Next-generation sequencing to investigate antimicrobial resistance

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Chapter 3

Surveillance and Genomic Characterization of Colistin-Resistant Gram-Negative Bacteria in Drains of Hospital High-Risk Units

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Abstract

The healthcare water environment, including sinks and drainage systems, can be a long-term reservoir of nosocomial pathogens. In this study, we aimed to investigate the presence of colistin-resistant Gram-negative (ColR-GN) bacteria in humid compartments of high-risk hospital units in the University Medical Center Groningen (UMCG). We found ColR-GN in all investigated units with *Enterobacter* spp. being the most abundant genus. Twelve isolates showed a colistin resistance phenotype (>2 µg/mL): *Enterobacter cloacae* complex (n=11) and *Klebsiella pneumoniae* (n=1). Chromosomal mutations in genes involved in lipopolysaccharide structure modifications, were the main resistance mechanisms contributing to colistin resistance in *Enterobacter* spp. and *Klebsiella* spp. (91.6%, 11/12). Only two *Enterobacter kobei* isolates carried mobile colistin resistance determinants, both the *mcr-4.3* and *mcr-9.1* genes. The presence and persistence of ColR-GN bacterial clones in the sink and shower drains of high-risk hospital units highlights the importance of monitoring such environment for antimicrobial resistant bacteria to identify reservoirs and prevent their further spread.

Introduction

Healthcare environmental contamination is responsible for up to 25% of healthcare-associated infections (HAIs) and an additional 20-40% of cases can be related to colonized healthcare workers[1]. Many studies have demonstrated cross-contamination of clinically relevant bacteria between the hospital environment, healthcare workers' hands and patients[2,3,4]. Hospital sink and shower drains and their surroundings can act as reservoirs of multidrug-resistant Gram-negative bacteria, contributing to HAIs[5,6]. Hand-washing frequency and other hygiene practices in sinks in intensive care units (ICUs) directly affect the ward environment, especially around the sink area, and sink usage impacts bacterial contamination[7].

Colistin is one of the last-resort antibiotics available to treat life-threatening infections caused by multidrug-resistant Gram-negative bacteria, especially carbapenem-resistant ones. Increasing use of colistin has led to resistance to this antibiotic, which is associated with colistin-resistant *Escherichia coli* and *Klebsiella pneumoniae* colonization and infection in hospitalized patients, and with increased in-hospital mortality[8,9]. Intrinsic/natural colistin resistance has been reported in species such as *Proteus mirabilis* and *Serratia marcescens* by modification of the lipopolysaccharides (LPS) structure. Similarly, acquired colistin resistance due to chromosomal mutations in many genes and operons involved in LPS modifications has been described in *Enterobacterales*, including mutations in *pmrA/pmrB, phoP/phoQ* two-component systems *and mgrB* gene[10]. However, since 2015, a new worrying plasmid-mediated colistin resistance mechanism has been reported worldwide and to date, up to ten *mcr* gene variants have been described. These genes can be acquired by horizontal gene transfer and rapidly disseminate to other pathogens, especially in an environment with high antibiotic selective pressure[11].

While most of the data available regarding colistin-resistant Gram-negative (ColR-GN) in the hospital is related to wastewater and environmental water-bodies surrounding the hospital, there is a lack of knowledge about ColR-GN in tap water

and humid-compartments from the hospital built-environment. Our study aimed i) to investigate the presence of ColR-GN bacteria in the humid compartments of high-risk hospital units, ii) to characterize their phenotypic and molecular resistance profiles, and iii) to investigate the persistence in time of colistin resistant bacterial clones in the hospital humid environment. In addition, to investigate whether environmental isolates were responsible for HAIs or colonization, we retrospectively reviewed hospital records searching for clinical colistin-resistant isolates from 2019 and 2020.

Materials and Methods

Sampling strategy

We collected samples once a month from different humid compartments during five consecutive months, from April until August 2019, using the SRK kit containing FLOQSwab (Copan, Brescia, Italy). We sampled sink-drains (n=9) from three different ICUs: adult, paediatric and neonatal; and sink-drains (n=5) and shower-drains (n=2) of a haematology unit (Table 1), where we selected three rooms: a single bedroom, a three beds room, and a "dirty utility room" (a room where cleaning and waste disposal procedures take place).

Colistin-resistant Gram-negative bacteria identification

Liquid containing swabs were cultured (100µl) onto selective agar-media CHROMID Colistin R (bioMérieux, Inc., Hazelwood, MO). Colonies were isolated and identified using MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry) (Bruker, Daltonik Gmbh, Bremen, Germany). We determined colistin minimum inhibitory concentrations (MIC) using broth microdilution (BMD), following European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations[12] (colistin MIC ≤2µg/ml, susceptible; colistin MIC >2 µg/ml, resistant). We included *E. coli* NCTC 13846 as a positive control. In addition, we

performed microdilution to determine the complete antibiotic resistance profile using the Vitek-2 system (AST-N344 cards) (bioMérieux, Inc., Hazelwood, MO). Meropenem disk-diffusion test and KPC&MBL&OXA-48 disc kit (Liofilchem, Italy) were used to confirm carbapenem susceptibility and carbapenemase production, respectively. We interpreted breakpoints according to EUCAST guidelines v9.0.

