (PBS) (Sigma-Aldrich, USA) to remove antibiotic residues. After a final resuspension in PBS, the donor and recipient were mixed at a 1:1 ratio, with a density of ~$1x10^8$ cells. The conjugation mixture was filtered through 0.45 µm pore filters, placed on Mueller-Hinton agar (Oxoid, UK) and incubated. The conjugation temperature and period were different depending on the objective of the experiment. In **Paper I**, we wanted to examine if the plasmid in question was transferrable and the plates were incubated at 37 ºC over night, while for **Paper III** the aim was to quantify plasmid transfer, and a strict protocol with an incubation temperature of 30 ºC and

incubation time of three h was used to assure that the experiments were repeatable. An incubation time of three h have previously been shown to yield relative stable and reproducible results (Jutkina et al., 2016). Conjugation was disrupted by placing the filter in Falcon tubes with 10 ml PBS and sterile glass beads. To select potential transconjugants, serial dilutions were spread on Mueller-Hinton Orientation (CHROMagar^{TM}, France) chromogenic agar selective for urinary tract pathogens supplemented with 50 µg/ml RIF, 50 µg/ml KAN and 100 µg/ml AMP. For **Paper III**, conjugation frequencies were calculated from the number of transconjugants obtained divided by the total number of recipients before mating. Transconjugants were re-streaked to obtain pure cultures and the resistance profiles of transconjugants were examined following the disk diffusion method as described in section 3.4.

4 Summary of papers

4.1 Paper I

In **Paper I**, we examined the prevalence of *K. pneumoniae* and related species in the marine environment using bivalve molluscs and sea urchins as indicators and further examined the antibiotic susceptibility of the obtained isolates.

In total, **Paper I** included 469 batch samples of bivalves and seven batch samples of the sea urchin *S. droebachiensis* collected along the Norwegian coast. Based on MALDI-TOF MS identification, we found *K. pneumoniae* to be present in 17 % (n=78) of the samples, and both in samples from locations for commercial and non-commercial production. *K. oxytoca* (n=41), *K. variicola* (n=33), *K. aerogenes* (n=1), *R. ornithinolytica* (n=38) and *R. planticola* (n=13) were also recovered from these samples. There was a higher frequency of samples positive for *Klebsiella* spp. and *Raoultella* spp. with increasing concentrations of *E. coli*.

Antibiotic susceptibility screening by disk diffusion revealed acquired resistance in eight (10 %) of the *K. pneumoniae* isolates (n=78). These eight isolates displayed resistance to amoxicillin-clavulanic acid (n=3), tetracycline (n=3), chloramphenicol (n=2), trimethoprim-sulfamethoxazole (n=2), nitrofurantoin (n=2), ciprofloxacin (n=1), cefotaxime (n=1) and cefuroxime (n=1). Five of these isolates were resistant to more than one agent. Additionally, one *K. pneumoniae* isolate was categorised as intermediate susceptible, increased exposure to aztreonam, while three isolates fell within the area of technical uncertainty for piperacillin-tazobactam. Phenotypic susceptibility to ampicillin was observed in *K. pneumoniae* (n=4), *K. oxytoca* (n=2), *K. variicola* (n=7) and *R. ornithinolytica* (n=3).

K. pneumoniae isolate 2016-1400 was recovered from blue mussels (*M. edulis*) collected from an area used for commercial production and displayed resistance to clinically important extended spectrum cephalosporins. To examine the determinants responsible for the observed resistance, this isolate was subjected to complete genome sequencing. Hybrid *de novo* assembly of the short- and long-reads, and subsequent bioinformatic analysis revealed that this isolate belonged to ST1035 and harboured a 191 744 bp IncFIB(K)/IncFII plasmid (CP065035) carrying $bla_{\text{CTX-M-3}}$ and $bla_{\text{TEM-1}}$ as well as genes encoding resistance to silver (*sil*), copper (*pco*) and arsenic (*ars*). This plasmid was not transferrable to *E. coli* by filter mating conjugation. Further analysis showed that the plasmid was highly similar (100% sequence coverage, >99.9% identity) to plasmid pC17KP0055-1 (CP052387) from *K. pneumoniae* ST1035 strain

C17KP0055 (CP052386) of clinical origin. Also, the chromosome of *K. pneumoniae* 2016-1400 (CP065034) was highly similar (99.6% sequence coverage, 99.4% identity) to the chromosome of *K. pneumoniae* C17KP0055.

4.2 Paper II

In **Paper I** we showed that *K. pneumoniae* was present in marine bivalves from the Norwegian marine environment. In **Paper II**, we examined a further 109 bivalve samples for the presence of *K. pneumoniae*, bringing the total number of bivalve samples up to 578. Additionally, **Paper II** included 53 fish samples, 24 sediment samples and 17 samples of seawater. The aim of this study was to examine the genetic diversity, resistome and pathogenic potential of *K. pneumoniae* isolated from marine sources using WGS.

From the samples included in **Paper I** and **Paper II**, a total of 99 isolates were presumptively identified as *K. pneumoniae* using MALDI-TOF MS. Of these isolates, 92 were recovered from bivalves and seven were isolated from seawater samples. All these isolates were short-read sequenced. Based on WGS data, 87 isolates were identified as *K. pneumoniae*, 81 from bivalves and six from seawater. Thus, *K. pneumoniae* was present in 14 % of the examined bivalve samples and 35 % of seawater samples. Overall, 12 isolates were identified as other members of the KpSC, nine *K. quasipneumoniae* subsp. *similipneumoniae* from bivalves, one *K. quasipneumoniae* subsp. *quasipneumoniae* from seawater, and two isolates from bivalves were identified as *K. quasivariicola* and *K. variicola* subsp. *variicola*, respectively.

Among the additional isolates included in **Paper II**, none displayed phenotypic antibiotic resistance to the agents included in the susceptibility screening. WGS revealed presence of acquired ARGs in six (7 %) *K. pneumoniae* isolates, including *K. pneumoniae* 2016-1400, and one *K. quasipneumoniae* subsp. *quasipneumoniae* isolate. The identified ARGs encoded resistance to aminoglycosides, penicillins, quinolones, cephalosporins, tetracycline, trimethoprim, sulphonamides, and chloramphenicol. All sequenced isolates harboured the intrinsic *bla*$_{SHV}$ gene. HMRGs were also common in *K. pneumoniae* from the examined samples, present in 62 (71 %) *K. pneumoniae* isolates and eight (66 %) isolates belonging to other species in the KpSC, also in isolates with acquired ARGs. Of the acquired virulence factors frequently associated with human infection, ten *K. pneumoniae* isolates carried the yersiniabactin siderophore on different ICEs.

Phylogenetic analysis showed high diversity among *K. pneumoniae* recovered from bivalves and seawater, with a total of 50 STs identified. Most of these were represented by one single isolate, while only ST200 (n=5) was present in both bivalves (n=4) and seawater (n=1). The identified STs also included globally disseminated STs associated with MDR-Kp (ST17, ST20, ST29 and ST37) and hvKp (ST25). Similarly, high genetic diversity was also seen among the *K. quasipneumoniae* subsp. *similipneumoniae* isolates with the nine isolates distributed in eight STs. In this work, we also performed an ST specific core genome SNP analysis of globally disseminated STs and found a close genetic relationship between *K. pneumoniae* ST25 isolate 2016-1200 and a clinical *K. pneumoniae* ST25 strain from Germany (ERR1217000) (David et al., 2019), differing by 24 core genome SNPs. These two isolates carried the exact same ARGs, HMRGs, virulence genes and plasmid replicons.

4.3 Paper III

In **Paper III** we combined short- and long-read sequencing to obtain complete genome sequences of antibiotic resistant *K. pneumoniae* isolated from marine bivalves in order to understand the genetic context of ARGs and HMRGs in these isolates.

The complete genome of these isolates ranged in size from 5.34 Mbp to 5.58 Mbp, with a mean GC content of 57.3 % and average chromosome size of 5.27 Mbp. In total, seven plasmids were detected, with size ranging from 2 667 bp to 265 616 bp. Five IncFIB plasmids, present in five different isolates, carried ARGs. The number of ARGs on the plasmids ranged from one to eight, with the highest number identified on IncFIB/IncFII plasmid pKp1200_1 (CP085034) from *K. pneumoniae* ST25 2016-1200 (CP085033). Furthermore, four of the plasmids encoding ARGs also carried HMRGs, with *sil*, *pco* and *ars* genes presented on all of these. IncFIB/IncH1B plasmid pKp1198 (CP085098) identified in *K. pneumoniae* ST2167 isolate 2016-1198 (CP085097) also harboured genes encoding resistance to mercury (*mer*). In **Paper III** we showed that plasmids encoding both ARGs and HMRGs also carried type II toxin-antitoxin systems. Only *K. pneumoniae* ST292 isolate 2019-1764 (CP085099) carrying IncFIB plasmid pKp1764 (CP085100), lacked HMRGs. This isolate was recovered from blue mussels (*M. edulis*) collected from an area used for recreational activities whereas the remaining antibiotic resistant isolates were recovered from commercial production locations.

In this work, we also showed that all plasmids carrying both ARGs and HMRGs carried the *sil* and *pco* operon in similar regions flanked by different transposases. Also, similar regions were found in plasmid CP065035 from CTX-M producing *K. pneumoniae* isolate 2016-1400

presented in **Paper I** and plasmid pKp848CTX (NC_024992) from *K. pneumoniae* strain Kp848 responsible for an outbreak at Stavanger University hospital in Norway (Löhr et al., 2015) (**Figure 11**). Further comparison of HMRG encoding plasmids revealed that plasmids pKp319 (CP085102), pKp1200_1 (CP085034), CP065035 and pKp848CTX (NC_024992) all carried the *sil* and *pco* operon in regions flanked by IS*5* and truncated IS*L3* transposases.

Filter mating experiments revealed that plasmid IncFIB plasmid pKp319 (CP085102) carrying ARGs encoding resistance to tetracycline and penicillins was transferable to *E. coli* strain CV601-GFP, yielding transconjugants with identical resistance pattern.

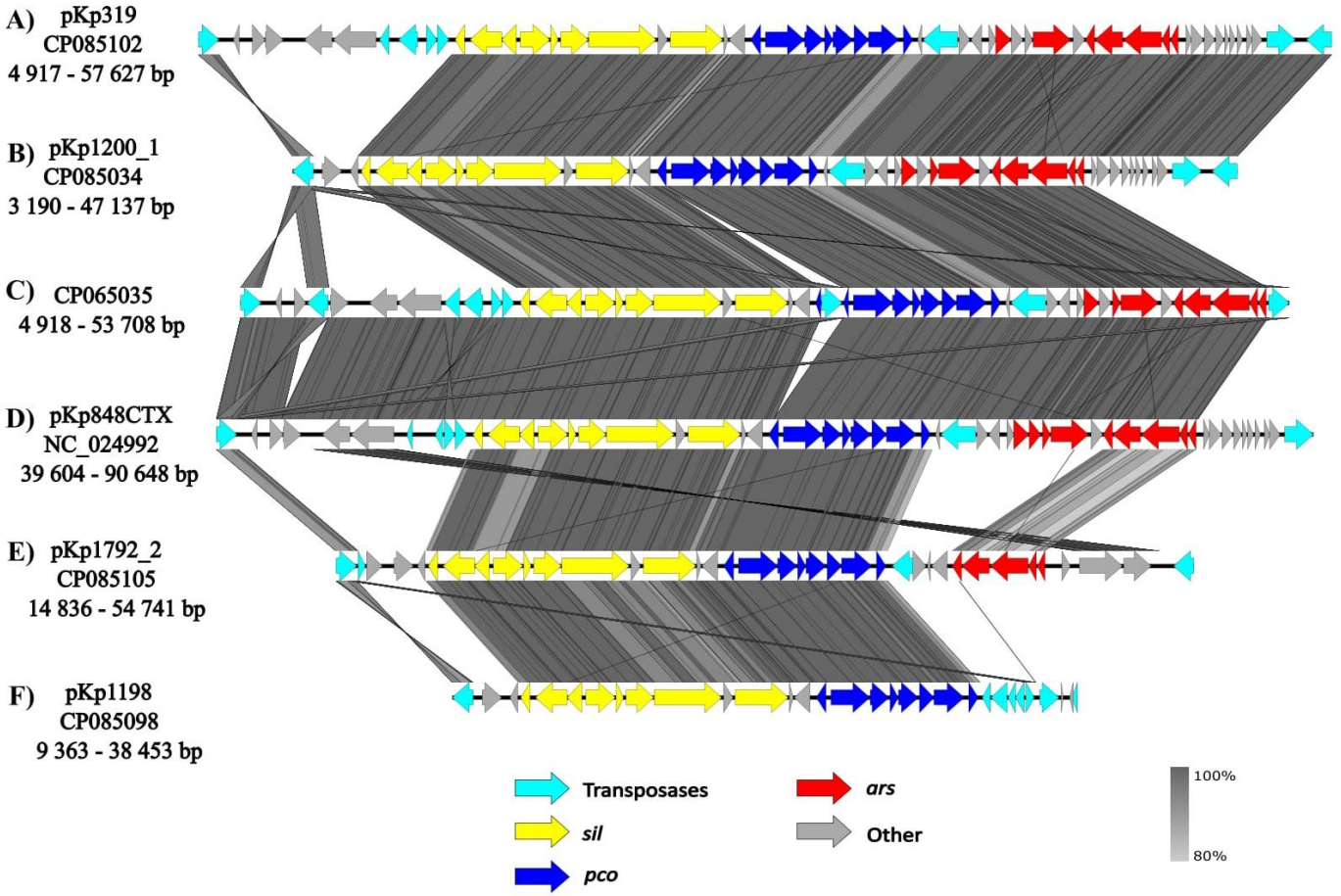

Figure 11.Alignment of plasmid regions carrying heavy metal resistance genes. A: plasmid pKp319 (CP085102), B: plasmid pKp1200_1 (CP085034), C: plasmid CP 065035, D: plasmid pKp848CTX (NC_024992), E: plasmid pKp1792_2 (CP085105), F: plasmid pKp1198 (CP085098). The *sil* operon is highlighted in yellow, the *pco* operon is coloured blue, *ars* genes are coloured red and insertion sequences and transposases are highlighted in cyan. Other genes present i the region are coloured grey.

5 Discussion

5.1 Prevalence of *K. pneumoniae* in the Norwegian marine environment

In **Paper I** and **Paper II**, we examined the prevalence of *K. pneumoniae* in samples collected from the Norwegian marine environment, including seafood organisms intended for human consumption. We reported presence of KpSC members in 16 % of examined bivalve samples. Without any obvious correlation, the frequency was similar to that found in fecal samples from community-based Norwegian adults (Raffelsberger et al., 2021). *K. pneumoniae* was the most frequent species isolated, present in 14 % (95 % confidence interval 11.2 % – 16.8 %) of the bivalve samples, followed by *K. quasipneumoniae* subsp. *similipneumoniae* (1.6 %). In the present work, *K. pneumoniae* was more common compared to what is seen among community-based carriers where *K. pneumoniae* was detected in ~10 % of the examined samples (Raffelsberger et al., 2021). However, in the study by Raffelsberger et al. (2021), both isolates identified as *K. pneumoniae* and *K. variicola* using MALDI-TOF MS were further analysed, while in the work presented in this thesis, only isolates identified as *K. pneumoniae* were subjected to further analysis. Therefore, considering all isolates identified as members of the KpSC recovered from marine bivalves using MALDI-TOF MS, the overall prevalence is slightly higher (19 %) than in adult Norwegian carriers.

Even though the presence of *K. pneumoniae* in food and its association with colonisation and infection is not well understood, the presence of *K. pneumoniae* in food is receiving interest from the scientific community and it has been shown that strains recovered from food can resemble those found in the clinic (Alonso et al., 2019;Rodrigues et al., 2022;Theocharidi et al., 2022;Hartantyo, 2020;Huynh et al., 2020;Wareth and Neubauer, 2021;Davis et al., 2015). Foodborne *K. pneumoniae* has also been suggested to be responsible for a nosocomial outbreak reported from Spain (Calbo et al., 2011). The global seafood consumption is increasing, and the production of seafood is the fastest growing animal protein food source (FAO, 2020). Presence of *K. pneumoniae* and *K. quasipneumoniae* subsp. *similipneumoniae* in retail seafood (Wareth and Neubauer, 2021;Sanjit Singh et al., 2017;Guo et al., 2016;Davis and Price, 2016;Sajeev et al., 2022;Sola et al., 2022), and marine bivalves have been reported previously (Bueris et al., 2022). However, to the best of our knowledge no larger studies on seafood organisms in their natural habitat have been conducted previously. Isolates recovered from seafood samples collected from retail markets may originate from different sources, *e.g.* due to insufficient hygienic practices during harvesting, processing, transport or handling in the

market (Wareth and Neubauer, 2021). Hence, one of the important findings in the current work is that *K. pneumoniae* can be present in the marine environment and may contaminate seafood organisms before harvest and before they reach the market.

