Mobile genetic elements and clonal dynamics of antibiotic-resistant hospital-acquired bacteria

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1. Introduction

Antimicrobial resistance (AMR), a defence mechanism used by bacteria to overcome antimicrobial drugs, has emerged as a global health threat in the last decades.[1, 2] Since the begin of the antibiotic era, marked with the discovery of the β-lactam penicillin in 1928 by Alexander Fleming, the use, misuse, and overuse of antibiotics in human and veterinary medicine is considered as the major driving force of AMR.[1, 3, 4] The first report of antimicrobial resistance became apparent when bacteria were growing in the presence of penicillin, in 1940 shortly after its discovery.[5] Ever since, the AMR rates are rapidly growing and have provoked the emergence of multidrug-resistant (MDR), extensive drug-resistant (XDR) or even pan drug-resistant (PDR) bacteria. MDR implies acquired non-susceptibility to one or more agents of at least three antimicrobial classes (Figure S1), XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial classes while, PDR implies non-susceptibility to all agents in all antimicrobial categories.[6]

1.1 ESKAPE organisms

Although many bacterial species are commensals to humans, as they can be found throughout the human body, there is a distinct group of bacteria which are increasingly prevalent in the healthcare setting and is able to escape the biocidal action of antibiotics. This group of pathogens are referred to "ESKAPE organisms" and encompass: vancomycin-resistant *Enterococcus faecium* (VREfm), methicillin-resistant *Staphylococcus aureus* (MRSA), and MDR *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species. The ESKAPE bugs not only cause the vast majority of nosocomial infections, but serve as paradigms of transmission and resistance.[7, 8] The World Health Organization (WHO) included ESKAPE pathogens in the list of 12 bacteria which present a great threat and for which new antibiotics are urgently needed.[9] Carbapenem-resistant *A. baumannii* (CRAb) and members of the family Enterobacterales, such as *K. pneumoniae*, have been classified as critical while, VREfm has been given high priority, according to the urgency of a need to develop new antibiotic treatment options and the levels of reported antimicrobial resistance.[10]

A. baumannii is an aerobic Gram-negative bacillus often involved in healthcare-associated infections, such as ventilator-associated pneumonia, but also skin and soft tissue infections,

wound, and urinary tract infections. Of major concern are the increasing trends of resistance to carbapenems, considered as antibiotics of last resort. Carbapenems, as all β-lactam antibiotics, targets the cell wall synthesis of growing cells by inhibiting the peptidoglycan crosslinking resulting in cell death.[11, 12] Of note is that the first CRAb was reported in 1993, but this organism was isolated in 1985, prior to the introduction of carbapenems.[13] Carbapenem resistance in *A. baumannii* is primarily mediated through acquired carbapenem-hydrolysing class D ß-lactamases, referred also as oxacillinases (OXAs). The most prevalent OXA in the species is $bla_{\text{OXA-23-like}}$ while, four more groups occur; $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-143-like}}$ and $bla_{\text{OXA-235-like}}$.[14-16] However, *A. baumannii* also has an intrinsic bla_{OXA} (51-like) and overexpression of certain variants confer carbapenem resistance.[17, 18] Besides the OXA-type carbapenemases a secondary mechanism of resistance occurs, such as outer membrane porin deficiencies affecting the influx of carbapenems into the bacterial cell, or changes in penicillin-binding proteins (PBPs) resulting in low binding affinity of the antibiotic.[19]

K. pneumoniae is a common colonizer of the gastrointestinal tract of healthy human and animals. Nevertheless, this Gram-negative bacterium has been isolated the first time in 1882 from the lung of a pneumonia patient and since then has been reported as the cause of a wide range of human infections, such as urinary tract infections or bacteraemias.[2, 20] *K. pneumoniae* is considered as a successful bacterium in terms of antimicrobial resistance.[2] Of particular concern are the steadily increasing rates of resistance against the third-generation cephalosporins and carbapenems observed in *K. pneumoniae*. This adds to the endemicity of MDR *K. pneumoniae* in certain regions, such as in Mediterranean countries.[21] Resistance to third-generation cephalosporins is mainly mediated through acquired extended-spectrum β-lactamases (ESBLs) while resistance to carbapenems is due to the presence of acquired carbapenemases, such as OXA-48 or KPC. However, outer membrane permeability alterations due to porin loss can contribute also to β-lactam resistance in this species.[2]