DNA extraction and whole-genome sequencing

DNA extraction of phenotypically confirmed colistin-resistant isolates was performed using the DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The concentration and purity of extracted DNA were determined using Qubit® 2.0 fluorometer and the dsDNA BR Assay Kit (ThermoFisher Scientific, Waltham, MA) and NanoDrop 2000C spectrophotometer (TYP-I0001 Nanodrop), respectively. Libraries were prepared using the Nextera XT Kit (Illumina, San Diego, CA, USA) and sequenced with a MiSeq Reagent Kit v2 500-cycles Paired-End in a MiSeq instrument (Illumina).

Data analysis

Raw reads were quality-trimmed and *de novo* assembled into contigs using Shovill v1.1.0 (https://github.com/tseemann/shovill). We evaluated the assemblies' quality by Quast v5.0.2[13], results are provided in the Supplementary Material (Datasheet 1). Draft genomes were screened for contamination using MASH v2.2.2 and annotated using Prokka v1.14.5[14].

We additionally analyzed *Enterobacter cloacae* complex assembled genomes by average nucleotide identity (OrthoANI) using the following reference genomes: *E. asburiae* ATCC 35953 (NZ_CP011863.1), *E. ludwigii* P101 (NZ_CP006580.1), *E. hormaechei* 20710 (NZ_CP030076.1), *E. kobei* (NC_018405.1), *E. cloacae* subsp. *cloacae* ATCC 13047 (NC_014121.1) and *E. bugandensis* EB-247 (NZ_LT992502.1). *Klebsiella pneumoniae* genome assemblies were analyzed using Kleborate v0.4.0[15]. We investigated antibiotic resistance, virulence, and plasmid replicon type genes in all

assembled genomes using ABRicate v1.0.1 (https://github.com/tseemann/abricate) and ResFinder, VFDB and PlasmidFinder databases (30-Mar-2020), respectively (coverage threshold ≥ 80%, identity threshold ≥ 70%).

We examined plasmid content using RFPlasmid[16], MOB-suite v3.0.0 (https://github.com/phac-nml/mob-suite) and PlasmidID (https://github.com/BU-ISCIII/plasmidID). RFPlasmid uses a Random Forest machine-learning model to predict the likely origin, plasmid or chromosomal, of contigs. MOB-suite is a set of modular tools for plasmid typing (replicon and relaxase) and plasmid reconstruction from draft assemblies. PlasmidID is a read mapping-based tool, which can also use assembly data, for plasmid identification and reconstruction (database of 36,762 plasmid reference sequences). This program provides a list of matching plasmid reference sequences and circular images for each plasmid reference sequence against the query genome. Several criteria are used to select the best match: plasmid features, i.e. Inc groups or antibiotic resistant genes presence, mapping percentage (MAPPING %, percentage of reference covered with reads) and fraction covered (ALIGN FR, total length of contigs aligned (complete) / reference sequence length) (https://github.com/BU-ISCIII/plasmidID/wiki/How-to-chose-the-right-plasmids).

The genetic backgrounds of *mcr*-9.1 were visualized using EasyFig v2.2.2[17].

In addition, we investigated chromosomal point mutations and gene alterations linked to colistin resistance in MgrB, PmrA-PmrB, and PhoP-PhoQ protein sequences using the following colistin-susceptible references genomes as wild-types: *E. roggenkampii* strain WCHER090065[18] (GenBank: GCA_009184765.2), *E. kobei* SECR18-0236[19] (GenBank: GCA_013390685.2) and *K. pneumoniae* KP51[20] (GenBank: GCA_022009035.1), and the tool Artemis v18.1.0[21].

Core-genome SNP analysis of *E. kobei* and *E. roggenkampii* isolates was performed by Snippy v4.6.0 (https://github.com/tseemann/snippy), using the reference genomes of *E. kobei* IB2020 (RefSeq assembly accession number: GCF_014041955.1) and *E. roggenkampii* MGH34 (accession number: NZ_KIS35616.1), respectively. "Core SNP genome" refers to only single nucleotide polymorphisms, ignoring other variant types such as insertions and deletions. We used the resulted whole genome SNP alignment (which includes invariant sites) as input for MEGA X v10.2.4[22] to generate a maximum-

likelihood phylogenetic tree using General Time Reversible (GTR) as nucleotide substitution model. A pairwise SNP distance matrix was created from the SNP alignment using the snp-dist script (https://github.com/tseemann/snp-dists). The phylogenetic tree and associated metadata were visualized using iTOL v5.6.3[23].

Comparison with retrospective clinical isolates

All patients hospitalized in the sampled units are screened for resistant Gram-negative bacteria by the infection prevention team every week. The results help to investigate possible transmission events from the hospital-wet environment to the patient population and *vice versa*. According to this, we searched for clinical ColR-GN isolates in the hospital records from January 2019 until October 2020 and found fifty-four colistin-resistant *E. cloacae* complex and three colistin-resistant *K. pneumoniae* isolates. Whole-genome sequencing (WGS) data of one strain, *E. roggenkampii* ST165 (named ECC1), was included in this study for comparison purposes.