From seawater samples, the frequency of samples positive for *K. pneumoniae* was high compared to bivalve samples (14 %), with six isolates recovered from 17 samples (35 %). However, *K. pneumoniae* was only present in water samples collected from coastal waters. Additionally, no *K. pneumoniae* isolates were recovered from fish intestine or sediments. This is in line with results from a large-scale metagenome study of the global ocean microbiome where *Klebsiella* spp. were very rarely detected in the open ocean (Sunagawa et al., 2015). The coastal marine environment has a various degree of anthropogenic influence, *e.g.* coastal waters, especially those close to wastewater treatment plant effluents, runoff from livestock farming and urban areas, are more likely to be affected by contamination than the open oceans. It is known that bacteria of faecal origin can be transferred to the marine environment through sewage effluents (Baquero et al., 2008), also in Norway (Grevskott et al., 2021). The results presented here are in accordance with the previous study (Sunagawa et al., 2015) and indicates that *K. pneumoniae* is mainly present in coastal marine environments.

5.2 Phenotypic antibiotic resistance

We found low levels of acquired phenotypic antibiotic resistance in *K. pneumoniae* isolated from marine sources (**Paper I and Paper II**). Overall, eight out of 87 *K. pneumoniae* isolates displayed acquired phenotypic resistance to one or more agents included in the study.

Among the 87 *K. pneumoniae* isolates, acquired phenotypic resistance was most frequently observed against tetracycline (~3 %). Due to lack of breakpoints for tetracycline, we applied no inhibition zone as criterion for classifying isolates as resistant to this agent. This may have caused underestimation of phenotypic tetracycline resistance. Tetracyclines is one of the most used classes of antibiotics in human and veterinary medicine, and in some countries, tetracycline is also used as growth promotors in food producing animals (Thaker et al., 2010;Hao et al., 2014). Due to the extensive use of tetracycline antibiotics, resistance to this class of antibiotics is widespread, also in indicator bacteria of faecal contamination from animals and food (Thaker et al., 2010;Li et al., 2015;EFSA and ECDC, 2021). Although the use of tetracyclines in animals and food production in Norway is low, especially in aquaculture, tetracycline resistance is one of the most frequently reported types of resistance in indicator bacteria from animals also in Norway (NORM/NORM-VET, 2022). Furthermore, tetracycline

resistance is the most common resistance determinant identified in *K. pneumoniae* from healthy Norwegian poultry (Franklin-Alming et al., 2021). Previously, tetracycline resistance has been identified as one of the most common types of resistance in *E. coli* isolated from marine bivalves collected in Norway (Grevskott et al., 2017;NORM/NORM-VET, 2017) and in *E. coli* recovered from sewage effluent in Norway (Grevskott et al., 2021). We also observed phenotypic resistance against chloramphenicol (~2 %), trimethoprim-sulfamethoxazole (~2 %) and nitrofurantoin (~2 %), which is similar to what was reported in *E. coli* in bivalves from the Norwegian marine environment (Grevskott et al., 2017). Resistance to clinically important antibiotics, like extended spectrum cephalosporins and quinolones was seen in two *K. pneumoniae* isolates, one isolate recovered from bivalves produced for human consumption and one isolate recovered from bivalves collected at a location used for recreational activities, indicating that seafood and the marine environment could serve as a vector for transmission of *K. pneumoniae* resistant to clinically important antibiotics to humans.

Looking at the whole isolate collection, a total of 225 isolates belonging to *Klebsiella* spp. (n=174) and *Raoultella* spp. (n=51) were subjected to antibiotic susceptibility screening by the disk diffusion method. Overall, acquired phenotypic resistance was only observed in the eight *K. pneumoniae* isolates (~4 %). Isolates belonging to other *Klebsiella* species and *Raoultella* species only displayed known intrinsic phenotypic resistance (Wyres et al., 2020;Yang et al., 2022;Hajjar et al., 2020;Passarelli-Araujo et al., 2019). Overall, the present work is in line with previous studies showing low prevalence of antibiotic resistance in the Norwegian marine environment (Grevskott et al., 2017;Håkonsholm et al., 2020), as well as the low prevalence of antibiotic resistance in the human sector in Norway (NORM/NORM-VET, 2022).

5.3 Acquired antibiotic- and heavy metal resistance genes and virulence factors

The results from **Paper I** and **Paper II** showed that six *K. pneumoniae* isolates and one *K. quasipneumoniae* subsp. *quasipneumoniae* carried acquired ARGs. However, no known acquired ARGs explaining phenotypic resistance to amoxicillin-clavulanic acid, piperacillin-tazobactam and/or nitrofurantoin were identified in isolates with reduced susceptibility to these agents. Three *K. pneumoniae* isolates were categorised as MDR according to the definition Magiorakos et al. (2012). The identified ARGs encoded resistance to tetracycline, third generation cephalosporins, penicillins, quinolones, amphenicol, aminoglycosides, trimethoprim and sulphonamides, of which most are considered important for the treatment of human infections (WHO, 2019).

With regards to acquired ARGs, the results corresponded well with results from phenotypic susceptibility screening, with some discrepancies. In **Paper I** we applied breakpoints for intravenous administration of amoxicillin-clavulanic acid, and some isolates were classified as resistant to this agent. However, in **Paper II** we applied breakpoints for oral administration of amoxicillin-clavulanic acid and all isolates were classified as susceptible, showing the importance of using relevant breakpoints when working with bacteria isolated from environmental sources. Additionally, we found no ARGs responsible for reduced susceptibility to piperacillin-tazobactam, however, increased production of the intrinsic SHV have previously been reported to cause resistance to this antibiotic in *K. pneumoniae* (Han et al., 2020). Furthermore, no acquired ARGs encoding resistance to nitrofurantoin were detected. However, overexpression of the intrinsic *oqxAB* genes can cause reduced susceptibility to nitrofurantoin (Li et al., 2019). In contrast, several genes encoding aminoglycoside modifying enzymes (*aadA1, aadA2, aph(3`)-Ia, aph(3``)-Ib* and *aph(6)-Id*) were detected, also in one *K. quasipneumoniae* subsp. *quasipneumoniae* isolate, without any observed phenotypic resistance against gentamicin. This could be explained by that these genes do not confer resistance to gentamicin as aminoglycoside modifying enzymes have different substrate profiles against different aminoglycosides (Ramirez and Tolmasky, 2010). The four *K. pneumoniae* isolates that displayed phenotypic susceptibility to ampicillin all carried the intrinsic bla_{SHV} gene, supporting the suggestion that ampicillin susceptibility in this species is caused by differential expression of these genes (Fu et al., 2007).

The most commonly observed ARGs were those encoding resistance to tetracycline (*tet(D)* and *tet(A)*), in line with results from disk diffusion. Sequence analysis revealed presence of one additional isolate carrying *tet(A)* not detected during disk diffusion. For this isolate, an inhibition zone of nine mm was measured, and it was therefore considered phenotypically susceptible to tetracycline. Acquired genes encoding resistance to penicillins ($bla_{\text{TEM-1}}$, $bla_{\text{SHV-1}}$) were detected three isolates. The $bla_{\text{TEM-1}}$ gene is frequently found in Gram-negative bacteria, both in clinics and in the environment (Korzeniewska et al., 2013;Grevskott et al., 2017;Cooksey et al., 1990;Bedenić and Meštrović, 2021). Penicillin antibiotics are the most commonly used agents in Norway, both in human and veterinary medicine (NORM/NORM-VET, 2022), thus the use of such agents could be the main driver for dissemination of genes conferring resistance to penicillins in Norway.

One isolate carried genes encoding resistance to third generation cephalosporins. *K. pneumoniae* isolate 2016-1400 was recovered from bivalves produced for consumption and

carried the *bla*$_{\text{CTX-M-3}}$ gene. The CTX-M genes originated in the environmental *Kluyvera* species but have since been mobilised and are now spread worldwide, limiting the available treatment options for infections caused by CTX-M producing pathogens (Cantón and Coque, 2006;Cantón et al., 2012). A recent study on ESBL producing clinical *K. pneumoniae* found *bla*$_{\text{CTX-M-15}}$ to be the dominating CTX-M variant, however, in the same study, one isolate carrying the *bla*$_{\text{CTX-M-3}}$ gene was isolated from the urine of a patient in Western Norway (Fostervold et al., 2021). Previous studies have shown presence of CTX-M producing *E. coli* in wastewater (Grevskott et al., 2021) and bivalves collected in Norway, but at low prevalence (Grevskott et al., 2017;NORM/NORM-VET, 2017), in accordance with results from the present work. In **Paper I** and **Paper II**, we show low prevalence of antibiotic resistance among *K. pneumoniae* isolated from bivalves and seawater. However, our results confirm the presence of isolates resistant to clinically important antibiotics in the Norwegian marine environment and in bivalves produced for human consumption.

HMRGs were common in *K. pneumoniae* isolated from the marine environment, especially *pco* and *sil* genes encoding resistance to copper and silver, respectively. High prevalence of HMRGs have also been reported in *K. pneumoniae* recovered from humans and animals (Zheng et al., 2022;Sütterlin et al., 2017). Heavy metals are frequently used in aquaculture. They are present in anti-fouling agents, used as additives in fish feed and are also naturally present in sediments and seawater (Grefsrud et al., 2021;Seiler and Berendonk, 2012). Furthermore, metal compounds are used in agriculture as antimicrobials and pesticides, fertilisers and in livestock feed (Grefsrud et al., 2021;Seiler and Berendonk, 2012;Silbergeld and Nachman, 2008;Pal et al., 2017). Therefore, heavy metals can be present in the marine environment both due to aquaculture activities and by run off from agriculture areas and potentially create a selection pressure for metal resistance in contaminated marine environments.

Even though our data shows low prevalence of acquired virulence factors associated with hvKp, ten (~11 %) *K. pneumoniae* isolates, of which eight were recovered from bivalve samples collected from commercial production locations carried the yersiniabactin locus. Comparing these results to recent Norwegian studies on *K. pneumoniae* of different origin, they are comparable to prevalence of yersiniabactin in *K. pneumoniae* from community-based carriers (~11 %) (Raffelsberger et al., 2021), but lower than in clinical isolates (~18 %) (Fostervold et al., 2021) and isolates recovered from broiler and turkey (~22 %) (Franklin-Alming et al., 2021). However, the mentioned studies also included isolates carrying the salmochelin and aerobactin siderophores (Fostervold et al., 2021;Raffelsberger et al., 2021;Franklin-Alming et

al., 2021). Hence, the prevalence of hvKp associated virulence factors in *K. pneumoniae* recovered from marine sources in Norway was lower compared to those recovered from human and animal sources. However, the yersiniabactin siderophore, detected in ten isolates, is the most common *K. pneumoniae* virulence factor associated with human infection (Martin and Bachman, 2018). Thus, in **Paper II** we show that *K. pneumoniae* isolates with a potentially increased virulence profile were present in seafood organisms and seawater sampled in Norway.

Although this work shows that *K. pneumoniae* present in bivalves and seawater carry ARGs (~7 %, n=6) and well characterised *K. pneumoniae* virulence factors (~11 %, n=10), the prevalence of such acquired genes could be underestimated. In this study, only one isolate identified as *K. pneumoniae* from each sample was selected for further characterisation. Sewage contains faecal material from a high number of households (Hutinel et al., 2019) and sewage discharge may therefore contain a high diversity of *K. pneumoniae* (Ludden et al., 2019). As a result, positive samples could potentially contain multiple different strains. Especially filter feeding organisms, like bivalves, which can retain bacteria from different sources simultaneously (Strand and Ferreira, 2019) could contain several strains. Thus, the true prevalence of antibiotic resistant and/or pathogenic *K. pneumoniae* in the marine environment may be higher or lower then reported in this work.

5.4 Diversity and phylogeny of *K. pneumoniae* from the marine environment

K. pneumoniae is a genetically diverse species (Wyres and Holt, 2018), and a high number of STs of different origin have been deposited in publicly available databases (https://bigsdb.pasteur.fr/klebsiella). In the present work, MLST analysis revealed that the 87 *K. pneumoniae* isolates belonged to 50 different STs (Simpson diversity index 97.6 %), and the majority of these (n=34, 68%) were represented by one single isolate. Furthermore, only one single ST, ST200, was identified in isolates from both marine bivalves and seawater. The high genetic diversity observed among *K. pneumoniae* isolates recovered from the marine environment is similar to observations in carriers (Lepuschitz et al., 2020;Huynh et al., 2020), hospital patients (Fostervold et al., 2021) and in studies on *K. pneumoniae* in animals (Paulin-Curlee et al., 2007;Yang et al., 2019), and might indicate that the isolates obtained from marine samples have numerous sources and originate from different niches. As discussed earlier, the study design used in this work is likely to underestimate the true diversity of *K. pneumoniae* in the marine environment as only one isolate from each positive sample was subjected to WGS.

Interestingly, the most common STs identified in our collection of *K. pneumoniae*, ST20 (n=8) and ST10 (n=7), are also common among community-based carriers in Norway (Raffelsberger et al., 2021) as well as among clinical isolates recovered from blood and urine samples from Norwegian patients (Fostervold et al., 2021). Thus, the presence of *K. pneumoniae* ST20 and ST10 in seafood organisms could indicate exchange of *K. pneumoniae* between the human and marine niches. However, more studies and data are required to further examine the directionality of the potential movement between these niches.

Among the 50 *K. pneumoniae* STs, we also identified globally disseminated STs, like ST17, ST20, ST25 and ST37, of which ST25 is associated with hypervirulence, whereas the remaining STs are associated with resistance to important antibiotics used in the treatment of infections among humans (Wyres et al., 2020). To examine if marine isolates belonging to such STs are related to those found in other environments, including the clinical environment, we performed an ST specific core genome SNP analysis. Most of the isolates belonging to such STs were not closely related to isolates recovered from other sources. However, our study showed that *K. pneumoniae* isolate 2016-1200 belonging to ST25 recovered from *M. edulis* produced for human consumption was closely related to a *K. pneumoniae* strain (ERR1217001) (David et al., 2019) isolated from the blood of a patient in Germany, differing by 24 SNPs. *K. pneumoniae* ST25 isolate 2016-1200 carried multiple ARGs as well as the yersiniabactin siderophore, also present in the clinical ERR1217001 strain. Thus, in **Paper II** we showed that *K. pneumoniae* closely related to clinical *K. pneumoniae* strains causing human infections were present in the marine environment, suggesting that clinically relevant *K. pneumoniae* isolates can potentially be transferred to humans via seafood organisms.

5.5 Complete genome sequences of antibiotic resistant *K. pneumoniae*

In **Paper I** and **Paper III**, we showed that all *K. pneumoniae* isolates carrying acquired ARGs harboured these on IncFIB plasmids, belonging to the IncF family. Plasmids belonging to the IncF group are the most frequently described plasmid type within the Enterobacteriaceae family, present in both humans and animals, and is often associated with ARGs (Rozwandowicz et al., 2018). Our results are in line with studies conducted in Italy where IncF (FII and FIB) plasmids were found to be common in antibiotic resistant *E. coli* isolated from marine bivalves (Citterio et al., 2020) and Brazil where IncF plasmids were common among CTX-M producing *E. coli* and *K. pneumoniae* recovered from wild-harvested marine bivalves (Bueris et al., 2022). Importantly, in five of the six antibiotic resistant *K. pneumoniae* isolates, ARGs and HMRGs

were co-localised on the same plasmid, thus indicating a potential for co-selection of ARGs in metal contaminated environments (Baker-Austin et al., 2006;Pal et al., 2017). In earlier work, it has been demonstrated that low concentrations of arsenic, like those that potentially can be found in different contaminated environments, was sufficient to select and maintain the MDR plasmid pUUH239.2, from a *K. pneumoniae* isolate responsible for a hospital outbreak in Sweden, also carrying genes encoding resistance to copper, silver and arsenic (Gullberg et al., 2014). Additionally, a recent metagenome study found a strong correlation between ARGs and metal resistance genes in the Black Sea, indicating that HMRGs are important in selection of ARGs in this sea (Sabatino et al., 2022).

One of the plasmids carrying both ARGs and HMRGs (pKp319) was transferred to the *E. coli* recipient via conjugation, showing potential for dissemination of such plasmids in the marine microbiota. Furthermore, in **Paper III** we show that all plasmids carrying both ARGs and HMRGs also harboured type II toxin-antitoxin systems, suggesting that these plasmids could be maintained even in the absence of selection pressure imposed by antibiotics and/or heavy metals (Carattoli, 2009;Yang and Walsh, 2017;Martinez, 2012).