E. faecium, first discovered in 1899, is a Gram-positive bacterium belonging to the family Enterococcaceae and is a colonizer of the gastrointestinal tract of humans and animals. Of major concern is the continuous increase of VREfm causing healthcare-associated infections.[22, 23] Since the 1980s when vancomycin-resistant enterococci were reported for the first time, VREfm have become a major public health threat.[24] In the US and Australia more than 50% of invasive infections are caused by VREfm (https://resistancemap.cddep.org/AntibioticResistance.php). Vancomycin resistance in

enterococci is conferred by acquired *van* operons (*A*/*B*/*D*/*E*/*G*/*L*/*M*/*N*), but the *vanA* and *vanB* are the predominant resistance determinants. Glycopeptides target the C-terminal D-Ala–D-Ala ending of the peptide side chain of the peptidoglycan cell wall precursor in enterococci. Both *vanA* and *vanB* inducible operons encode a ligase altering the target binding site of vancomycin and eliminate the high-affinity precursors produced normally by the host. The *vanA* operon replaces the terminal D-Ala of peptidoglycan precursors with D-Lac resulting in high-level vancomycin resistance, while the *vanB* operon exchanges the D-Ala terminal with D-Ser, producing low-level vancomycin resistance. In addition to vancomycin, VanA-type isolates exhibit high-level of inducible resistance to another glycopeptide, teicoplanin, while VanB-type isolates are susceptible to teicoplanin.[25, 26]

1.2 Clonal spread

In the last decades some bacteria, such as MRSA or VREfm, have become major nosocomial pathogens. A key factor facilitating the success of antibiotic-resistant bacteria in the nosocomial environment is their clonal expansion. Nosocomial outbreaks, clonal expansion in excess of normal expectancy, of MDR bacteria compose a serious threat especially to susceptible patient populations. In particular, intensive care unit (ICU) patients are prone to bacterial infections because of indwelling devices, such as intravascular catheters, or intratracheal tubes for mechanical ventilation, and severe underlying illnesses. Moreover, the prolonged overuse of antibiotics, insufficient nurse-to-patient ratios, overcrowding and poor hand-hygiene contribute to the clonal dissemination of MDR bacteria thriving in the healthcare settings and increasing the number of bacterial outbreaks.[27, 28]

Numerous studies have reported the clonal dissemination of isolates within or between hospitals of the same country.[28] Clonal spread is tightly linked with the intrahospital or interhospital transfer of colonized patients or healthcare workers. For instance, a recent study about a sharp increase of VREfm and the detection of two major clones in a hospital in southwest Germany demonstrated the continuous import of the outbreak clones from other local hospitals as the likely intrahospital transmission routes.[29] In addition, clonal expansion is of particular concern as it is often coupled with acquired antibiotic resistance genes (ARGs). For instance, the worldwide dissemination of *K. pneumoniae* sequence type (ST) 258 arose with the acquisition of a carbapenemase-encoding (bla_{KPC}) plasmid.[2, 30]

1.3 Horizontal gene transfer

Bacteria have a remarkable genome plasticity which allows them to adapt and survive under continuously altering environmental conditions, e.g. the presence of antimicrobial compounds.[31] Bacterial pathogens use two major strategies to escape the lethal action of antibiotics: (a) mutations in genes associated with the mechanism of action of the antimicrobial compound, and (b) transfer and acquisition of new genetic information coding for ARGs between genomes, referred as horizontal gene transfer (HGT).[32, 33] The latter mechanism of adaptation is considered as the most important driving force for the dissemination of ARGs in prokaryotes and results in bacteria thriving in certain environments and to the emergence of hard-to-treat MDR infections.[32] Understanding the genetic basis and dissemination of AMR is essential for combatting this public health challenge.