Results

Bacteria identification and antimicrobial susceptibility testing

We confirmed a colistin-resistant phenotype (>2 μg/mL) in twelve environmental isolates: *Enterobacter cloacae* complex (n=11, MICs= 16-64mg/L) and *Klebsiella pneumoniae* (n=1, MIC= 64 mg/L) (Table 1). Based on WGS data, environmental isolates belonging to the *Enterobacter cloacae* complex were identified as *E. kobei* (n=2) with >98.90% ANI identity (NCBI Reference Sequence: NC_018405.1) and *E. roggenkampii* (n=9) with >98.50% ANI identity (NCBI Reference Sequence: NZ_CP058637.1). The four clinical *Enterobacter cloacae* complex isolates were identified as *E. roggenkampii* (ECC1), *E. bugandensis* (ECC2), *E. cloacae* (ECC3) and *E. hormaechei* (ECC4).

We observed high colistin resistance levels (MIC >32 mg/L) in 33.3% (4/12) of isolates belonging to *E. roggenkampii*, *E kobei* and *K. pneumoniae* species. All *Enterobacter* spp. and *Klebsiella* spp. had a multi-drug-resistant profile, defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. In particular, *E. roggenkampii* ero3 (colistin MIC= 32 mg/L), ero1 (colistin MIC= 16 mg/L) and ero4 (colistin MIC= 32 mg/L) were resistant for 14 out of 16 antibiotics tested, including ampicillin (AMP), amoxicillin/clavulanic acid (AMC), piperacillin/tazobactam (TZP), cefuroxime (CXM), cefoxitin (FOX), cefotaxime (CTX), ceftazidime (CAZ), gentamicin (GEN), tobramycin (TOB), ciprofloxacin (CIP), fosfomycin (FOS), nitrofurantoin (NIT), trimethoprim (TMP), trimethoprim/sulfamethoxazole (SXT). All strains were susceptible to carbapenems, except for *Enterobacter cloacae* complex isolates belonging to ST54 (eko1 and eko2) and ST486 (ero8), which showed intermediate susceptibility to imipenem. However, these three isolates were carbapenem-susceptible when using meropenem disk-diffusion test and KPC&MBL&OXA-48 disc kit.

Table 1. Antibiotic susceptibility profile of colistin-resistant Gram-negative bacteria isolated, between April and August 2019, from the sink- and shower-drains of high-risk hospital units.

Hospital Unit	Date of isolation	Site of isolation	Isolate	Species ID	ST	Colistin MIC (mg/L)	AMP	AMC	TZP	CXM	FOX	CTX	CAZ	IMP	MEM	GEN	TOB	CIP	FOS	NIT	TMP	SXT
Haematology (multiple room)	April	sink1	ero1	*E. roggenkampii*	165	16	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
	May	sink1	ero2	*E. roggenkampii*	165	32	R	R	R	R	R	R	R	S	S	S	S	S	R	R	S	S
	June	sink1	ero3	*E. roggenkampii*	165	32	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
	July	sink1	ero4	*E. roggenkampii*	165	32	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
	August	sink3	kpn1	*K. pneumoniae*	12	64	R	R	R	S	S	S	S	S	S	R	R	S	S	R	R	R
Adult ICU	April	sink2	ero5	*E. roggenkampii*	595	64	R	R	R	R	R	R	R	S	S	R	R	S	R	R	R	R
Neonatal ICU	April	sink1	ero8	*E. roggenkampii*	486	16	R	R	R	R	R	R	R	I	S	S	S	S	S	R	S	S
	July	sink1	ero6	*E. roggenkampii*	486	32	R	R	R	R	R	R	R	S	S	S	S	S	S	R	S	S
	August	sink1	ero7	*E. roggenkampii*	486	32	R	R	R	R	R	R	R	S	S	S	S	S	S	R	S	S
Pediatric ICU	June	sink3	eko1	*E. kobei*	54	64	R	R	R	R	R	S	S	I	S	S	S	S	R	R	S	S
	July	sink2	ero9	*E. roggenkampii*	965	32	R	R	R	R	R	R	R	S	S	R	R	S	R	R	S	S
	August	sink3	eko2	*E. kobei*	54	64	R	R	R	R	R	S	S	I	S	R	R	R	R	R	S	S

Abbreviations: S, susceptible; I, intermediate; R, resistant; ST, sequence-type. AMP, ampicillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam, CXM, cefuroxime; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; IMP, imipenem; MEM, meropenem; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; FOS, fosfomycin; NIT, nitrofurantoin; TMP, trimethoprim; SXT, trimethoprim/sulfamethoxazole.

Molecular mechanisms of colistin resistance isolates

Mobile colistin resistance. Two *E. kobei* isolates (eko1 and eko2) had both mobile colistin resistance *mcr-4.3* (100% coverage, 100% identity) and *mcr-9.1* (100% coverage, 100% identity) genes. Comparative analysis of eko2 *mcr-9.1* carrying contig and reference plasmid pN1566-2 (best hit using Blastn search, GenBank: CP048299.1; 100% identity and 100% coverage) showed a match region of 7,794 bp (100% identities). This region consisted of the following putative gene products: IS5 family transposase (IS*903*), lipid A phosphoethanolamine transferase (*mcr-9.1*), hypothetical protein of the cupin superfamily (*wbuC*), QseB/C two-component system (*qseB/C*), ATPase, and IS*481* family transposase (Figure 1). Downstream the transposon region, we observed the following chromosomal putative gene products: adaptive-response sensory-kinase SasA (*sasA*), copper-binding protein PcoE (*pcoE*), hypothetical protein (hp) and transcriptional repressor RcnR (*rcnR*). Comparative analysis of eko1 *mcr-9.1* carrying contig with the same reference plasmid, we observed a match region of 6,819 bp with 100% identity, IS5 element was missing in this contig. RFPlasmid and MOB-suite tools predicted these two *mcr-9.1* carrying contigs as chromosomal (RFPlasmid votes chromosomal contig90 eko1= 0.963/1 and contig13 eko2= 0.996\1; MOB-suite: chromosomal) (Supplementary Material, datasheet 2).