Interestingly, several of the plasmids obtained during this work carried the *sil* and *pco* operons in similar genetic surroundings, with IS*5* and truncated IS*L3* flanking this region, possibly indicating presence of a composite transposon. Similar findings, especially the association between IS*L3* and *pco*, have been found in antibiotic resistant *K. pneumoniae* recovered from clinical settings and wastewater, where the IncFIB replicon was among the plasmid replicon types identified in the genomes of these isolates (Rocha et al., 2022;Löhr et al., 2015). Thus, suggesting presence of a potential composite transposon carrying heavy metal resistance genes in *K. pneumoniae* isolated from different sources.

6 Conclusion

The work described in this thesis represents the first comprehensive study on the prevalence of *K. pneumoniae* in the Norwegian marine environment. It provides important insights into the genetic diversity, antimicrobial resistance and the pathogenic potential of *K. pneumoniae* present in these settings.

K. pneumoniae was present in marine bivalve molluscs and seawater collected along the Norwegian coast, whereas its absence in samples obtained from the open ocean, indicates that this species is not commonly there. The overall prevalence of antibiotic resistance among *K. pneumoniae* and related species was low. However, some of the *K. pneumoniae* isolates carried clinically relevant ARGs, including those encoding resistance to extended spectrum cephalosporins ($bla_{\text{CTX-M-3}}$), aminoglycosides (*aph(3')-Ia, aph(3'')-Ib, aph(6)-Id, aadA1, aadA2*) and quinolones (*qnrS1*), as well as the yersiniabactin siderophore associated with human infection. All *K. pneumoniae* isolates carrying acquired ARGs harboured these on plasmids belonging to the IncF family and most of these plasmids also harboured HMRGs. Thus, suggesting a potential for co-selection of antibiotic resistance by heavy metals. Additionally, plasmids carrying both ARGs and HMRGs harboured type II toxin-antitoxin systems, indicating that these plasmids may be maintained even in the absence of a selection pressure. This study further reveals high genetic diversity among *K. pneumoniae* from marine sources, and the presence of globally disseminated STs associated with hypervirulence and MDR in bivalves. Also, the most common STs present in the examined samples are commonly found in humans, possibly indicating cross-sectoral transmission between the human and marine niches. However, it is not possible to determine the directionality of such transmission based on this study. Finally, presence of *K. pneumoniae* carrying clinically relevant ARGs and virulence factors genetically related to clinical isolates in bivalves may indicate potential for seafood to serve as a vector for transmission of such isolates to humans.

Overall, the prevalence of ARGs and virulence genes was low among *K. pneumoniae* isolated from the Norwegian marine environment. However, this study illustrates the potential of seafood and the marine environment to serve as a dissemination route for potentially pathogenic *K. pneumoniae* and ARGs to humans. It further highlights the importance of, and the need for, surveillance of pathogens and antibiotic resistance in the marine environment and seafood.

7 Future perspectives

Even though the work presented in this thesis provides a comprehensive account of *K. pneumoniae* in the Norwegian marine environment, there is still a need for more knowledge regarding this opportunistic pathogen in the marine setting and its relevance for seafood safety.

In this work, a qualitative method was used for detection of *K. pneumoniae*, and it is therefore not possible to determine the amount of this species present in the examined samples. For future work, a quantitative method for isolation of *K. pneumoniae* from marine sources should be developed. This would provide data on the total number of *K. pneumoniae* present in samples and enable better comparison with fecal indicator bacteria and further provide valuable data on the correlation between *E. coli* and *K. pneumoniae* in such environments.

Even though this work provides additional evidence that *K. pneumoniae* is present in the marine environment, there is a lack of knowledge on the ecology of *K. pneumoniae* in this compartment. Therefore, future studies should explore the ability of *K. pneumoniae* to survive and grow in marine settings under different environmental conditions. Furthermore, this work is part of an ongoing project which amongst other, aims to explore human, animal and environmental reservoirs of *K. pneumoniae*. Within this project, a comparative analysis of marine, human and animal *K. pneumoniae* isolates will be conducted. Thus, this work will examine if marine *K. pneumoniae* isolates carry accessory genes involved in the survival in, and adaptation to, the marine environment.

As discussed in this thesis, the study design may underestimate the prevalence of antibiotic resistant *K. pneumoniae* in the marine environment. Therefore, using selective methods for isolation of *K. pneumoniae* resistant to clinically important antibiotics, *e.g.* extended spectrum cephalosporins could provide a better estimate on the prevalence of such strains in the marine environment.

Similar to previous studies on *K. pneumoniae* from other sources (Sütterlin et al., 2017;Zheng et al., 2022), this study shows that HMRGs are common in *K. pneumoniae* recovered from seawater and marine environment and we therefore discuss the potential for co-selection of ARGs in metal contaminated environments. However, this work does not include experiments focusing on phenotypic resistance to heavy metals, and this together with further studies on the potential for co-selection should therefore be examined.

Although we have shown that *K. pneumoniae* is present in marine bivalves from commercial rearing localities, there is no data on *K. pneumoniae* in seafood from retail markets in Norway. Thus, domestically produced seafood from the markets should be examined to further elucidate the relevance of this opportunistic pathogen for seafood safety.

Finally, it is important to gain more knowledge on the transmission of *K. pneumoniae* to, from and within the marine environment. As mentioned, this work is part of a larger study on *K. pneumoniae* in different niches in Norway that will provide further and important data on the exchange of *K. pneumoniae* between these niches.

8 References

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Papers

Paper I

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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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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 $bla_{\text{CTX-M-3}}$ and $bla_{\text{TEM-1,}}$ 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 ($bla_{\text{CTX-M}}$) 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, MO, 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), cefoxitin (FOX, 30 µg), cefuroxime (CXM, 30 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 + effbaf84 (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*/*mpA2* 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 bla_{SHV}

To confirm the absence of bla_{SHV} *K. pneumoniae* isolate 2016-1400 was subjected to PCR amplification of the bla_{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 bla_{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, St. Louis, MO, 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, St. Louis, MO, 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, St. Louis, MO, 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, St. Louis, MO, 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

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

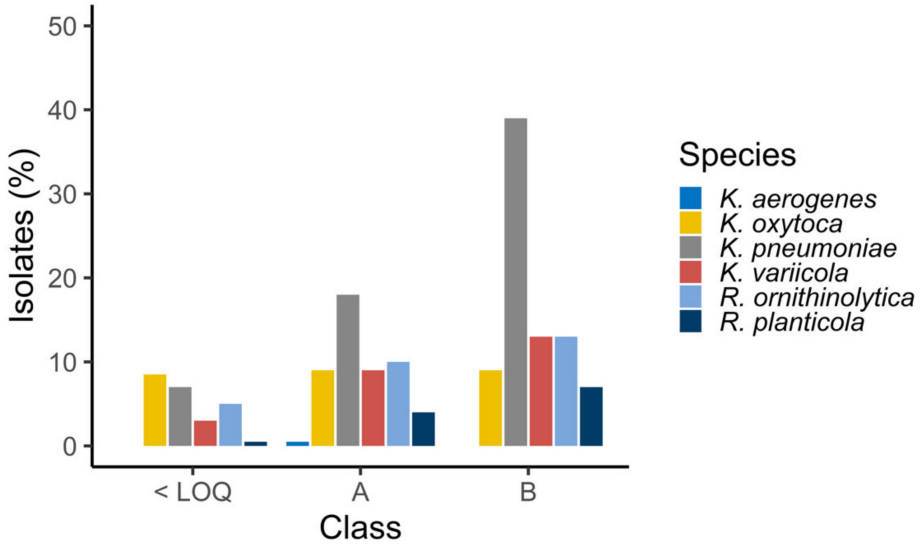

Figure 1. Distribution (%) of *Klebsiella* spp. and *Raoultella* spp. isolates recovered from areas of increasing *E. coli* load according to the EU classification A, B and C. < LOQ: < 18 MPN *E. coli*/100 g, A: 18-230 MPN *E. coli*/100 g, B: 230-4600 MPN *E. coli*/100 g. No results corresponded to *E.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

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 $bla_{\text{CTX-M-3}}$, $bla_{\text{TEM-1}}$, *oqxA*, *oqxB*, *fosA* 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-harbouring *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 $bla_{\text{SHV-1}}$, 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 2. Prevalence of antibiotic resistance among *Klebsiella* spp. and *Raoultella* spp. isolated form marine bivalves.

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

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	$bla_{\text{CTX-M-3}}$, $bla_{\text{TEM-1}}$, *oqxA*, *oqxB*, *fosA*, *erm(D)*	*pcoA*, *pcoB*, *pcoC*, *pcoD*, *pcoR*, *pcoS*, *pcoE*, *arsB*, *arsA*, *arsD*, *arsR*, *arsC*, *arsH*, *SilE*, *SilS*, *SilR*, *SilC*, *SilF*, *SilB*, *SilA*, *SilP*	*ent*, *fep*, *ecp*, *mgt*, *ompA*	CP065034, CP065035

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

$bla_{\text{CTX-M-3}}$ and $bla_{\text{TEM-1}}$, as well as the heavy metal resistance genes were carried on a IncFIB(K)/IncFII plasmid highly similar to the190 582 bp non-conjugstiveCP052387.1 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.

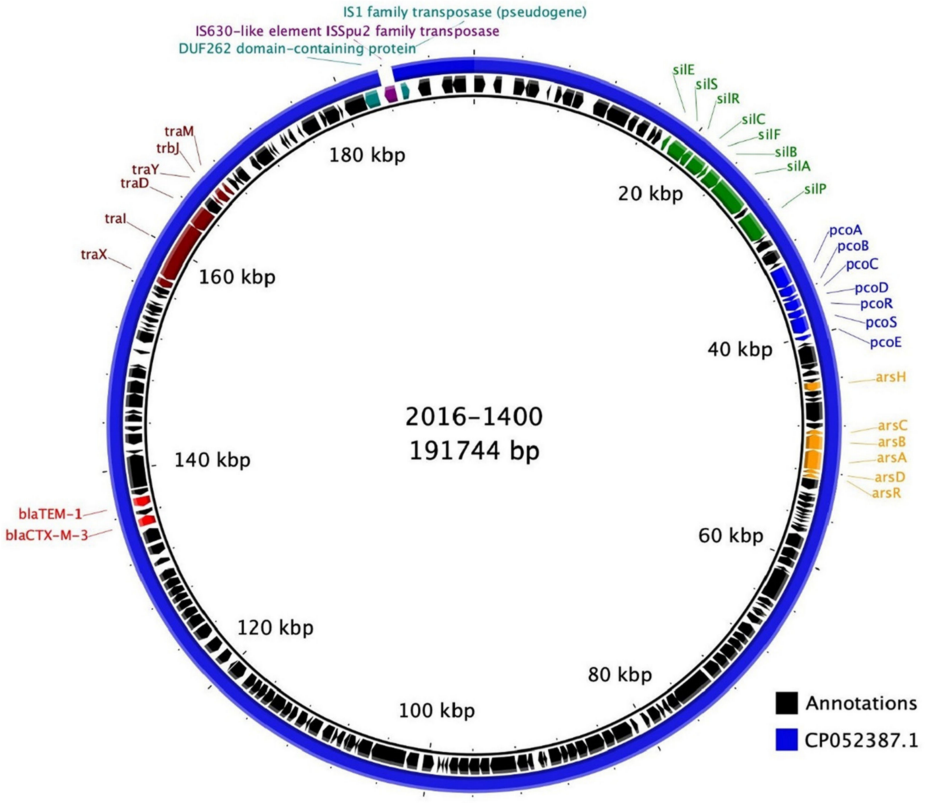

Figure 2. BLAST Ring Image Generator (BRIG) comparison of the *K. pneumoniae sensu stricto* 2016-1400 plasmid (accession number CP065035) and the plasmid from *K. pneumoniae* strain C17KP0055 (accession number CP052387.1). Ring 1 (innermost) shows the positions in the 2016-1400 plasmid, ring 2 shows its gene annotations and ring 3 shows the BLASTn result of CP052387.1 against the 2016-1400 plasmid. The locations of $bla_{\text{CTX-M-3}}$, $bla_{\text{TEM-1}}$, 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 $bla_{\text{CTX-M-3}}$ 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 $bla_{\text{TEM-1}}$ 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 $bla_{\text{CTX-M}}$ genes ($bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$) 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 http://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.

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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.

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Paper II

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Insights into the genetic diversity, antibiotic resistance and pathogenic potential of *Klebsiella pneumoniae* from the Norwegian marine environment using whole-genome analysis

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ABSTRACT

Klebsiella pneumoniae (Kp) can cause hospital- and community acquired infections. Although, Kp is widespread in the environment, very little is known about the genetic diversity and pathogenicity of Kp from the marine environment. The aim of our study was to understand the genetic diversity, resistome and pathogenic potential of 87 Kp isolates from the Norwegian marine environment, using whole-genome sequencing. We identified 50 sequence types, including globally disseminated sequence types associated with multidrug resistance or hypervirulence. Ten isolates carried the yersiniabactin loci. Acquired antibiotic resistance genes were identified in six Kp isolates. Heavy metal resistance genes were widespread among the isolates, with 71% carrying genes encoding resistance to copper, silver, arsenic, nickel and/or mercury. Co-occurrence of antibiotic resistance genes and heavy metal resistance genes was seen in five Kp isolates. Phylogenetic analysis revealed a close genetic relationship between Kp 2016-1200 ST25 isolated from blue mussels (*Mytilus edulis)* and a clinical isolate reported in Germany. To the best of our knowledge, this study provides the first comprehensive account of genetic diversity among Kp from the marine environment. Our study reveals high diversity of Kp in the Norwegian marine environment and seafood, including globally disseminated pathogenic sequence types carrying clinically relevant antibiotic resistance genes and virulence factors, as well as several heavy metal resistance genes.

1. Introduction

Klebsiella pneumoniae (Kp) can cause nosocomial as well as community acquired infections (Paczosa and Mecsas, 2016). In addition to the clinical environment, Kp is widespread in nature and can be found in surface waters, soil, on plants and in the gut of healthy humans and animals (Brisse et al., 2006; Bagley, 1985; Podschun et al., 2001). However, the primary reservoirs of Kp are not well understood (Davis and Price, 2016).

Recently, whole-genome sequencing has revealed the existence of five closely related species, of which two include subspecies, that together constitute the *Klebsiella pneumoniae* species complex (KpSC). The KpSC consists of *K. pneumoniae* sensu stricto, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. variicola* subsp. *variicola*, *K. quasipneumoniae* subsp. *similipenumoniae*, *K. variicola* subsp. *tropica, K. quasivariicola*, and *K. africana* (Wyres et al., 2020a). Of the KpSC members, Kp is responsible for the majority of human infections (Wyres et al., 2020a).

Kp is well known for its ability to acquire genetic material through horizontal gene transfer (Wyres and Holt, 2018), and the acquisition of mobile genetic elements have led to the development of two Kp groups, hypervirulent Kp (hvKp) and multidrug resistant Kp (MDR-Kp) (Russo and Marr, 2019). The hvKp carry plasmids and integrative conjugative elements (ICEs) encoding siderophores (*iro, iuc* and *ybt*), the colibactin toxin (*clb)* and/or genes responsible for a mucoid phenotype (*rmpA/rmpA2*) and are able to cause infections in otherwise healthy

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Abbreviations

ARGs	antibiotic resistance genes
BSI	blood stream infection
clb	colibactin
GI	gastrointestinal
HMRGs	heavy metal resistance genes
hvKp	hypervirulent *Klebsiella pneumoniae*
ICE	integrative conjugative element
iro	salmochelin
iuc	aerobactin
K	capsule
KL	capsule locus
Kp	*K. pneumoniae*
KpSC	*Klebsiella pneumoniae* species complex
MDR	multidrug resistant
MLST	multilocus sequence typing
SNP	single nucleotide polymorphism
ST	sequence type
ybt	yersiniabactin

individuals (Russo and Marr, 2019). In most cases *ybt* is chromosomally encoded and mobilised by ICEs, whereas the remaining virulence factors associated with hvKp are normally carried on plasmids (Wyres et al., 2020a). MDR-Kp is a common cause of hospital acquired infections (Pomakova et al., 2012; Russo and Marr, 2019). Both groups are associated with specific sequence types (STs), but recently convergence between the two groups has been observed (Wyres et al., 2020a).

Kp is a frequent coloniser of the human gastrointestinal (GI) tract and colonisation represents a significant risk for subsequent development of infections in immunocompromised individuals (Martin et al., 2016; Podschun and Ullmann, 1998; Martin and Bachman, 2018). Large variations in GI carriage rates of Kp have been reported worldwide. It has been found to be 16% in Norway and 6% in Australia, while in Asia, carriage rates as high as 88% in healthy adults have been reported (Gorrie et al., 2017; Lin et al., 2012; Raffelsberger et al., 2021).

