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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.

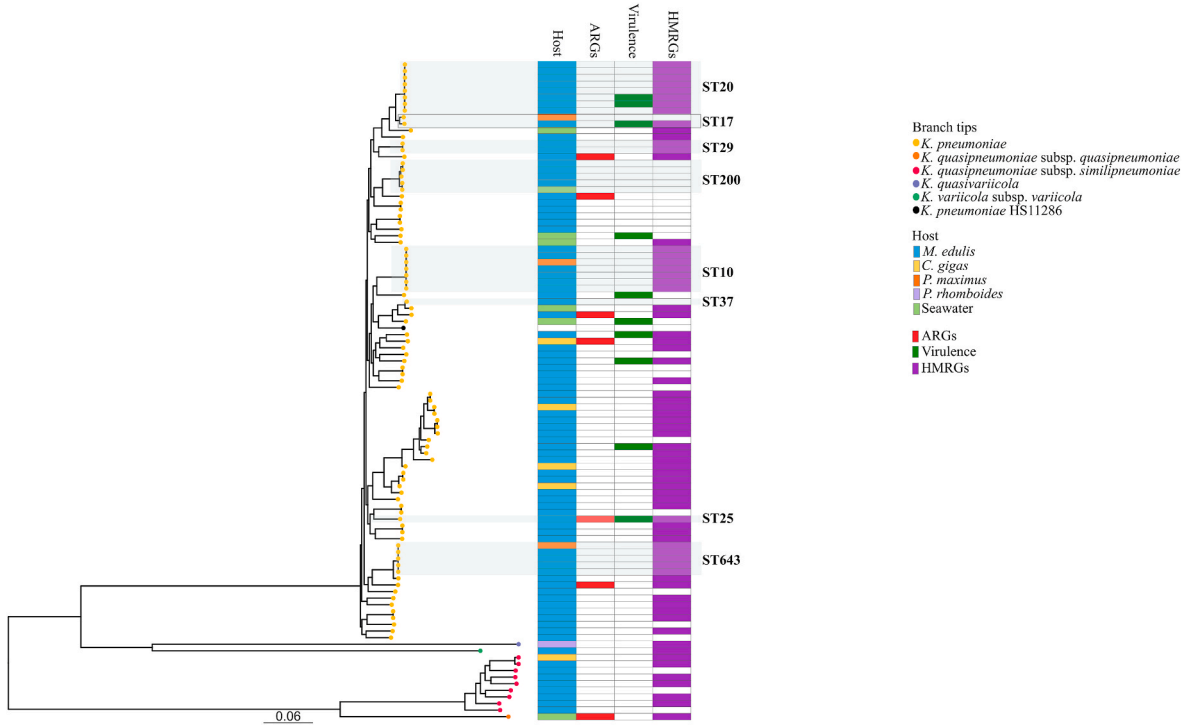

Fig. 1. Midpoint-rooted core genome phylogeny of 87 *Klebsiella pneumoniae,* nine *K. quasipneumoniae* subsp. *similipenumoniae*, one *K. quasipneumoniae* subsp. *quasipneumoniae*, one *K. quasivariicola* and one *K. variicola* subsp. *variicola* isolated from the marine environment. In total, 218 281 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

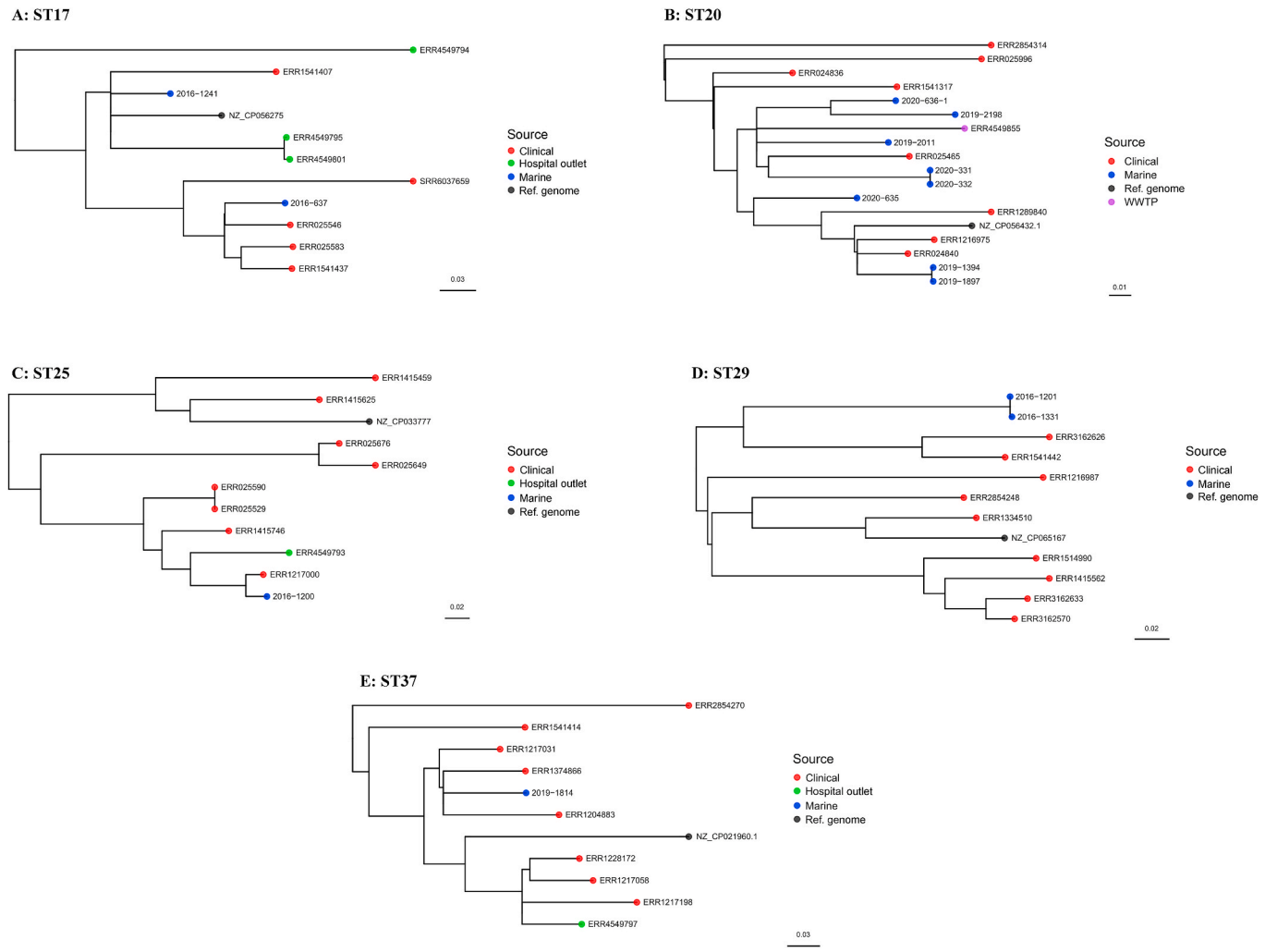

Fig. 2. Comparison of globally disseminated *Klebsiella pneumoniae* sequence types (STs) isolated from the marine environment and isolates of the same STs of human origin. A: ST17, B: ST20, C: ST25, D: ST29, E: ST37.

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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