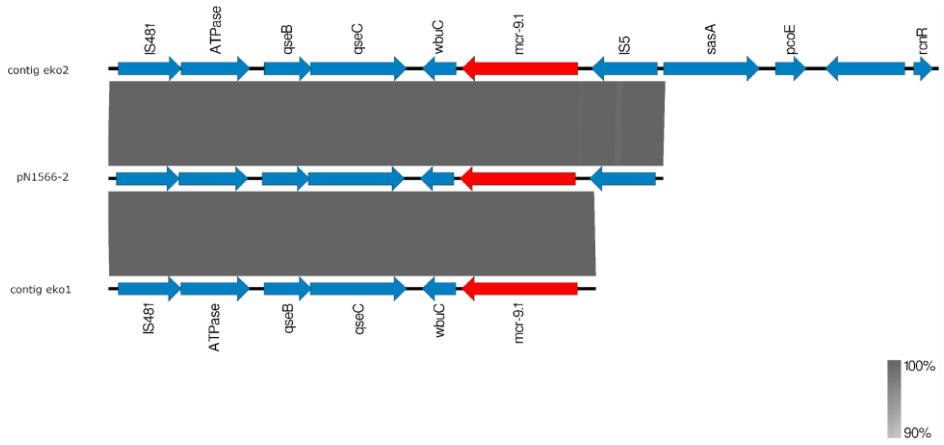

Figure 1. Comparison of the genetic environment of the *mcr-9.1* gene between the reference plasmid pN1566 (CP048299.1) and the annotated genomes of *E. kobei* eko1 (BioSample:

SAMN20867095) and eko2 (BioSample: SAMN20867096) isolates (figure created using EasyFig v2.2.2).

We observed the *mcr-4.3* gene in a contig of 7,764 bp in both isolates (100 % sequence identity). RFPlasmid predicted these two *mcr-4.3* carrying contigs as plasmid-borne (contig88-eko 1 plasmid votes = 0.868/1 and contig97-eko 2 plasmid votes = 0.868/1), being typed as a mobilizable ColE10 plasmid. MOB-suite predicted contig88-eko 1 as plasmid and contig97-eko 2 as chromosomal (Supplementary Material, datasheet 2), but the latter was confirmed as plasmid-borne using plasmidID. PlasmidID results are summarized in supplementary material datasheet 3. We used plasmidID to identify and reconstruct possible *mcr-4.3* carrying plasmids in *E. kobei* genomes. Considering matches that included the contig harbouring the *mcr-4.3* gene and the highest mapping %, the best match was the reference plasmid pIB2020_ColE_MCR (NZ_CP059482.1) for both *E. kobei* isolates (Supplementary Material, datasheet 3). The genetic surrounding of the *mcr-4.3* gene consisted of the following putative gene products: conjugal transfer protein TraD (*tra*D), plasmid mobilization proteins (*mobA* and *mobX*), IS110 family transposase, IS110 family transposase, ISKpn26 family transposase, tyrosine-type recombinase/integrase, IS26 family transposase, lipid A phosphoethanolamine transferase (*mcr-4.3*), helix-turn-helix transcriptional regulator, type II toxin-antitoxin system RelE/ParE family toxin (*relE*), hypothetical protein and replication initiation protein (Figure 2A, Figure 2B).