Although not a classic foodborne pathogen, food has been identified as a risk factor for GI colonisation with Kp (Huynh et al., 2020; Lepuschitz et al., 2020; Raffelsberger et al., 2021). Kp has been isolated from several food sources, such as meat, street food, vegetables and seafood (Sanjit Singh et al., 2017; Guo et al., 2016; Davis et al., 2015; Falomir et al., 2013). Furthermore, it has been shown that strains isolated from food and the environment resemble clinical strains (Davis et al., 2015; Struve and Krogfelt, 2004).

Since the 1960s, the consumption of seafood has more than doubled worldwide (FAO, 2018). Consumption of contaminated seafood is a possible cause of GI infections. Seafood can be contaminated with pathogenic microorganisms in the environment, or it can be contaminated during transport and/or processing (Elbashir et al., 2018). Bivalve molluscs are filter feeders that retain and concentrate particles, including bacteria and viruses of both marine and terrestrial origin (Bernard, 1989). As a result, bivalves are well known to cause foodborne disease, and species traditionally consumed raw or lightly conserved, such as oysters (*Crassostrea gigas*), frequently cause food borne infections (Potasman et al., 2002; Elbashir et al., 2018). Due to the active accumulation of microorganisms and exposure to chemical pollutants, bivalves are also good indicators of faecal and chemical contamination in a given marine environment (Kibria et al., 2016; Grevskott et al., 2017).

Kp is extensively studied in clinical settings but the prevalence in the environment, especially the marine environment, is not well known (Manges, 2015). There are numerous transmission routes of pathogenic bacteria like Kp to the marine environment, *e.g.* through run-off from land and wastewater (Baquero et al., 2008; Marathe et al., 2017). Although we have shown the presence of Kp in marine bivalve molluscs collected along the Norwegian coast (Håkonsholm et al., 2020), there is a lack of knowledge on the genetic diversity and pathogenic potential of Kp isolated from the marine environments. The aim of this study was to understand the diversity, resistome and pathogenic potential of Kp strains isolated from the marine environment using whole-genome sequencing. We further examined the genetic relatedness of marine isolates of specific STs to isolates of human origin, including clinical isolates.

2. Materials and methods

2.1. Sampling, isolation and identification of presumptive Klebsiella pneumoniae

All samples included in the study were collected in 2016, and 2019–2020. In total, 578 batch samples of bivalve molluscs were examined. Of these, 563 samples covering production locations, depurated bivalves and wild populations were collected from 79 locations through the national surveillance programme of bivalve molluscs conducted by the Norwegian Food Safety Authority (NFSA), while 15 batch samples were collected from six locations not covered by the national surveillance programme.

The bivalve samples comprised 476 blue mussels (*Mytilus edulis*), 58 oysters (*Crassostrea gigas*), 31 scallops (*Pecten maximus*), five horse mussels (*Modiolus modiolus*), three ocean quahogs (*Arctica islandica)*, two carpet shells (*Politapes rhomboides*), two cockles (*Cerastoderma edule*) and one sand gaper (*Mya arenaria*). Although not bivalves, the samples also included seven batch samples of sea urchins (*Strongylocentrotus droebachiensis*) from two locations. A total of 53 fish samples were examined, 40 herring (*Clupea harengus)* and five mackerel (*Scomber scombrus*) collected by commercial fishing vessels in the North- and Norwegian Sea, three pollack (*Pollachius pollachius*), two cusk (*Brosme brosme*), two ling (*Molva molva)* and one hake (*Merluccius merluccius*) caught from coastal waters. Additionally, 17 samples of surface water from 13 different locations collected using a Van Dorn water sampler (KC Denmark, Denmark), and 24 sediment samples from nine locations were collected using a Van Veen Grab (KC Denmark, Denmark) were included. All samples were collected in sterile plastic containers (VWR, USA) or sterile plastic bags (VWR, USA) and kept at 4 °C until analysis.

Isolation of Kp from bivalve molluscs was performed as previously described (Håkonsholm et al., 2020). From each seawater sample, 1–5 l water was filtered through three separate 0.45 μm filters (Merck Millipore, Germany) using the EZ-fit Manifold 3-place system (Merck Millipore, Germany). The three filters used per sample were folded with sterile forceps and transferred to 100 ml buffered peptone water (BPW) (VWR, USA). From fish, 10 g of intestinal contents were weighed into sterile plastic bags (VWR, USA), homogenised for 2.5 min, diluted 1:10 with BPW and homogenised for 30 s. Sediment samples were diluted 1:10 in BPW in sterile plastic bags and homogenised for 30 s. Incubation conditions for all samples and further processing of enrichment cultures followed the methods described previously (Håkonsholm et al., 2020). All presumptive Kp isolates were identified using MALDI-TOF MS (Bruker, Germany). A complete list of isolates is provided in Supplementary Table S1.

2.2. Antibiotic susceptibility testing

Antibiotic susceptibility profiles for 70 Kp isolates included in the study have been reported previously (Håkonsholm et al., 2020). Antibiotic susceptibility testing of the additional isolates included in the present study was done with disk diffusion following the protocol described previously (Håkonsholm et al., 2020). The inhibition zones were interpreted following EUCAST breakpoints for Enterobacterales (https://www.eucast.org/clinical_breakpoints/). For tetracycline (TET), no breakpoints were available, and no inhibition zone was used to

classify the isolates as resistant. Measured inhibition zones for all isolates are presented in Supplementary Table S2.

2.3. DNA extraction and whole-genome sequencing

DNA was extracted from freshly grown isolates using MagNA Pure 96 and Viral Small volume kit with the Pathogen Universal 200 4.0 purification protocol (Roche Applied Science, 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.

2.4. Whole-genome sequence analysis

Raw short reads were adapter- and quality trimmed using Trim Galore v0.6.4 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and *de novo* assembled with Unicycler v0.4.8 (Wick et al., 2017). Species identification, multilocus sequence typing (MLST) and identification of the key virulence factors yersiniabactin (*ybt*), salmochelin (*iro*), aerobactin (*iuc*), colibactin (*clb*) and the regulator genes of a mucoid phenotype (*rmpA* and *rmpA2)*, and antibiotic resistance genes (ARGs) was done using Kleborate v2.1.0 (Lam et al., 2021), while serotype prediction was done with Kaptive v0.7.3 (Wyres et al., 2016). Plasmid replicons were identified with Plasmid Finder v.2.1(Carattoli et al., 2014). Further identification of ARGs, heavy metal resistance genes (HMRGs) and virulence genes was done using AMRFinderPlus v3.9.8 (Feldgarden et al., 2019), the BIGSdb-Kp database (https://bigsdb.pasteur.fr/klebsiella) and VFDB v2021-4-8 (Chen et al., 2016) via ABRicate v1.0.1 (https://github.com/tseemann/abricate). All bioinformatic tools were run using default settings. Novel STs were assigned by submitting sequence data to the Kp MLST database (https://bigsdb.pasteur.fr/klebsiella). The assembled genomes were annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). In isolates where intrinsic virulence genes were not identified in the assemblies, the annotated files were manually searched. A complete list of identified ARGs, virulence genes and HMRGs are provided in Supplementary Table S3.

2.5. Colistin MIC determination

Isolates with substitutions in the *pmrB* gene were subjected to MIC testing by broth microdilution using the Sensititre EUVSEC panel (Thermo Scientific, USA) following the protocol described earlier (Grevskott et al., 2021) and results interpreted according to EUCAST breakpoints for Enterobacterales (https://eucast.org/clinical_breakpoints/).

2.6. String test

All isolates possessing the yersiniabactin locus or belonging to serotypes associated with invasive infections were subjected to the string test to identify the hypermucoid phenotype associated with systemic infections (Catalán-Nájera et al., 2017). The isolates were grown on MacConkey agar (Sigma-Aldrich, USA) over night at 37 °C. A 10 μm loop was used to stretch a single colony, and a hypermucoviscous phenotype was defined as the formation of a string ≥ 5 mm (Catalán-Nájera et al., 2017).

2.7. Phylogenetic analysis

The RedDog pipeline v1beta.11 (https://github.com/katholt/RedDog) was used to create a core genome single nucleotide polymorphism (SNP) phylogeny of Kp isolated from the marine environment. Isolates belonging to other species of the KpSC were also included in the analysis. The raw reads were aligned to the Kp ST11 HS11286 chromosome (NC_016845.1) using BowTie2 v2.2.9 (Langmead and Salzberg, 2012) and SNPs identified with SAMtools v1.9 (Danecek et al., 2021). A core chromosomal SNP phylogeny was inferred with FastTree v2.1.10 (Price et al., 2010).

To examine the genetic relatedness between isolates belonging to selected Kp STs (ST17, ST20, ST25, ST29 and ST37) isolated from the marine environment and isolates of human origin, including clinical isolates, isolates from a hospital outlet and from waste water treatment plant, were used for ST specific core genome SNP analysis, performed as described above. The following genomes were used as references, NZ_CP056275.1 (ST17), NZ_CP056432.1 (ST20), NZ_CP033777.1 (ST25), NZ_CP065167.1 (ST29) and NZ_CP021960.1 (ST37). Gubbins v2.4.1 (Croucher et al., 2014) was used to remove SNPs in recombination sites. The total number of SNPs in the aligned core genomes were extracted with SNP-sites (Page et al., 2016), and SNP-dists (https://github.com/tseemann/snp-dists) was used to create pairwise SNP distance matrices. The SNP matrices are presented in Supplementary Table S4. RAxML v8.2.12 (Stamatakis, 2014) was used to infer maximum likelihood phylogenies from the core SNP alignments. The public available Kp genomes included in the core genome SNP analyses were downloaded from the European Nucleotide Archive and are listed in Supplementary Table S5.

3. Results

3.1. Prevalence and genetic diversity of Klebsiella pneumoniae in the marine environment

In total, 99 isolates from all samples were identified as Kp using MALDI-TOF MS and were whole-genome sequenced. The sequenced genomes were *de novo* assembled into an average of 133 contigs (35–343) with a mean genome length of 5 423 501 bp (5 009 383–5 854 074) and an average GC content of 57.35% (56.68%–58.07%).

Based on Kleborate analysis of whole-genome sequences, 87 of these isolates were identified as Kp, nine as *K. quasipneumoniae* subsp. *similipenumoniae*, one isolate was identified as *K. quasipneumoniae* subsp. *quasipneumoniae*, one isolate as *K. variicola* subsp. *variicola* and one isolate was identified as *K. quasivariicola*. Kp was recovered from 81 (14%) bivalve samples collected from 43 locations. Of these, 34 locations were used for commercial production of bivalves for human consumption. Kp was isolated from 74 samples of *M. edulis*, four batch samples of *C. gigas* and three *P. maximus* samples. Six isolates were found in water samples from six different locations (35%). For the other members of the KpSC, *K. quasipneumoniae* subsp. *similipenumoniae* was recovered from nine (2%) bivalve samples collected from eight locations, of which seven were used for commercial production of bivalves, one *K. variicola* subsp. *variicola* and one *K. quasivariicola* isolate was isolated from bivalves from two separate locations (0.2%), and the single *K. quasipneumoniae* subsp. *quasipneumoniae* isolate was recovered from a water sample (6%). No isolates were recovered from bivalves cleared for market, fish or sediment samples.

The Kp isolates belonged to 50 different STs, of which 34 were only represented by one single isolate. The most common STs were ST20 (n = 8), ST10 (n = 7), ST200 (n = 5) and ST643 (n = 5). ST200 was the only ST isolated from both bivalve molluscs and seawater (Fig. 1). Four isolates belonged to novel STs (ST4675, ST4676, ST5676 and ST5696). The nine *K. quasipneumoniae* subsp. *similipenumoniae* isolates belong to eight different STs.

Among all Kp isolates, 34 different capsule loci (KL) were identified, with KL28 (n = 10), KL102 (n=7) and KL62 (n = 6) being the most common. For six isolates no KL was assigned. ST specific combinations of KL and O types were seen in the ST20 (n = 8), ST10 (n = 7), ST416 (n = 3), ST110 (n = 2), ST1867 (n = 2), ST1966 (n = 2), ST2441 (2), ST27 (n = 2) and ST29 (n = 2) isolates. The remaining STs with more than one isolate differed in KL and/or O-type. One *K. quasipneumoniae* subsp. *similipneumoniae* isolate belonged to KL1.

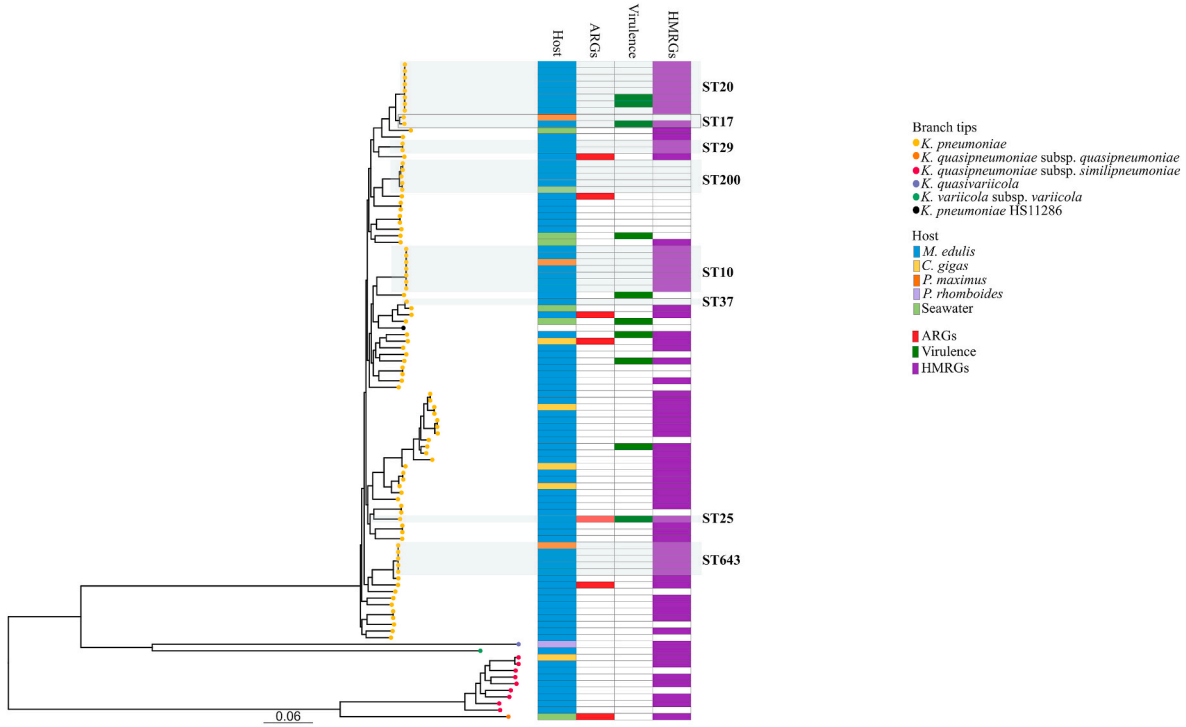

Fig. 1. Midpoint-rooted core genome phylogeny of 87 *Klebsiella pneumoniae,* nine *K. quasipneumoniae* subsp. *similipenumoniae*, one *K. quasipneumoniae* subsp. *quasipneumoniae*, one *K. quasivariicola* and one *K. variicola* subsp. *variicola* isolated from the marine environment. In total, 218 281 SNPs were identified in the aligned core genome of the marine Kp isolates. Branch tips are coloured according to the species the isolates belong to. The phylogeny is visualised alongside the marine host the isolates were recovered from, acquired antibiotic resistance genes (ARGs), virulence factors (yersiniabactin) and heavy metal resistance genes (HMRGs). Sequence types (STs) that are frequently reported in clinical settings and the most common STs isolated in this study are highlighted.

3.2. Phenotypic antibiotic resistance

Among the isolated Kp, we observed phenotypic resistance to tetracycline (~3%, n = 3), chloramphenicol (~2%, n = 2), nitrofurantoin (~2%, n = 2), trimethoprim-sulfamethoxazole (~2%, n = 2), ciprofloxacin (~1%, n = 1), cefotaxime (~1%, n = 1) and cefuroxime (~1%, n = 1). In total, ~3% (n = 3) of the isolates were susceptible to ampicillin. Resistance to amoxicillin-clavulanic acid was observed in three isolates (~3%) according to breakpoints for intravenous administration. However, these isolates remained susceptible while applying breakpoints for oral administration. No phenotypic resistance to agents other than ampicillin was seen among other species within the KpSC.

3.3. Acquired antibiotic resistance genes, heavy metal resistance genes and plasmid replicons

Among the 87 Kp genomes, 17 different acquired ARGs were identified. The ARGs were detected in six isolates, of which three were MDR as defined by Magiorakos et al. (2012). The identified ARGs included five genes encoding resistance to aminoglycosides (*aph(3″)-Ib, aph(3')-Ia, aph(6)-Id, aadA1* and *aadA2*) and three genes encoding resistance to sulphonamides (*sul1, sul2* and *sul3*), while the most prevalent ARGs was $bla_{\text{TEM-1}}$ (n = 3) and *tet*(D) (n = 3) (Table 1). As previously described, Kp 2016-1400 carried $bla_{\text{CTX-M-3}}$ and $bla_{\text{TEM-1}}$ on a non-conjugative plasmid and lacked the chromosomal $bla_{\text{SHV-1}}$ gene (Håkonsholm et al., 2020). Further, the three ampicillin susceptible

Table 1
Sequence types (STs), acquired antibiotic resistance genes (ARGs), heavy metal resistance genes (HMRGs) and plasmid replicons identified in antibiotic resistant *Klebsiella pneumoniae* and *K. quasipneumoniae* subsp. *quasipneumoniae* isolated from the marine environment.

Isolate	Species	ST	ARGs	HMRGs	Plasmid replicons
2016–1200	*K. pneumoniae*	ST25	*dfrA14, sul1, sul2, aph(3″)-lb, aph(6)-ld, aph(3')-la, tet(D),* $bla_{\text{TEM-1}}$	*silABCEFPRS, pcoABCDRS, arsABDR*	IncFIB(K), IncFII(K)
2016–1400	*K. pneumoniae*	ST1035	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-3}}$	*silABCEFPRS, pcoABCDERS, arsABDR*	IncFIB(K), IncFII (pKP91)
2016–1198[a]	*K. pneumoniae*	ST2196	*sul2, tet(D), catA2*	*silABCEFPRS, pcoABCDERS, merDEFPRT*	IncFIB(K)(pCAV1099-114), IncHI1B(pNDM-MAR)
2016–319	*K. pneumoniae*	ST556	*tet(D)*	*silABCEFPRS, pcoABCDERS, arsABDR*	IncFIB(K)
2019–1792[b]	*K. pneumoniae*	ST4267	*tet(A)*	*silABCEFPRS, pcoABCDRS, arsABCDR*	IncFIB(K), IncFII(K)
2019–1764	*K. pneumoniae*	ST292	*dfrA12, sul3,* $bla_{\text{TEM-1}}$, *cmlA1, qnrS1, aadA1, aadA2*	–	IncFIB(pKPHS1)
2019–1836	*K. quasipneumoniae* subsp. *quasipneumoniae*	ST5648	*aph(3″)-Ib, aph(6)-Id,*	*silABCEFPRS, pcoABCDRS*	IncFIB(K), IncR

Note; bla_{SHV}, *fosA* and *oqxAB* are intrinsic and therefore not presented in the table. a; Two copies of *merP, merR* and *merT* identified on separate contigs, b; : Two copies of *arsA, arsB, arsD* and *arsR* identified on separate contigs.

isolates all carried the intrinsic bla_{SHV}-gene. Additionally, one *K. quasipneumoniae* subsp. *quasipneumoniae* isolate carried the *aph(3″)-Ib* and *aph(6)-Id* genes encoding resistance to aminoglycosides (Table 1). A single amino acid substitution in the *pmrB* gene (R256G) associated with colistin resistance (Xiaoliang et al., 2019) was identified in six Kp isolates. However, MIC for colistin was <1 μg/ml for these isolates.

HMRGs were widespread in isolates from the marine environment, with 71% (n = 62) of the Kp isolates carrying genes encoding resistance to silver (*sil*), copper (*pco*), mercury (*mer*), nickel (*ncr*) and/or arsenic (*ars*). Among the nine *K. quasipneumoniae* subsp. *similipenumoniae* isolates, 44% (n = 4) carried *sil* and *pco* genes, 33% (n = 3) harboured genes conferring resistance to arsenic while 1% (n = 1) carried *ncr* or *mer* genes. Silver and copper resistance genes were identified in the single *K. quasipneumoniae* subsp. *quasipneumoniae* isolate, while the *K. quasivariicola* isolate carried mercury resistance genes. Both HMRGs and ARGs were present in five Kp isolates and one *K. quasipneumoniae* subsp. *quasipneumoniae* isolate.

Plasmid replicons were found in 84% (n = 83) of the isolates with 22 different replicon types identified. The most common plasmid replicon was IncFIB(K) (n = 59) followed by IncFII(K) (n = 48) and IncR (n = 23). More than one replicon type was found in 72 (73%) isolates. IncFIB(K) or IncFIB(K)(pCAV1099-114) replicons were found in all strains carrying both ARGs and HMRGs (Table 1).

3.4. Virulence genes

The type 3 fimbriae (*mrk*) cluster was present in all except one Kp isolate, while the type 1 fimbriae (*fim*) cluster was present in all except two isolates. The intrinsic enterobactin (*ent)* siderophore was identified in all isolates. The previously described CTX-M producing Kp 2016-1400 lacked both the *mrk* and *fim* clusters.

The yersiniabactin locus (*ybtAEPQSTUX-fuyA-irp1-irp2*) was detected in 11% (n = 10) of the Kp isolates. Five distinct integrative conjugative elements (ICEKps) and six *ybt* lineages were identified among the 10 ybt positive isolates, of which ICEKp5 (n = 5) and *ybt*14 (n = 4) were the most common. One *ybt* positive isolate (2019-1349), carried a novel *ybt* lineage (*ybt*18) and a new structural variant of ICEKp (ICEKp15). No other complete siderophore loci or hypermucoidity-encoding genes were identified in the marine Kp isolates. One isolate carried partial *iro*N and *iro*C genes on the same contig, this may be due to a deletion of the locus as described previously (Lam et al., 2018). Two of the Kp isolates carried the KL2 and KL57 locus, while one *K. quasipneumoniae* subsp. *similipenumoniae* isolate harboured the KL1 locus. These KLs encode capsule types associated with hypervirulence or invasive infections (Russo and Marr, 2019). All examined isolates were negative for the hypermucoviscous phenotype (Table 2). Genes encoding allantoinase (*all*) was present in two Kp isolates, one isolate carried *allABCDRS*, while Kp 2016-1400 harboured *allARS*. Genes involved in ferric iron uptake (*kfu*) and/or capsule formation (*kvg*) were found in 5% (n = 4) of the isolates. *kfu* genes were common in *K. quasipneumoniae* subsp. *similipenumoniae*, present in 78% (n = 7) of the isolates, while *all* genes were identified in four (44%) of the *K. quasipneumoniae* subsp. *similipneumoniae* isolates. *kfu* genes were also present in *K. variicola* subsp. *variicola* (n = 1) and *K. quasipneumoniae* subsp. *quasipneumoniae* (n = 1).

Table 2
Strain characteristics of *Klebsiella pneumoniae* with yersiniabactin isolated from the marine environment.

Isolate	ST	*ybt*	ICEKp	KL	String test
2016–1200	ST25	*ybt* 6	ICEKp5	KL2	–
2016–637	ST17	*ybt* 15	ICEKp11	KL25	–
2019–604	ST111	*ybt* 9	ICEKp3	KL63	–
2019–1349	ST866	*Ybt* 18	ICEKp15	KL46	–
2019–1394	ST20	*ybt* 14	ICEKp5	KL28	–
2019–1497	ST45	*ybt* 10	ICEKp4	KL43	–
2019–1897	ST20	*ybt* 14	ICEKp5	KL28	–
2019–1898	ST3403	*ybt* 16	ICEKp12	KL43	–
2019–2010	ST1307	*ybt* 14	ICEKp5	KL127	–
2020–749	ST704	*ybt* 14	ICEKp5	KL31	–

ST: Sequence type, *ybt*: yersiniabactin lineage, ICEKp: *Klebsiella pneumoniae* integrative conjugative element variant, KL: capsule (K) locus, -: negative string test.

3.5. ST specific phylogenetic analyses

The ST specific phylogenetic analyses identified 2 131, 3 700, 938, 3 010 and 2 988 SNPs in the aligned recombination-free core genomes of ST17, ST20, ST25, ST29 and ST37 isolates, respectively.

The marine isolates of ST17 and ST20 were intermingled with isolates of human origin, while the two ST29 isolates from bivalves clustered together with only two core genome SNPs between them (Fig. 2A, B, D). The single ST37 isolate clustered closest to a clinical urine isolate (232 SNPs) (Fig. 2E). Comparison of the MDR and *ybt* positive Kp 2016-1200 ST25 isolate to clinical isolates revealed a close genetic relationship to Kp ERR1217000 isolated from a patient with blood stream infection (BSI) in Germany in 2013, differing by only 24 core genome SNPs (Fig. 2C), with 94.5% of the Kp 2016-1200 genome and 95.9% of the ERR1217000 genome mapped to the ST25 NZ_CP033777 reference chromosome. Further, Kp 2016-1200 ST25 and ERR1217000 shared the same ARGs, HMRGs, virulence genes and plasmid replicons (*aph(3′)-Ia, aph(3″)-Ib, aph(6)-Id,* bla_{TEM-1}*, dfrA14, sul1, sul2, tet(D), silABCEFPRS, arsABDR, pcoACDRS, ybt,* IncFIB(K) and IncFII(K)), suggesting that these isolates are clonally related.

4. Discussion

During recent years, the environment has emerged as a potential reservoir for transmission of Kp and antibiotic resistance to humans (Wyres et al., 2020a). To the best of our knowledge, this study provides the first comprehensive account of genetic diversity among Kp from the marine environment. Our results show high genetic diversity of Kp and the presence of Kp carrying clinically relevant ARGs and virulence genes in the marine environment. Further, phylogenetic analysis of globally disseminated STs revealed a close genetic relationship between Kp isolated from blue mussels (*M. edulis*) and a clinical isolate, suggesting a potential transmission route for Kp from the marine environment to humans via seafood.

A high ST diversity of Kp was observed in Norwegian coastal waters and bivalve molluscs, including globally disseminated STs, like ST17, ST20, ST25, ST29 and ST37, associated with MDR or hypervirulence (Wyres et al., 2020a; David et al., 2019). Carbapenem resistant Kp ST17, ST20 and ST29 have been reported from a range of geographical locations, including Africa (Strydom et al., 2020), Asia (Safavi et al., 2020) and Europe (Aires-de-Sousa et al., 2019). High genetic diversity is also frequently reported from studies on Kp carriage in healthy individuals (Lepuschitz et al., 2020; Huynh et al., 2020), among clinical isolates (Fostervold et al., 2021) as well as studies on Kp in animals (Runcharoen et al., 2017; Gibbon et al., 2021; Paulin-Curlee et al., 2007), potentially indicating various sources of origin for the isolates from the marine environment. Interestingly, a recent study on Kp carriage in humans in Norway also found ST20 as the most common ST (Raffelsberger et al., 2021), possibly indicating exchange of Kp between the human population and the marine environment and/or vice versa. Large-scale metagenome analyses of the global marine environment have shown low abundance of *Klebsiella* in open oceans (Sunagawa et al., 2015), and the absence of Kp in samples of fish, seawater and sediments collected from open waters are in accordance with the previous study. Our study indicates that Kp may be largely present in the marine environments influenced by anthropogenic activities. Kp may follow numerous transmission routes to the marine environment, including sewage pollution, animal faeces, marine mammals and run-off from land, especially during periods with heavy rainfall (Jang et al., 2010; Roe

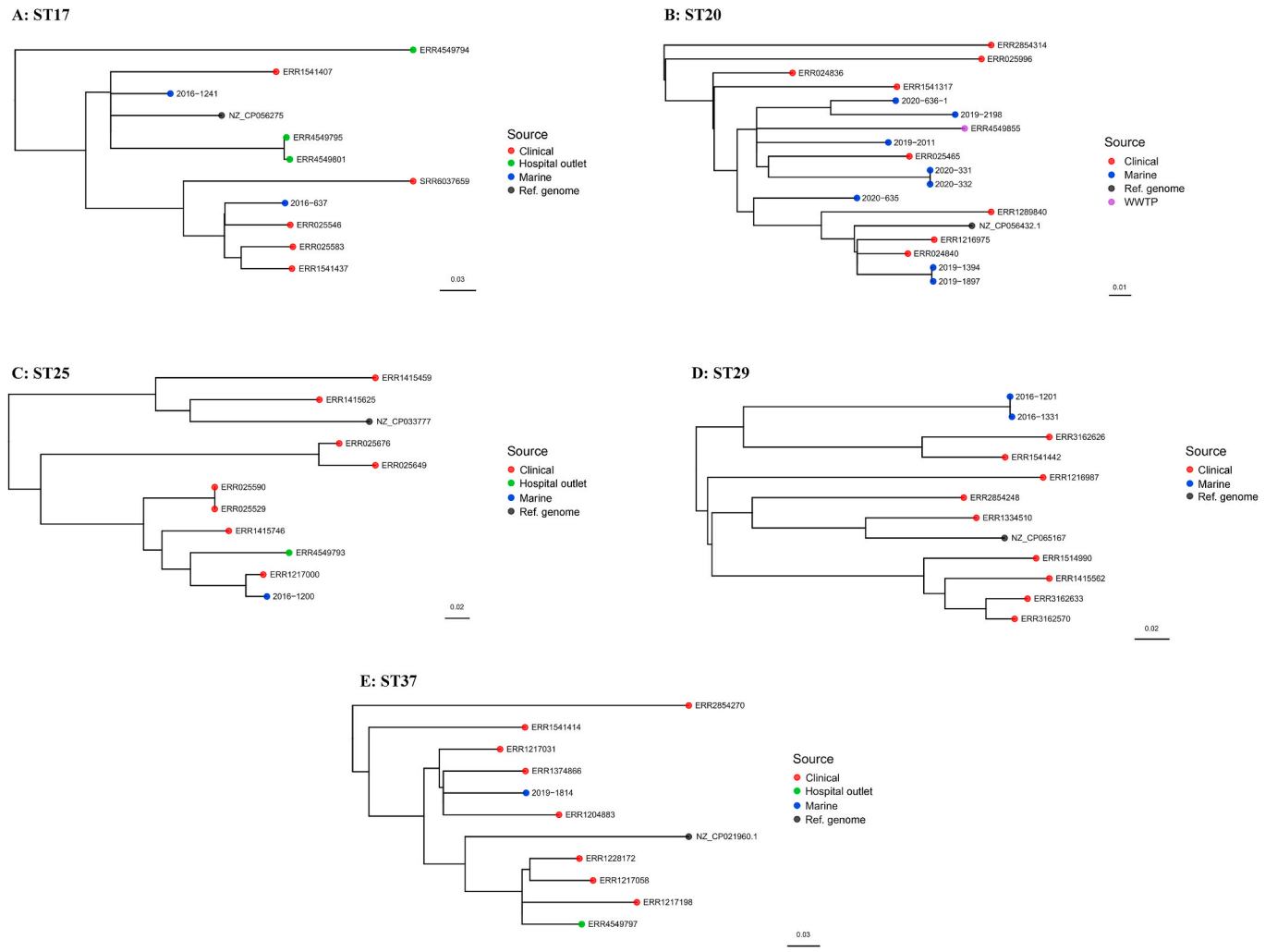

Fig. 2. Comparison of globally disseminated *Klebsiella pneumoniae* sequence types (STs) isolated from the marine environment and isolates of the same STs of human origin. A: ST17, B: ST20, C: ST25, D: ST29, E: ST37.

et al., 2015). This may explain the high genetic diversity of Kp observed in our study.

Our study included an ST specific comparison of a small set of marine isolates belonging to globally disseminated STs with human/clinical isolates. ST17, ST20 and ST37 isolates from the marine environment differed from isolates of human origin by 149–588 SNPs, indicating that isolates belonging to these STs recovered from the marine environment are not clonally related to the human/clinical genomes included in the study. Kp isolate 2016-1200 isolated from *M. edulis* collected from a production location in the middle of Norway carried multiple ARGs as well as genes encoding the yersiniabactin siderophore associated with human infection. We found a close genetic relationship between this isolate, belonging to ST25, and a clinical isolate from Germany causing BSI (24 SNPs). Further comparison revealed that the two isolates shared the same ARGs, HMRGs, virulence genes and plasmid replicons. Kp ST25 is associated with infections in both humans and animals (Bidewell et al., 2018; Wyres et al., 2020a, 2020b; Struve et al., 2015). The presence of MDR Kp with acquired virulence genes in bivalves reared for human consumption is especially worrisome, both with regards to transmission of pathogenic bacteria to the human population and the spread of ARGs and virulence genes in the food-production chain.