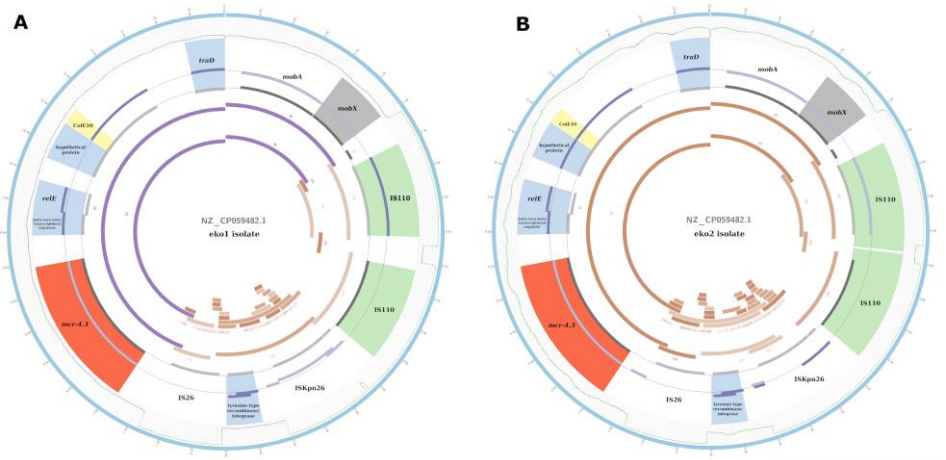

Figure 2. Reconstruction of plasmids carrying *mcr-4.3* gene in *E. kobei* eko1 (A) and eko2 (B) isolates using plasmidID. Circular image based on plasmid reference sequence NZ_CP059482.1 as best match considering plasmid features, *mcr-4.3* and ColE10 replicon genes presence, and plasmid coverage. From outer to inner track 1) The outer blue ring represents the reference plasmid from the database. 2) Graph line represents the number of reads mapped in each position of the reference plasmid. Green colour indicates more than 200 reads mapped, orange indicates less than 20 reads mapped and red indicates no reads mapped (grey axes lines space every 50 reads). 3) Two layers for contig and plasmid annotation results, each purple and grey box indicates a predicted named gene or cds, for contigs and plasmid database respectively. Genes over the line and darker are located in + strand, the rest in – strand. Identified sequences with more than 90% identity and 80% coverage are highlighted in colour: antibiotic resistance genes (orange), plasmid replicon genes (yellow), insertion sequences (green), mobilizable proteins (grey) and others (blue). 4) This layer represents only local alignments of contigs that match plasmid reference regions. Contigs are coloured depending on its number, which is displayed above the contig. 5) The inner layer represents the full length of contigs that had at least 20% of their size homologous to the reference plasmid.

Chromosomal-mediated colistin resistance. Table 2 summarizes colistin-resistance mechanisms detected in *Enterobacterales* isolates. We identified five colistin-resistant *Enterobacter* spp. and one *Klebsiella* spp. isolates having a mutated *mgrB* gene (50%, 6/12). *E. roggenkampii* ero6 and ero8 isolates harboured a missense mutation 4A>C (K2Q) in *mgrB* gene, while ero7 isolate harboured an insertion (Ins4C). The analysis of the *mgrB* gene in *E. kobei* isolates eko1 and eko2 showed an insertional inactivation at nucleotide 106 by a partial IS*1* (122 bp).

All *Enterobacter roggenkampii* isolates had mutated PmrB and PhoQ proteins. In PmrB we observed the following mutations: A13P and S260A (ero1, ero2, ero3 and ero4); F110Y, A221T and D272E (ero5); F110Y and S260A (ero6, ero7 and ero8); S60A (ero9). In PhoQ protein we observed the L286M mutation in all isolates, and E112K mutation was present only four isolates (ero1, ero2, ero3 and ero4).

Regarding *K. pneumoniae* isolate kpn1, we identified an insertional inactivation at nucleotide 52 by a partial IS*3* sequence (125 bp) in the *mgrB* gene. In addition, kpn1 isolate had mutations in PmrA (D97G) and in PmrB (R256G) proteins (Table 2).

Table 2. Colistin resistance molecular mechanisms detected in *Enterobacterales* isolates: mobile colistin resistance (*mcr)* genes, chromosomal point mutations and gene alterations in *mgrB* gene, PmrA-PmrB and PhoP-PhoQ two-component regulatory systems.

				Chromosomal alterations				
				Gene	Protein			
Isolate	Species ID	Colistin MIC(mg/L)	*mcr* genes	*mgrB*	PmrA	PmrB	PhoP	PhoQ
kpn1	*K. pneumoniae*	64	-	partial IS*3* at nt 52	D97G	R256G	wt	wt
eko1	*E. kobei*	64	*mcr-4.3, mcr-9.1*	partial IS*1* at nt 106	wt	wt	wt	wt
eko2	*E. kobei*	64	*mcr-4.3, mcr-9.1*	partial IS*1* at nt 106	wt	wt	wt	wt
ero1	*E. roggenkampii*	16	-	wt	wt	A13P, S260A	wt	E112K, L286M
ero2	*E. roggenkampii*	32	-	wt	wt	A13P, S260A	wt	E112K, L286M
ero3	*E. roggenkampii*	32	-	wt	wt	A13P, S260A	wt	E112K, L286M
ero4	*E. roggenkampii*	32	-	wt	wt	A13P, S260A	wt	E112K, L286M
ero5	*E. roggenkampii*	64	-	wt	wt	F110Y, A221T, D272E	wt	L286M
ero6	*E. roggenkampii*	32	-	4A>C	wt	F110Y, S260A	wt	L286M
ero7	*E. roggenkampii*	32	-	Ins4C	wt	F110Y, S260A	wt	L286M
ero8	*E. roggenkampii*	16	-	4A>C	wt	F110Y, S260A	wt	L286M
ero9	*E. roggenkampii*	32	-	wt	wt	S260A	wt	L286M

Abbreviations: wt, wild-type; nt, nucleotide.

Virulence factors, plasmid replicon type genes and other antibiotic resistance genes

Figure 3 shows the complete distribution of antimicrobial resistance, virulence and replicon type genes in clinical and environmental colistin-resistant isolates included

in this study. Besides mobile colistin resistance genes, we found other plasmid-mediated resistance determinants in four (33.3%, 4/12) *E. roggenkampii* isolates (er01, er03, er05, er09), most of them being related to beta-lactams, fluoroquinolones, aminoglycosides, tetracycline resistance and folate pathway antagonist antibiotics. We detected extended-spectrum ß-lactamase (ESBL) genes $bla_{\text{CTX-M-15}}$ (n=2), $bla_{\text{OXA-21}}$ (n=1), and narrow-spectrum beta-lactamases $bla_{\text{TEM-1B}}$ (n=3), $bla_{\text{OXA-1}}$ (n=2) in *E. roggenkampii* isolates. *K. pneumoniae* isolate harboured $bla_{\text{TEM-1B}}$ and $bla_{\text{SHV-107}}$ genes. In addition, we identified chromosomal genes encoding AmpC enzymes (bla_{ACT} or bla_{MIR}) in all *Enterobacter* spp. isolates.