Overall, the frequency of acquired ARGs was low in Kp and other members of the KpSC isolated from the marine environment. Five of the antibiotic resistant Kp isolates also carried genes encoding resistance to heavy metals (*pco, sil, mer* and/or *ars*). This was also seen in the single *K. quasipneumoniae* subsp. *quasipneumoniae* isolate with acquired ARGs. These isolates also carried IncFIB(K) or IncFIB(K)(pCAV1099-114) plasmid replicons. Recently, we reported the co-occurrence of $bla_{CTX\text{-}M\text{-}3}$, $bla_{TEM\text{-}1}$, *pco, sil* and *ars* genes on an IncFIB(K)/IncFII(pKP91) plasmid in Kp from bivalves (Håkonsholm et al., 2020). HMRGs were common in our collection of Kp isolated from marine sources. This has also been reported in Kp from cattle suffering from mastitis, where *pco, sil* and *ars* genes were commonly found. The same study also showed lower frequencies of HMRGs in strains of human and environmental origin (Zheng et al., 2021). Heavy metals, especially copper, is commonly used in anti-fouling agents in aquaculture, and is also present in fish feed (Burridge et al., 2010; Grefsrud et al., 2021). Further, heavy metals are used in fertilisers in agriculture (Seiler and Berendonk, 2012), and may thus be introduced to the marine environment through run-off from land. Low concentrations of heavy metals are sufficient to select for and maintain the presence of antibiotic resistant bacteria in the environment (Gullberg et al., 2014). Thus, Kp isolates carrying heavy metal resistance genes may persist in metal contaminated marine environments and potentially contribute to dissemination of clinically important antibiotic resistance genes and related plasmids in the environment.

Yersiniabactin is one of the major virulence factors in Kp associated with human infections (Holt et al., 2015). In our study, *ybt* was identified in ten isolates, suggesting that Kp with pathogenic potential are present in bivalves produced for human consumption. Additionally, two isolates had capsule loci encoding capsule types associated with hvKp (K2 and

K57) (Russo and Marr, 2019). These findings suggest that potentially pathogenic Kp strains are present in the marine environment.

The presence of Kp in food and its association with human colonisation and infection is not well understood (Wareth and Neubauer, 2021). Since several studies on Kp in food have focused on retail food or food from markets (Hartantyo et al., 2020; Aguilar-Bultet et al., 2020), it is difficult to know where in the food-production chain the contamination has occurred (Huynh et al., 2020). Although no Kp were recovered from bivalves cleared for market, our study shows that bivalves from commercial production locations and coastal waters can carry Kp. Furthermore, we show close genetic relatedness between isolates from the marine environment and clinical isolates associated with human infections and MDR in bivalves produced for human consumption. Our study therefore supports the notion that consumption of raw or undercooked bivalves potentially may represent a risk of GI colonisation by Kp.

5. Conclusions

Our study reveals high genetic diversity among Kp isolated from seawater and bivalve molluscs collected from the Norwegian marine environment, including globally disseminated STs associated with MDR and hypervirulence. Along with ARGs, HMRGs were widespread in Kp from the marine environment, suggesting potential for co-selection of antibiotic resistance. Further, we show that Kp carrying clinically relevant ARGs and virulence genes genetically related to clinical isolates were present in bivalves, indicating potential for seafood borne transmission of Kp to humans. Our study thus indicates that the marine environment, especially the coastal environment, is a potential source of Kp, and further illustrates the need for environmental monitoring of pathogens and antimicrobial resistance.

Authorship contribution statement

Fredrik Håkonsholm., Nachiket P. Marathe, Bjørn Tore Lunestad, Iren H. Löhr and Cecilie S. Svanevik contributed to the design and conception of the study. Fredrik Håkonsholm performed the experiments, bioinformatic analyses were done by Fredrik Håkonsholm and Marit A.K. Hetland. Fredrik Håkonsholm 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.

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Data availability

The raw reads, genome assemblies and annotations are available under BioProject PRJNA591480. BioSample accession number and GenBank accession number for the individual genomes included in the study are presented in Table S1.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.113967.

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Paper III

Co-localisation of clinically relevant antibiotic- and heavy metal resistance genes on IncFIB plasmids in *Klebsiella pneumoniae* from marine bivalves

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Abstract

Background

Klebsiella pneumoniae is an opportunistic pathogen frequently associated with antibiotic resistance and present in a wide range of environments, including the marine environment. However, little is known about the development and persistence of antibiotic resistance in such environments. The aim of this study was to obtain complete genome sequences of antibiotic-resistant *K. pneumoniae* isolated from marine bivalves to determine the genetic context of antibiotic- and heavy metal resistance genes in these isolates.

Methods

Five antibiotic-resistant *K. pneumoniae* isolates, of which four also carried heavy metal resistance genes, were selected for complete genome sequencing using the Illumina MiSeq platform and the Oxford Nanopore Technologies GridION device. Conjugation experiments were conducted to examine the transfer potential of selected plasmids carrying antibiotic- and heavy metal resistance genes to a GFP-tagged *Escherichia coli* recipient strain.

Results

The complete genomes of *K. pneumoniae* isolates ranged in size from 5.34 Mbp to 5.58 Mbp, with an average length of 5.48 Mbp and mean chromosome size of 5.27 Mbp (5.20 Mbp – 5.31 Mbp). Overall, seven plasmids ranging in size from 2 667 to 265 616 bp were detected in the five antibiotic-resistant isolates. Five IncFIB plasmids carried clinically relevant antibiotic resistance genes like *qnrS1, aph(6)-Id* and *aph(3`)-Ia, aadA1* and *aadA2*. In addition, four of these plasmids carried genes encoding resistance to copper (*pco*), silver (*sil*) and arsenic (*ars*). One plasmid carrying *tet(D*) and $bla_{\text{SHV-1}}$ as well as *pco, sil* and *ars* genes was transferable to *E. coli* via conjugation.

Conclusion

We show co-occurrence of antibiotic- and heavy metal resistance genes on a transferable IncFIB plasmid from *K. pneumoniae* from marine bivalves. Our study highlights the importance of the marine environment and seafood as a possible dissemination route for antimicrobial resistance and provides insights into the potential for co-selection of antibiotic resistance genes by heavy metals.

Keywords: *Klebsiella pneumoniae*, plasmids, acquired antibiotic resistance, heavy metal resistance genes, co-selection

Background

Klebsiella pneumoniae is an opportunistic pathogen and a common cause of nosocomial infections. *K. pneumoniae* is often associated with antibiotic resistance, and strains resistant to clinically important antibiotics are considered a critical threat to public health (1). *K. pneumoniae* is commonly found in the gastrointestinal tract of humans and animals but can also be isolated from a range of environments, including soil, plants, surface waters and marine organisms (1-3).

Increased resistance to antibiotics is one of the greatest threats in modern medicine (4). Infections caused by antibiotic-resistant bacteria were estimated to be the direct cause of 1.27 million deaths globally in 2019 (5). *K. pneumoniae* is considered an important contributor to the spread of antibiotic resistance (6). Resistance to broad-spectrum cephalosporins and carbapenems is increasingly reported in clinical *K. pneumoniae* isolates in the WHO European region, with 44 % of countries reporting resistance rates of ≥ 50 % to third generation cephalosporins in 2020, particularly in southern and eastern European countries. However, the occurrence is still low in the Scandinavian countries with an average of 8.1 % of invasive *K. pneumoniae* isolates resistant to third generation cephalosporins (7).

Horizontal gene transfer is one of the primary drivers of antibiotic resistance, and the spread of antibiotic resistance genes (ARGs) is driven by conjugative plasmids (8). Plasmids can be classified according to their incompatibility (Inc), which refers to their inability to co-exist stably in the same cell line over time. In general, closely related plasmids are often incompatible, while those more distantly related more often are compatible. Overall, 28 Inc groups have been reported within the Enterobacterales family, and some of these are frequently associated with ARGs, *e.g.* extended-spectrum β-lactamase (ESBL) and carbapenemase encoding genes are commonly found on IncF plasmids (9, 10). In *K. pneumoniae*, most of the ARGs are present on large conjugative plasmids, and most acquired ARGs are carried on plasmids belonging to the IncFII, IncN, IncR and/or IncX3 groups (1, 6).

The environment is recognised as an important source and dissemination route of antibiotic resistance (11, 12). Although overuse of antibiotics is the major driver of antibiotic resistance, other compounds such as heavy metals and biocides can cause co-selection of antibiotic-resistant bacteria. Unlike antibiotics, metals in the environment are not degraded, and their presence could therefore represent long-term selection pressure (13).

In a previous study, we have shown that antibiotic-resistant *K. pneumoniae* carrying heavy metal resistance genes (HMRGs) are present in bivalve molluscs and seawater from the Norwegian marine environment (3). The aim of the present study was to obtain complete genome sequences of *K. pneumoniae* isolates carrying ARGs and HMRGs using a combination of long- and short-read whole genome sequencing, to determine the genetic context of antibiotic- and heavy metal resistance genes in this setting. We show co-occurrence of ARGs and HMRGs on transferrable IncFIB plasmids in these isolates.

Results

Complete genome sequences of *K. pneumoniae* isolates encoding ARGs and HMRGs

The size of the assembled genomes ranged from 5.34 Mbp to 5.58 Mbp, with a mean GC content of 57.3 % (57.1 % - 57.4 %) and an average chromosome size of 5.27 Mbp (5.20 Mbp – 5.31 Mbp). Overall, seven plasmids were identified in the assembled genomes, ranging in size from 2 667 to 265 616 bp (Figure S1). Five plasmids, identified in five separate isolates, carried acquired ARGs, while four of these also carried genes encoding resistance to heavy metals. All acquired ARGs and HMRGs were co-located on IncFIB plasmids (Table 1).

K. pneumoniae ST556 isolate 2016-319 (CP085101) was recovered from a pooled sample of *Crassostrea gigas* collected from a commercial production location and carried IncFIB plasmid pKp319 (CP085102), which encoded all acquired ARGs and HMRGs. Plasmid pKp319 carried tetracycline- (*tet(D)*) and penicillin resistance genes ($bla_{\text{SHV-1}}$) within a 13 867 bp (73 169 – 87 036 bp) region flanked by IS*26* transposases. Furthermore, plasmid pKp319 carried copper (*pco),* silver (*sil*) and arsenic resistance genes (*ars),* as well as genes related to heat tolerance (*clpK, hsp20*) in a region flanked by IS*5* transposases (IS*903* and IS*Kpn26*). Plasmid pKp319 also carried additional copies of the *arsB, arsC* and *arsH* genes (Figure 1A). Additionally, this plasmid encoded a type II toxin-antitoxin (TA) system (RelE/ParE family toxin, Phd/YefM family antitoxin) involved in plasmid maintenance (14). Comparing plasmid pKp319 to other publicly available plasmid sequences, it was identical to plasmid tig00001208_pilon (CP036443) (99 % coverage and >99.9 % identity) from a clinical *K. pneumoniae* ST45 strain ABFPV (CP036442) from the USA isolated in 2014. However, plasmid pKp319 carried accessory regions, including a 3 337 bp region encoding the betT choline transporter, as well as a 4 329 bp region carrying plasmid maintenance genes

like *psiB* and an additional copy of the ParB/RepB/Spo0J family partition protein coding genes, absent in plasmid tig00001208_pilon.

IncFIB/IncHI1B plasmid pKp1198 (CP085098), identified in *K. pneumoniae* ST2167 isolate 2016-1198 (CP085097) was recovered from a rearing facility in northern Norway, and carried genes encoding resistance to tetracycline (*tet(D)*), sulphonamides (*sul2*),chloramphenicol (*catA2*), mercury (*merACDEPRT*), additional copies of *merAPRT*, a truncated copy of *merD* and *arsBCH* on a ~43 200 bp region (185 828 – 229 073 bp) flanked by IS*26* transposases. In plasmid pKp1198, the *pco* and *sil* locus, *clpK* and *hsp20* were present in a region flanked by truncated and complete IS*5* family transposases (Figure 1B). Furthermore, plasmid pKp1198 carried multiple type II TA systems (HigB family toxin, VapC family toxin, VapB family antitoxin and phd/YefM). This plasmid was highly similar (98 % coverage and > 99.9 % identity) to plasmid p59062CZ_IncFIB (CP085732) encoding resistance to tetracycline (*tet(D)*) and chloramphenicol (*catA1*) from a clinical *K. pneumoniae* ST54 strain isolated in the Czech Republic in 2020. Both plasmids carried ARGs and HMRGs, however, pKp1198 carried *sul2* within this region, as well as *merDEF* all absent in plasmid p59062CZ_IncFIB. Also, plasmid pKp1198 harboured other accessory genes including a ~8 000 bp region encoding the *fec* system (*fecIRABCDE*) flanked by IS*100* transposases.

K. pneumoniae ST25 isolate 2016-1200 (CP085033) was isolated from *Mytilus edulis* collected from a commercial production location. This isolate carried all ARGs and HMRGs on IncFIB/IncFII plasmid pKp1200_1 (CP085034). The ARGs were located within a ~28 500 bp (82 483 – 110 975 bp) region, which contained genes encoding resistance to sulphonamides (*sul1, sul2*), aminoglycosides (*aph(3')-Ia, aph(3'')-Ib, aph(6)-id*), tetracycline (*tet(D)*), trimethoprim (*dfrA14*) and penicillins ($bla_{\text{TEM-1}}$). Within the region, one complete and one truncated class 1 integron with gene cassettes carrying *sul1, aph(3')-Ia* and *dfrA14* were identified. All ARGs were located in a region flanked by IS*26* and IS*5075* transposases. Similar to plasmid pKp319, plasmid pKp1200_1 carried multiple genes encoding resistance to silver (*sil*), copper (*pco*) and arsenic (*ars*), in addition to *clpK* and *hsp20* on a ~48 000 bp region flanked by IS*5* transposases (Figure 1C). Overall, plasmid pKp319 from isolate 2016-319 and plasmid pKp1200_1 from isolate 2016-1200 shared 76 % sequence coverage and >99 % identity. Plasmid pKp1200_1 carried the RelE/ParE and phd/YefM type II TA system. Blast searches against publicly available plasmid sequences showed that plasmid pKp1200_1

was genetically similar to several other *K. pneumoniae* plasmids (>80 % sequence coverage, >99% identity).

K. pneumoniae ST292 isolate 2019-1764 (CP085099) was isolated from *M. edulis* collected from an area used for recreational activities and was the only antibiotic-resistant isolate lacking HMRGs. IncFIB plasmid pKp1764 (CP085100) carried all ARGs on a region located between position 1 – 23 546 bp. A class 1 integron with a gene cassette containing ARGs encoding resistance to trimethoprim (*dfrA12*), aminoglycosides (*aadA1, aadA2*) and chloramphenicol (*cmlA1*) was identified within this region. Furthermore, pKp1764 harboured genes encoding resistance to penicillins ($bla_{\text{TEM-1}}$) on a Tn*3* transposon and also carried *qnrS1* and *sul3* (Figure 1D) encoding resistance to quinolones and sulphonamides, respectively. Several phage related genes were identified in the plasmid sequence, and the backbone of plasmid pKp1764 was similar (80 % coverage, 99.97 % identity) to the phage-like pSID3 plasmid (CP066514), from the clinical *K. pneumoniae* ST893 strain Kp36336 (CP066511) isolated from a Belgian patient in 2019, and an unnamed *K. pneumoniae* plasmid (CP063431) of human origin reported from Singapore (15). However, the resistance region on plasmid pKp1764 was absent on plasmid pSID3 and CP063431, but identical (100 % sequence coverage, >99 % identity) to segments present on the chromosome of two *Escherichia coli* strains recovered from pork in China (CP037903) in 2017 and Cambodia (CP044291) in 2016. The resistance region on plasmid pKp1764 was also similar (>99 % identity) to segments in an *E. coli* plasmid recovered from wastewater in the UK (CP056847) in 2017 which also carries a similar class 1 integron (92 % coverage, >99 % identity).

K. pneumoniae isolate 2019-1792 (CP085103) belonging to ST4267 carried two plasmids, IncFII plasmid pKp1792_1 (CP085104) and IncFIB plasmid pKp1792_2 (CP085105), of which plasmid pKp1792_2 carried the *tet(A)* tetracycline resistance gene on a *TnaS1* transposable element (Figure 1E). Similar to other plasmids identified in *K. pneumoniae* from marine bivalves collected in Norway, plasmid pKp1792_2 harboured the *sil, pco* and *ars* genes, the *clpK* and *hsp20* genes, as well as a type II TA system (RelE/ParE, phd/YefM). Plasmid pKp1792_2 also carried the *mrkABCDFJIH* fimbriae genes flanked by IS*110* transposases, that are also present on the chromosome of *K. pneumoniae* (1). BLASTn analysis showed that plasmid pKp1792_2 was similar (86 % sequence coverage and 99.9 % identity) to plasmid pK039_3 (CP034362) from a *K. pneumoniae* ST403 isolate (CP034359) from a carrier in Tanzania.

Identical plasmids could not be identified in the draft genomes of other isolates from our collection carrying the same combination of HMRGs as well as the IncFIB(K) replicon type, but lacking acquired ARGs (3). However, *K. pneumoniae* isolates ST337 2020-586 and ST337 2020-584/2 showed >90 % sequence coverage of plasmid pKp792_2 from *K. pneumoniae* ST4267 isolate 2019-1792, while *K. pneumoniae* isolates ST220 2016-729 and ST39 2019-400/1 had >80 % sequence coverage of plasmid pKp319 present in *K. pneumoniae* ST556 isolate 2016-319, indicating presence of similar plasmids in these isolates recovered from *M. edulis*.

Comparison of HMRG regions

The HMRG regions of the different plasmids all carried the *pco* and *sil* operon in similar regions but flanked by different transposases. Similar regions were also identified in previously published plasmid CP065035 from *M. edulis* and the $bla_{\text{CTX-M-15}}$ encoding plasmid pKp848CTX from *K. pneumoniae* ST17 (Kp848) causing an outbreak at Stavanger University Hospital in 2009 (16) (Figure 2). In pKp1200_1 and pKp319 *sil* and *pco* genes were located together with the *ars* genes as well as genes related to heat tolerance in a region flanked by IS*5* family transposases, similar to plasmid CP065035 (17). In plasmid pKp1792_2, the *sil, pco, ars, clpK* and *hsp20* genes were flanked by an IS*5* transposase, whereas plasmid pKp1198 carried the *sil* and *pco* operon and heat tolerance genes in a region containing several different transposases and *ars* and *mer* were clustered with ARGs in a separate region flanked by IS*26*.

Conjugation assay

Plasmid pKp319 from *K. pneumoniae* isolate 2016-319 was transferable to *E. coli* CV601-GFP strain, yielding transconjugants with identical resistance patterns (AMP^R, TET^R) at a transfer frequency of 5.1×10^{-4} transconjugants per recipient cell. Even though we predicted plasmid pKp1200_1 to be conjugative based on the genotype, we were not able to verify conjugative transfer of this plasmid to the *E. coli* recipient in repeated experiments, indicating either a very low transfer frequency or inability of pKp1200_1 to transfer to *E. coli*.

Discussion

In the present study, we report complete genome sequences of antibiotic-resistant *K. pneumoniae* isolated from bivalve molluscs collected along the Norwegian coast. We show

co-localisation of ARGs and HMRGs on IncF plasmids, suggesting potential for co-selection of ARGs by heavy metals in the marine environment.

Five of the identified plasmids carried genes encoding resistance to aminoglycosides, sulphonamides, cephalosporins, tetracycline, quinolones and/or amphenicols, all considered to be important for treatment of human infections by the WHO (18). All of the plasmids carrying ARGs belonged to the IncF group, the most frequently described plasmid type, and commonly found in bacteria of both human and animal origin (9). Previous studies have also shown that IncF plasmid replicons are common in Norwegian clinical *K. pneumoniae* isolates as well as in *K. pneumoniae* from healthy Norwegian carriers (19, 20). Furthermore, a study on antibiotic-resistant *E. coli* in marine sediments and clams collected in Italy found IncF type plasmids as the most common plasmid type carrying ARGs, while another study found IncF plasmids among CTX-M producing *E. coli* and *K. pneumoniae* isolated from marine bivalves in Brazil, consistent with our results for *K. pneumoniae* (21, 22). Plasmids belonging to this Inc group are often associated with ARGs and are recognised as important contributors to the spread of antibiotic resistance, especially quinolone resistance genes, ESBLs, carbapenemases and genes encoding resistance to aminoglycosides (9). This is in accordance with our results, where three of the five resistance plasmids carried genes involved in resistance to quinolones, aminoglycosides or β-lactam antibiotics, suggesting that such plasmids may be important in dissemination of ARGs also in the marine environment.

Only one plasmid, plasmid pKp319 encoding resistance to tetracycline and ampicillin, was transferred to *E. coli* recipient via conjugation. The presence of ARGs and HMRGs on a conjugative plasmid indicates the potential for dissemination of such plasmids in the marine environment. However, we were not able to show transmissibility of plasmid pKp1200_1, which carried multiple ARGs and genes related to conjugation, to the recipient used in this study. This is in accordance with a previous study showing inability of a CTX-M encoding IncFIB(K)/IncFII(K) plasmid (pKp848CTX), carrying a conserved transfer region, to transfer to an *E. coli* recipient in broth and filter mating experiments (16). Further experiments using recipients belonging to different species/genera, like *Klebsiella* spp., may be necessary to confirm that plasmid pKp1200_1 is not self-transferable. Additionally, pKp1200_1 carried genes encoding Klebicin B, a bacteriocin with nuclease activity, possibly reducing the number of recipient cells (23, 24).

Even though the plasmids reported in this study carry clinically relevant ARGs, one of the important findings of our study is the co-localisation of ARGs and HMRGs on the same plasmids in *K. pneumoniae* isolated from marine bivalves. HMRGs are frequently reported in clinical *K. pneumoniae* isolates as well as isolates from aquatic and marine environments, including marine seafood organisms (3, 25, 26). Interestingly, most plasmids included in the present study carried similar regions harbouring the *sil* and *pco* operon, and also genes encoding heat tolerance (*hsp20, clpK*), indicating that this region could be common in *K. pneumoniae* plasmids belonging to the IncFIB group. Furthermore, in plasmids pKp319, pKp1200_1, CP065035 and pKp848CTX the *sil* and *pco* operons were flanked by *IS*5 and truncated *IS*L3 transposases, possibly indicating that this composite transposon is important in dissemination of heavy metal resistance, also in clinical settings (16, 26). Overall, HMRGs were associated with different types of transposases in all plasmids, including members of the IS*5* and IS*26* families, indicating potential for mobilisation of these genes (27).

Norway has a low prevalence of antibiotic resistance, and the use of antibiotics in both human and veterinary medicine, including food producing animals, is low (28). However, heavy metals, such as copper, are used in the aquaculture industry, both in antifouling agents and as additives in fish feed (29, 30). As a result, copper can contaminate the marine environment through faecal material, spilled feed and leakage from metal impregnated fish farm nets (29). Copper is also naturally occurring in marine sediments and seawater (29). Additionally, metal compounds, including arsenic, are used in livestock feed and in agriculture as pesticides, fertilisers and antimicrobials (30-32), and can therefore be spread to the marine environment through run-off from agricultural land. It has been suggested that *pcoA* and *pcoB* alone are able to confer copper resistance, however, *pcoC*, *pcoD* and *pcoE* are required for full copper resistance (33). The plasmids detected in our study, harboured *pcoABCDE*, *silABCEFPRS* and arsenic resistance genes. Previously, Gullberg et al. have shown that low concentrations of copper and especially arsenic were sufficient to maintain the multi-drug resistance pUUH239.2 plasmid (NC_016966), carrying *ars* and *pco* genes, from a *K. pneumoniae* strain responsible for a nosocomial outbreak in Sweden (34). Thus, our results indicate the potential for co-selection of ARGs in *K. pneumoniae* in metal contaminated marine environments.

Furthermore, all plasmids encoding both ARGs and HMRGs characterised in the present study carried type II TA systems, responsible for the killing, or growth-inhibition, of plasmid-free progeny cells (14). These TA systems thus, ensure that the plasmids are maintained and

disseminated in bacterial populations even in environments without selection pressure imposed by antibiotics and/or heavy metals (35). These plasmids can potentially persist in such environments and be transferred to human microbiota through *e.g.* seafood or direct contact via recreational activities.

Conclusion

In the present study, we report complete genome sequences of antibiotic-resistant *K. pneumoniae* isolated from marine bivalve molluscs collected along the Norwegian coast. We show co-localisation of ARGs and HMRGs on IncFIB plasmids present in *K. pneumoniae* isolated from the marine bivalves. We further show that one of the plasmids carrying ARGs and HMRGs is transferrable to *E. coli* via conjugation. Our study therefore shows the potential for co-selection of ARGs and/or antibiotic-resistant *K. pneumoniae* in the marine environment by heavy metals. It also demonstrates the importance of the marine environment and seafood as dissemination routes for ARGs and pathogens and highlights the needs for surveillance of antibiotic resistance in the marine environment.

Materials and methods

Bacterial isolates

Five *K. pneumoniae* sensu stricto isolates with acquired ARGs recovered from marine bivalves were selected for complete genome sequencing (3). Four isolates were recovered from blue mussels (*M. edulis*) and one from oysters (*C. gigas*).

Whole genome sequencing, hybrid *de novo* assembly and bioinformatic analyses

The short-read sequencing was performed as described previously (3). For the long-read sequencing, DNA was extracted manually using the Beckman Coulter Life science GenFind V3 with the protocol: "DNA extraction from Bacteria using GenFindV3" (Beckman Coulter, USA). Library preparation was done with the SQK-LSK-109 kit (Oxford Nanopore Technologies, UK), DNA libraries were loaded onto a MINion flow cell (R9.4.1) and sequencing was done using the Oxford Nanopore Technologies GridION device. Basecalling was performed with Guppy v4.2.2 + effbaf84 (https://community.nanoporetech.com) and quality filtering using FiltLong v0.2.0 (https://github.com/rrwick/Filtlong).

Hybrid *de novo* assembly of the short- and long-read sequences was done with Unicycler v0.4.8 (36). The assemblies were analysed with AMRFinder plus v3.9.8 (37) and

PlasmidFinder v2.1 (database version 2021-11-29) (38). The assembled genomes were annotated through the NCBI prokaryotic genome annotation pipeline v5.3 (39). Both the assembled and annotated genomes were screened for virulence factors using the VFanalyzer available through the Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi) (40). BLASTN v2.13.0+ (41) was used to compare the plasmid sequences to previously described plasmids. Circular plasmid maps were created using the Proksee server (https://proksee.ca) and alignments of plasmid regions carrying HMRGs were generated with Easyfig v2.2.5 (42) using a minimum sequence identity of 80 %.

Previously published Illumina reads from isolates carrying the same HMRGs (*sil*, *pco* and *ars* (n=13), or *sil*, *pco*, *ars* and *mer* (n=2)) and IncFIB plasmid replicons were mapped against the complete genome sequences generated for the present study using the RedDog pipeline (https://github.com/katholt/RedDog) (3). Single nucleotide polymorphism (SNP) matrices were generated with SNP-dists v0.7.0 (https://github.com/tseemann/snp-dists). The criteria proposed by Hawkey et al. (43) (> 80 % mapping cover and < 10 SNPs) were used to determine presence of the closed plasmids in draft genomes. Mapping statistics and SNP matrices are available in Table S1 and S2, respectively.

Conjugation experiments

K. pneumoniae isolate 2016-319 and 2016-1200 carried resistance plasmids encoding multiple genes involved in conjugal transfer and were subjected to conjugation experiments by filter-mating following a previously described method (44). A kanamycin (KAN) and rifampicin (RIF) resistant *gfp* marked *E. coli* CV601-GFP strain was used as recipient. The recipient strain was grown over night in Muller Hinton broth (MHB) (Oxoid, UK) supplemented with 50 µg/ml KAN (Glentham Life Sciences, UK) at 30 ºC with shaking (200 rpm), while the donors were grown in MHB supplemented with 100 µg/ml ampicillin (AMP) (Sigma-Aldrich, USA) in the same conditions. Donor and recipient mixtures were washed twice in phosphate buffered saline (PBS) (Sigma-Aldrich, USA) and mixed at a ratio of 1:1 before filtering through 0.45 µm pore filters and placing the filter on Mueller Hinton plates (Oxoid, UK). The plates were incubated aerobically at 30 ºC for 3 hrs. and the mating was disrupted by vortexing the filters in tubes with 10 ml PBS and sterile glass beads. Serial dilutions were prepared in PBS and 100 µl of the 10^{-2} and 10^{-3} dilutions were spread on CHROMagar Orientation plates (CHROMagar, France) supplemented with KAN (50 µg/ml), RIF (50 µg/ml) (Glentham Life Sciences, UK), and AMP (100 µg/ml) and incubated at 35 ºC

for ~36 hrs. The antibiotic sensitivity patterns of transconjugants were examined by disk diffusion following the EUCAST method (45). Transfer frequencies were calculated using the total number of recipients before mating.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Data availability

Genome assemblies and annotations are available in GenBank (https://www.ncbi.nlm.nih.gov/genbank) under BioProject PRJNA769247. GenBank accession numbers for the individual genomes and plasmid are presented in Table 1.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Fredrik Håkonsholm, Nachiket P. Marathe, Iren H. Löhr and Bjørn Tore Lunestad contributed to the design and conception of the study. Fredrik Håkonsholm performed the experiments, bioinformatic analyses were done by Fredrik Håkonsholm and Marit A.K. Hetland. Fredrik Håkonsholm 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.

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Figure 1. Genomic maps of the resistance plasmids from *K. pneumoniae* isolated from the marine environment. A; pKp319, B; pKp1198, C; pKp1200_1, D; pKp1764, E; pKp1792_2. Genes are coloured according to function: Red; antibiotic resistance genes (ARGs), orange; Heavy metal resistance genes (HMRGs), green; Transposases, blue; Heat tolerance, purple; Conjugation, pink; Integrons, black; Virulence. GC skew is indicated in green and purple. Truncated genes are indicated with Δ. ARGs, HMRGs and the transposases associated with them are labelled.

Figure 2. Alignment of plasmid regions carrying heavy metal resistance genes. A; position 4 917 to 57 627 bp of plasmid pKp319 (CP085102), B; position 3 190 – 47 137 bp of plasmid pKp1200_1 (CP085034), C; position 4 918 – 53 708 bp of plasmid CP065035, D; position 39 604 – 90 648 bp of plasmid pKp848CTX (NC_024992), E; position 14 836 – 54 741 bp of plasmid pKp1792 (CP085105), F; position 9 363 – 38 453 of plasmid pKp1198 (CP085098). The *Sil* operon is highlighted in yellow, the *pco* operon is coloured blue, *ars* genes are coloured red and insertion sequences and transposases are highlighted in cyan. Other genes present in the region are coloured grey.

Figure S1. Complete genomes of *Klebsiella pneumoniae* isolated from marine bivalves. A; isolate 2016-319, B; isolate 2016-1198, C; isolate 2016-1200, D; isolate 2019-1764, E; isolate 2019-1792.

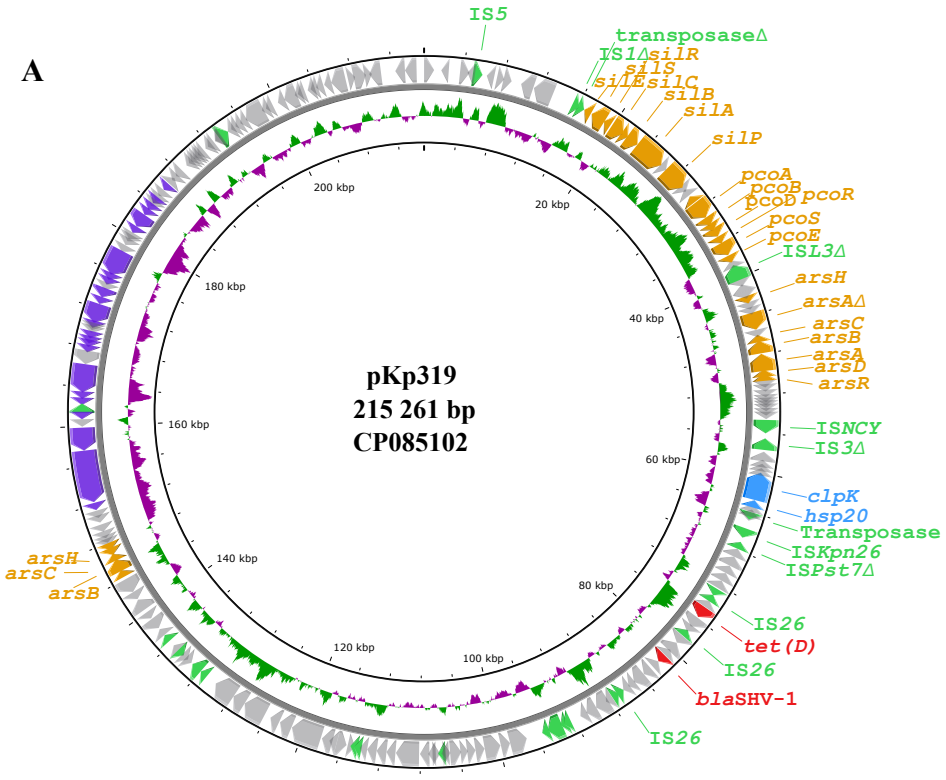
Table 1. Complete genome sequences of *Klebsiella pneumoniae* isolates included in the study, acquired antibiotic resistance genes (ARGs), heavy metal resistance genes (HMRGs), acquired virulence genes and plasmid replicons.