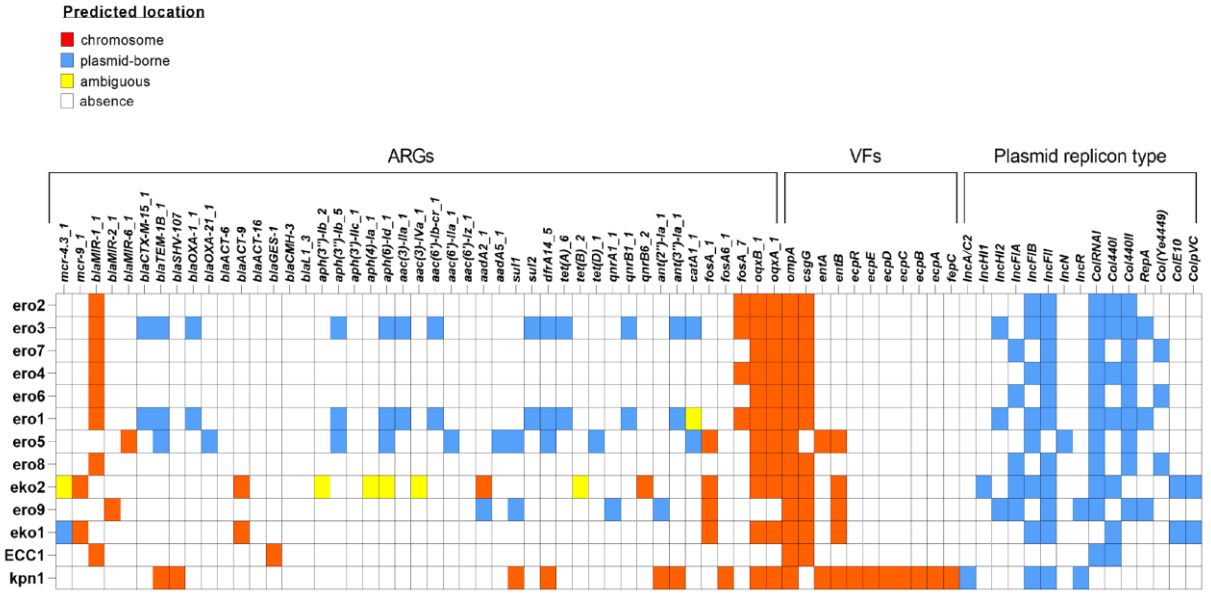

Figure 3. Heat map showing the distribution of antibiotic resistance, virulence and plasmid replicon type genes in environmental and clinical colistin-resistant isolates (ResFinder, VirulenceFinder and PlasmidFinder databases). Gene predicted location based on RFPlasmid and MOB-suite results is shown in the legend: red (chromosomal), blue (plasmid-borne), yellow ('ambiguous', meaning non-consensus results) and white cells indicate gene absence. Abbreviations: ECC, *Enterobacter cloacae* complex; ARGs, antibiotic resistance genes; VFs, virulence factors.

Phylogeny of *E. cloacae* complex isolates

The eleven *Enterobacter cloacae* complex environmental isolates corresponded to five STs: ST165 (n=4) in the haematology unit, ST54 (n=2) and ST965 (n=1) in the pediatric-ICU, ST486 (n=3) in the neonatal-ICU, and ST595 (n=1) in the adult-ICU. The clinical isolate (ECC1) belonged to ST165.

The core-genome SNP analysis of *Enterobacter roggenkampii* isolates revealed two clusters: one cluster of *E. roggenkampii* ST165 (n=4, haematology-unit) and one cluster of *E. roggenkampii* ST486 (n=3, neonatal-ICU) (Figure 4).

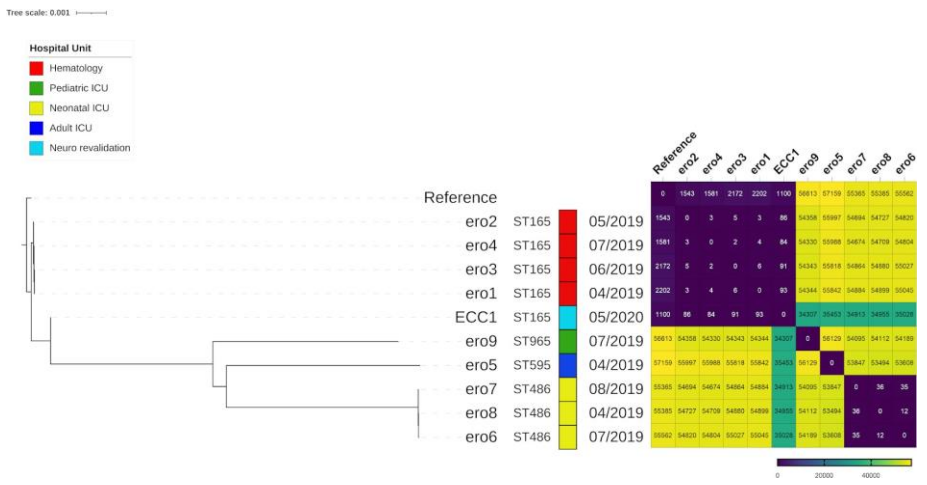

Figure 4. Maximum likelihood phylogenetic tree of *E. roggenkampii* isolates (nine environmental isolates and one clinical isolate) based on core-genome SNP analysis using the reference genome *E. roggenkampii* MGH34 (RefSeq accession number: NZ_KIS35616.1). The coloured distance matrix shows the number of SNP differences between isolates.

The ST165 cluster consisted of four environmental isolates recovered from the haematology unit (sink 1-drain, room 2) with 2 to 6 core SNP differences among these isolates. ST165 *E. roggenkampii* environmental isolates differed from the ST165 *E. roggenkampii* clinical isolate ECC1 in 84 to 93 core SNPs. The ST486 cluster consisted of three environmental isolates, recovered from sink 1-drain in the neonatal-ICU, differing from each other between 12 and 36 core SNPs.

We downloaded available *E. kobei* ST54 draft genomes from NCBI to compare with our isolates: three environmental, E35 (SRA: SRS5108102), STRAIN 17 (SRA: SRS17718234), RIVM C014549 (SRA: ERS4134515); and one clinical, ARLG 455 (SRA: SRS7252809). The core-genome SNP analysis of *E. kobei* ST54 showed two environmental *E. kobei* isolates recovered from the sink 3- drain in the pediatric-ICU differing from each other in 14 core SNPs (Figure 5).

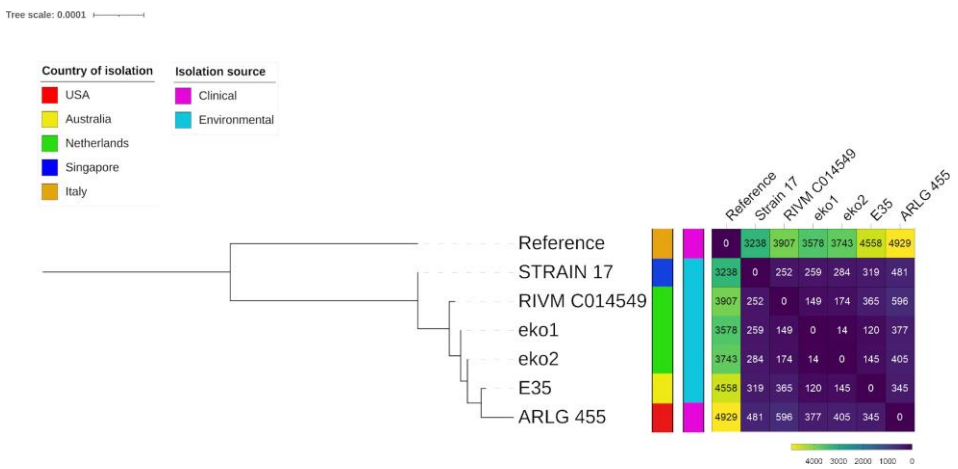

Figure 5. Maximum likelihood phylogenetic tree of *E. kobei* ST54 isolates from our study and available draft genomes from NCBI, based on core-genome SNP analysis using the reference genome *E. kobei* IB2020 (RefSeq accession number: GCF_014041955.1). The coloured distance matrix shows the number of SNP differences between isolates.

Environmental persistence

We observed possibly related isolates, from the same ST and with less than 14 core SNPs, in three of the four investigated hospital units. In the multiple-patient room of the haematology unit, four *E. roggenkampii* ST165 isolates (2 to 6 core SNPs) were recovered from the same sink-drain in four different time-points (April, May, June and

July 2020) (Figure 4). Regarding the neonatal ICU, we recovered two *E. roggenkampii* ST486 isolates (12 core SNPs) in April and July 2020. In the pediatric-ICU, we found two *E. kobei* ST54 isolates (14 core SNPs) in sink 3-drain in June and August 2020 (Figure 5).

Discussion

Our study aimed to monitor the presence of colistin-resistant Gram-negative bacteria in hospital drains of high-risk units, characterized the molecular mechanisms behind and investigated their phylogenetic relationships, the latter including available clinical isolates. Environmental monitoring revealed the presence of eighteen colistin-resistant Gram-negative bacteria along five months of screening, being *Enterobacter cloacae complex* isolates and *E. roggenkampii* species the most prevalent bacteria isolated in hospital sink-drains.

E. roggenkampii, a new clade of the *Enterobacter cloacae* complex[24], has been recently described as carrying the novel plasmid-born colistin-resistance *mcr-10* gene in clinical and environmental isolates, three of them detected in hospital sewage water[25]. Mobile colistin resistance frequency was low among our environmental isolates (2/12, 16%). Both *mcr* genes, *mcr-4.3* and *mcr-9*, have been previously described in clinical and environmental *E. kobei* ST54 strains from different countries (Singapore, Australia, USA, France, Spain, Italy and the Netherlands), revealing a rapid global emergence of this clone[26,27]. Although the co-occurrence of *mcr* genes is generally rare, other studies have reported different *mcr* variants in the same strain[28,29].