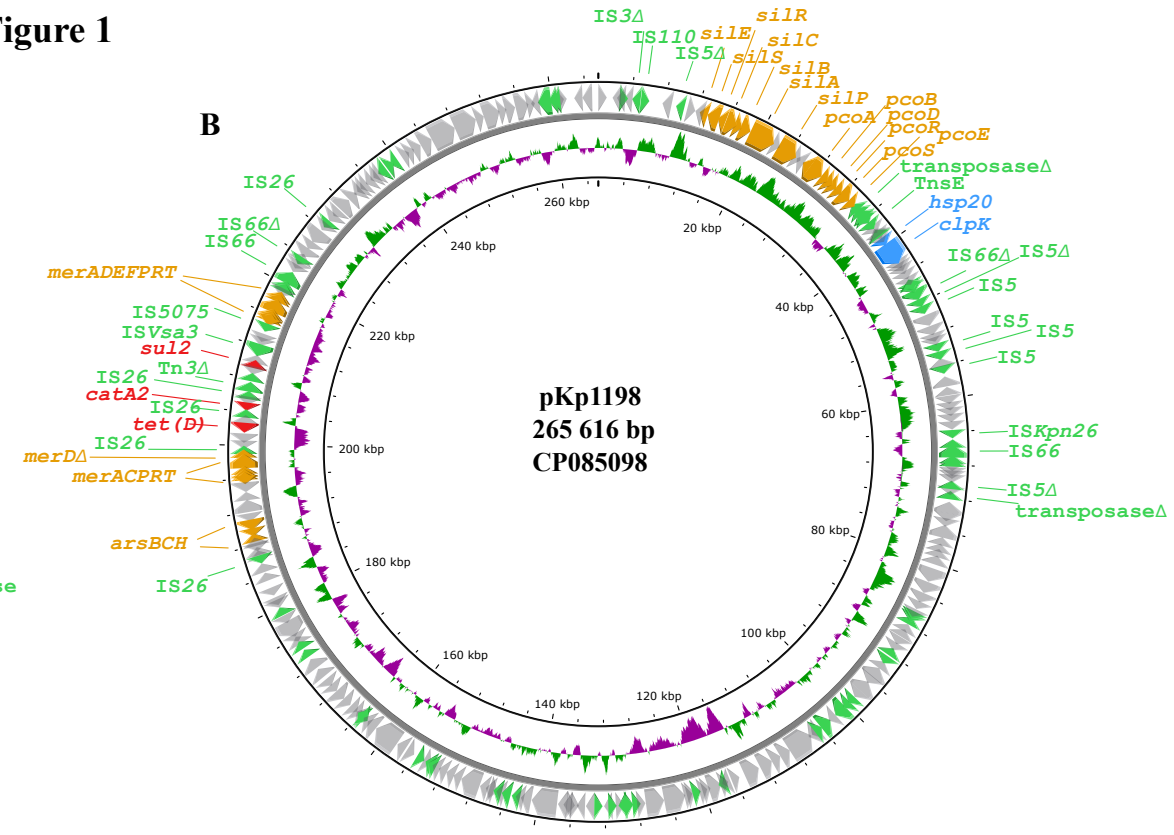
Isolate	Sequence type	Contig	Size (bp)	Accession no.	ARGs	HMRGs	Virulence	Plasmid replicons
2016-319	ST556	Chromosome	5 273 997	CP085101	-	-	-	-
		pKp319	215 261	CP085102	*tet(D)*, *bla*$_{SHV-1}$	*silABCEFPRS*, *pcoABCDERS*, *arsABCDHR*[a]	-	IncFIB(K)
2016-1198	ST2167	Chromosome	5 245 077	CP085097	-	-	-	-
		pKp1198	265 616	CP085098	*tet(D)*, *catA2*, *sul2*	*silABCEFPRS*, *pcoABCDERS*, *arsBCH*, *merACDEFPRT*[b]	-	IncFIB(K)(pCAV1099-114), IncHI1B(pNDM-MAR)
2016-1200	ST25	Chromosome	5 312 007	CP085033	-	-	*ybt*	-
		pKp1200_1	187 807	CP085034	*sul1*, *aph(3')-Ia*, *dfrA14*, *tet(D)*, *bla*$_{TEM-1}$, *aph(6)-Id*, *aph(3'')-Ib* *sul2*	*silABCEFPRS*, *pcoABCDERS*, *arsABCDHR*	-	IncFIB(K), IncFII(K)
		pKp1200_2	2 667	CP085035	-	-	-	-
2019-1764	ST292	Chromosome	5 197 806	CP085099	-	-	-	-
		pKp1764	137 603	CP085100	*dfrA12*, *aadA1*, *aadA2*, *cmlA1*, *bla*$_{TEM-1}$, *qnrS1*, *sul3*	-	-	IncFIB(pKPHS1)
2019-1792	ST4267	Chromosome	5 303 093	CP085103	-	-	-	-
		pKp1792_1	151 942	CP085104	-	-	-	IncFII(K)
		pKp1792_2	122 654	CP085105	*tet(A)*	*silABCEFPRS*, *pcoABCDERS*, *arsABCDR*	*mrkABCDFJIH*	IncFIB(K)

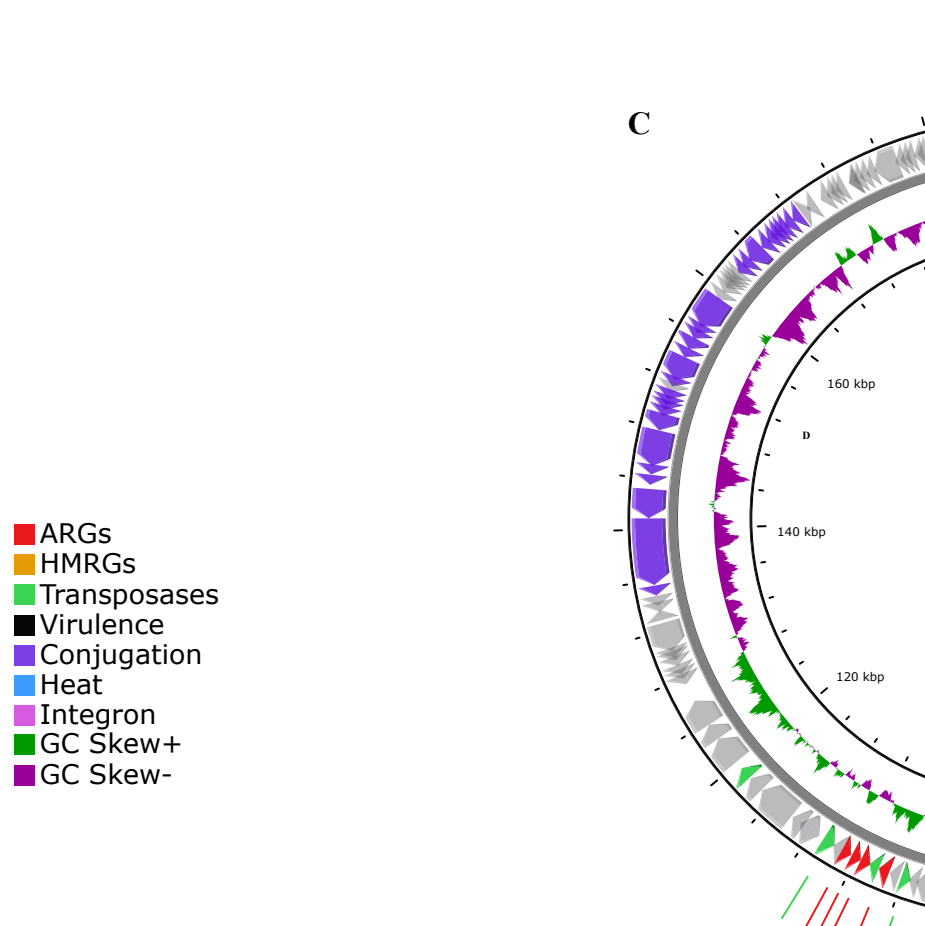
[a] pKp319 carried two copies of *arsH*, *arsC*, *arsB* and one truncated copy of *arsA*

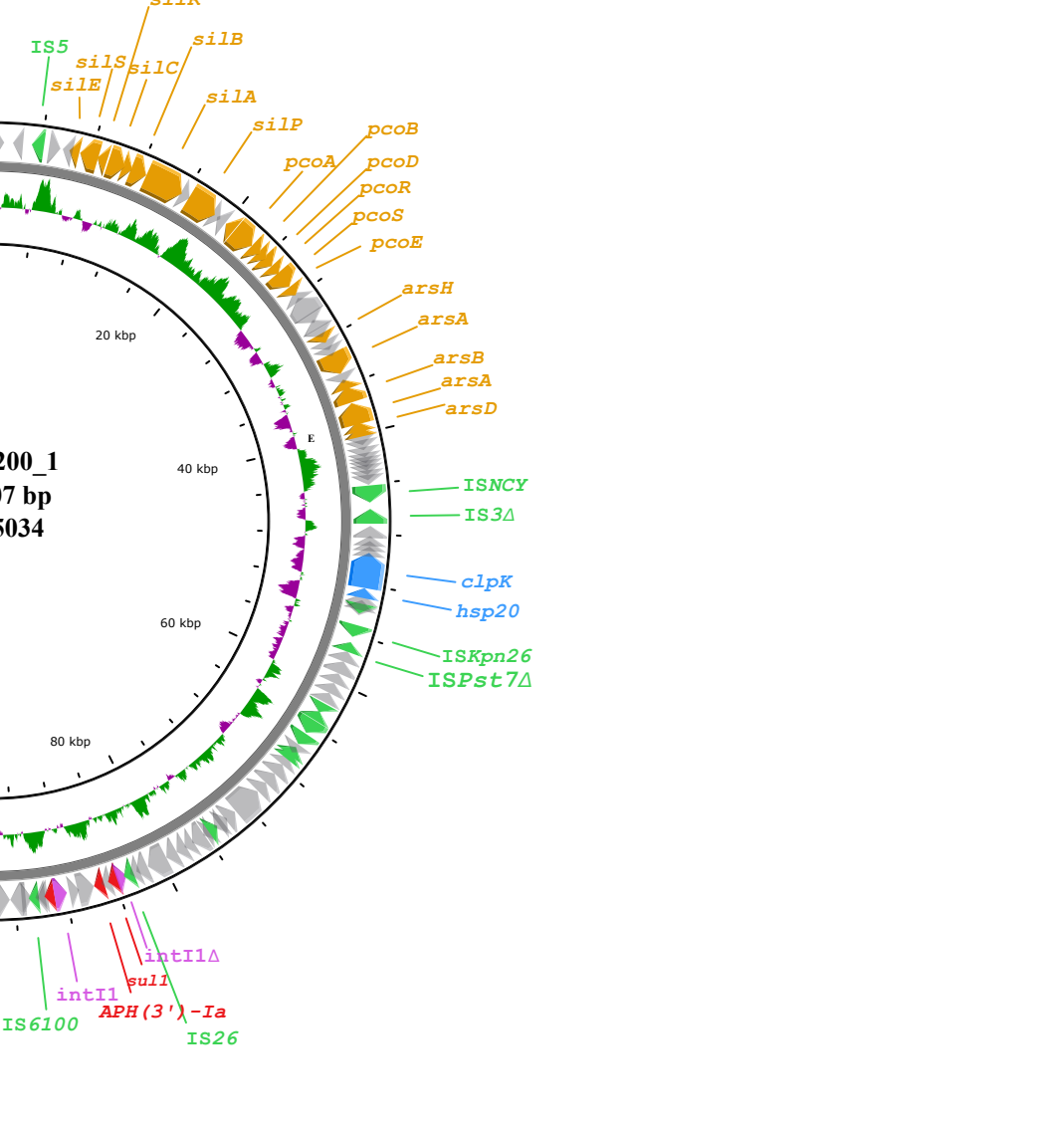
[b] pKp1198 carried two copies of *merT*, *merP*, *merR* and one truncated copy of *merD*

Figure 1









ARGs
HMRGs
Transposases
Virulence
Conjugation
Heat
Integron
GC Skew+
GC Skew-

Figure 2

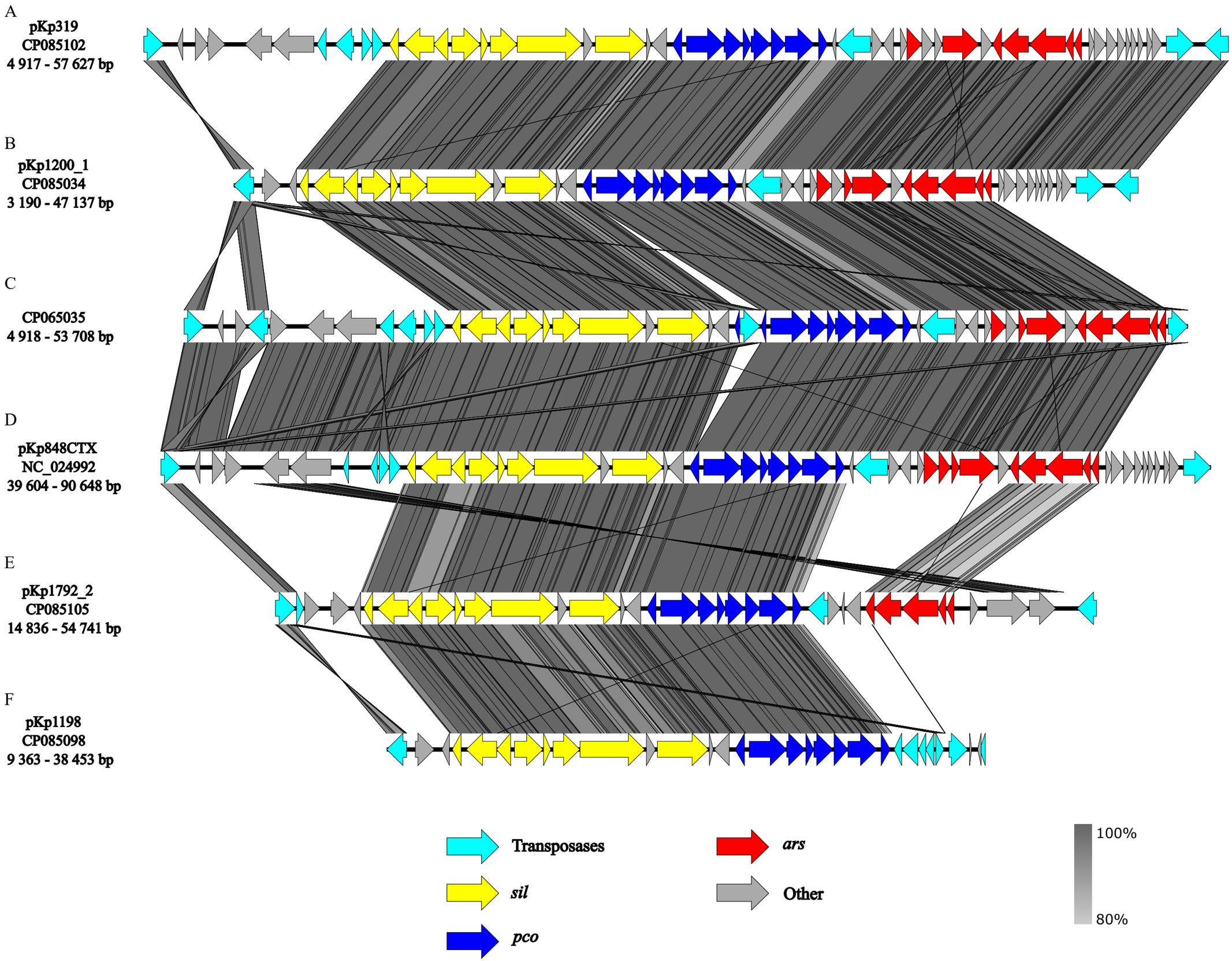

A
pKp319
CP085102
4 917 - 57 627 bp
B
pKp1200_1
CP085034
3 190 - 47 137 bp
C
CP065035
4 918 - 53 708 bp
D
pKp848CTX
NC_024992
39 604 - 90 648 bp
E
pKp1792_2
CP085105
14 836 - 54 741 bp
F
pKp1198
CP085098
9 363 - 38 453 bp
Transposases
ars
sil
Other
pco
100%
80%

Figure S1

A)
2016-319
Chromosome
5 273 997 bp
pKp319
215 261 bp
B)
2016-1198
Chromosome
5 245 077 bp
pKp1198
265 616 bp
C)
2016-1200
Chromosome
5 312 007 bp
pKp1200_1
187 807 bp
pKp1200_2
2 667 bp
D)
2019-1764
Chromosome
5 197 806 bp
pKp1764
137 603 bp
E)
2019-1792
Chromosome
5 303 093 bp
pKp1792_1
151 942 bp
pKp1792_2
122 654 bp
CDS
tRNA
rRNA
misc_feature
ncRNA
regulatory
repeat_region
tmRNA