The first identification of an *mcr-4.3* gene was in a ColE10 plasmid type[30], the same plasmid replicon type as the two *E. kobei mcr-4.3* carrying contigs from our study. This plasmid has been described as not self-conjugative and with a broad host range, being able to replicate in different bacterial species and genera[31,32]. The *mcr-9* gene has been described as both plasmid and chromosomally located[33,34]. These two *E. kobei* isolates harbouring *mcr* genes, additionally had several altered genes related to colistin resistance. The *mcr-4.3* gene alone is not able to confer high-level resistance to colistin[35]. Moreover, the authors that first described the *mcr-9* gene proposed that the

gene is not related to colistin resistance but may be silent or inducible in clinical isolates of *Enterobacterales*[33]. The two-component QseCB system, including a sensor (*qseC*) and a response regulator (*qseB*), play a role in the expression of *mcr-9*. Nevertheless, the *mcr-9* and the QseCB system have been described in both colistin-susceptible and colistin-resistant *Enterobacter* spp. strains[33]. The exact mechanism behind the regulation of *mcr-9* expression is therefore yet to be determined. Our results suggest that bacteria may need to combine several mechanisms to reach a high-level of colistin resistance.

High-level colistin resistance is usually associated with chromosomal-mediated mechanisms, such as mutations in the PmrA-B or PhoP-Q two-component regulatory system (TCS) and MgrB inactivation[36,37]. In our study, mutations in PmrB and PhoQ proteins were the primary resistance mechanisms observed in *E. cloacae* complex and *Klebsiella* spp. isolates (83.3%, 10/12), with colistin MICs ranging between 16 and 64 mg/L.

Mutations and inactivation changes in *mgr*B gene, encoding the negative regulator of PhoP/Q, have been identified to play an important role in colistin resistance, mainly in *K. pneumoniae*[38,39]. Fewer studies described *mgrB* modifications in *Enterobacter* spp., and Mhaya A *et al*.[40] confirmed for the first time the role of MgrB inactivation in colistin resistance *Enterobacter* spp. To the best of our knowledge, we here describe for the first time the truncation of the *mgrB* gene by IS element in an *Enterobacter cloacae* complex isolate, *E. kobei*, which was associated with the highest levels of colistin resistance.

Unique phylogenetic analysis of different *E. cloacae* complex species is often performed due to the difficulties in proper identification, and studies are focused on the most frequently species reported in the clinical setting: *Enterobacter aerogenes*, *E. cloacae*, and *E. hormaechei* [24]. These aspects make difficult to assess dissemination routes of clones between patients/healthcare workers, and between patients/healthcare workers and the hospital environment. Recent studies using (SNP)-based phylogenetic analysis in *E. cloacae* complex (ECC) described less overall SNP diversity than *Klebsiella* spp[41]. Harada S *et al* reported 0-3 SNPs between isolates within the same clade and 100-325 SNPs between different clades among ST78 ECC

isolates[42] Another study of SNP-based clustering of *E. cloacae complex* isolates revealed intra-cluster variability at most one SNP, whereas inter-cluster SNP difference was at least 180 SNPs[43]. Too many SNPs between colistin-resistant environmental isolates and the only available clinical *E. roggenkampii* isolate from the same ST in our study indicates lack of clonal relationship. Nevertheless, the isolation of *E. roggenkampii* ST165 isolates from different sources, hospital sink-drain and patient rectal swab, suggests a possible common origin, and highlights the ability of *E. roggenkampii* ST165 to not only contaminate and persist in the hospital water environment but also to colonize the human gastrointestinal tract. Besides, larger, and temporally matching collections of clinical and environmental isolates should be compared to better elucidate existing transmission.

On the other hand, the low number of SNP differences between *E. roggenkampii* ST165 environmental isolates (2-6) likely indicates clonality. Furthermore, we are likely losing actual representativeness by using selective agar media for resistant bacteria. Extended persistence of multidrug resistant bacteria on intensive care unit surfaces was previously reported in a year-long investigation of bacterial colonization at a tertiary hospital in Pakistan[44]. In this study, single lineages dominated *A. baumannii* and *E.faecium* populations whereas *K. pneumoniae* isolates belonged to multiple sequence types as described in other reports[45]. The presence of beta-lactam plasmid-mediated genes ($bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$) in hospital environmental *E. roggenkampii* ST165 isolates, especially in high-risk units, is worrying due to their potential transmission to other pathogenic bacteria via horizontal gene transfer and the limiting treatment options. Bacteria surviving in the built environment has been widely described in relation to biofilm formation which can help to escape from disinfectant procedures [44,46] Furthermore, biofilms are often polymicrobial contributing to the spread of multiple lineages within a species and boosting the exchange of antimicrobial resistance[1,46].

Our results showed the presence of colistin-resistant Gram-negative bacteria, mostly *Enterobacter* spp., in drains of high-risk hospital wards, throughout five months of the sampling period. These findings represent a healthcare concern and a potential threat for patients and healthcare workers. Monitoring the hospital water-related

environment for multidrug resistant bacteria is an essential tool to identify reservoirs of relevant pathogens and synchronized screening in hospitalized patients would provide insights into transmission routes to design appropriate control measures and prevent further dissemination

Data Availability

The datasets generated during the current study are available in the Sequence Read Archive (SRA), BioProject PRJNA756469, BioSample numbers SAMN20867095-SAMN20867107.

Electronic Supplementary Material

1. Datasheet 1 (Quast assemblies report)
2. Datasheet 2 (RFPlasmid, MOB-suite and ResFinder combined results)
3. Datasheet 3 (PlasmidID results summary)

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Competing Interest

JWAR is currently employed by IDbyDNA. All other authors declare no conflict of interest.

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