The contribution of HGT in the propagation of AMR is evidenced by the continuous report of new ARGs in new organisms, in new genetic locations, on new vehicles, and in new geographic locations. HGT includes the movement of deoxyribonucleic acid (DNA) mainly by three mechanisms: conjugation, transduction and transformation (Figure 1). Bacterial conjugation (or mating), first described in 1946, is the unidirectional and contact-dependent exchange of genetic material. During conjugation one bacterium serves as donor and another bacterium acts as recipient, while DNA transfer is mediated by plasmids and integrative conjugative elements (ICEs). Transduction is another form of DNA exchange facilitated by bacteriophages; independently replicating bacterial viruses. Bacteriophages can package fragments of host DNA and inject this into a new host. Unlike conjugation and transduction, bacterial transformation does not require cell-to-cell contact and refers to the natural ability of bacteria to take up exogenous DNA. A group of competence genes is involved in the phenomenon of transformation coded by some naturally transformable bacteria.[32, 33]

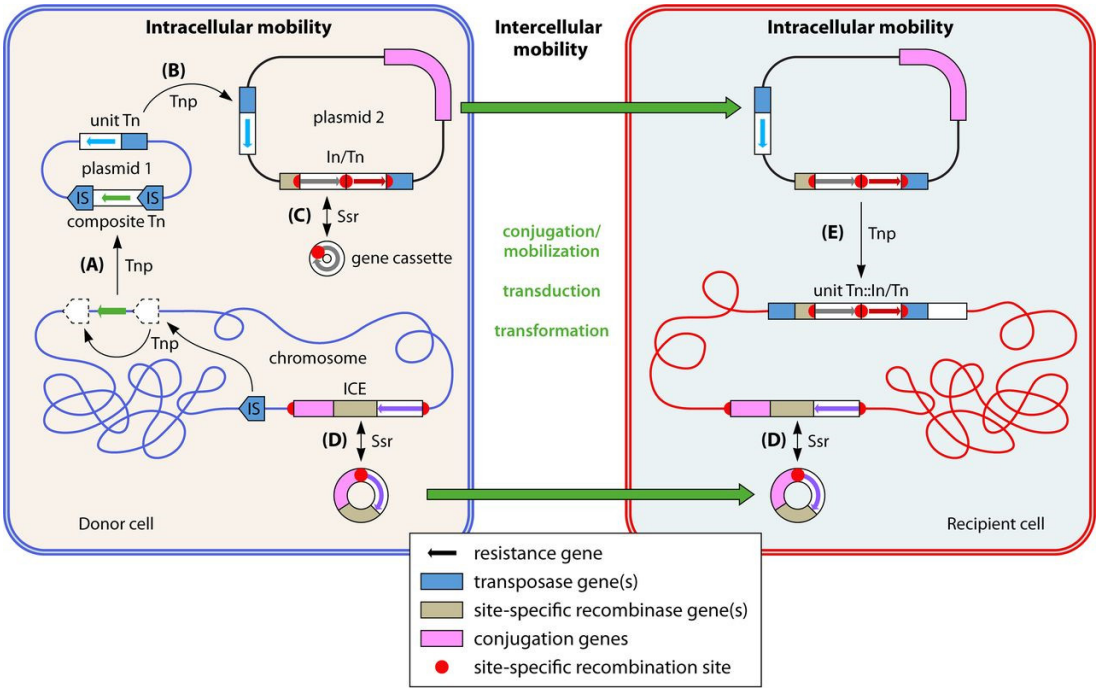

Figure 1. HGT, MGE and intracellular mobility or intercellular transfer of ARGs. (A) Example of a composite transposon, capturing e.g., an ARG, moves from chromosome to a plasmid. (B) A transposon carrying a resistance gene is transposed between two plasmids or from a plasmid to the chromosome. (C) A gene cassette may move between an integron (a class 1 In/Tn represented here) by a circular intermediate. (D) An ICE can be integrated into the chromosome at a specific recombination site or removed as a circular element that can then conjugate into a recipient cell and integrate. A plasmid encoding conjugative genes can mediate its own transfer from the donor to the recipient cell by conjugation or, if it lacks the conjugative apparatus, it can be mobilized by another plasmid or, even move horizontally by transduction or transformation. (E) Finally, transposons or integrons linked to ARGs on a plasmid, newly introduced into a recipient cell, could move into the chromosome or other plasmid(s). The figure was reprinted from the Journal of Infectious Diseases.[34]

1.4 Mobile genetic elements

Apart from the different types of HGT used by bacteria, it is also of great importance to understand the key agents catalysing the DNA mobilization; plasmids, and transposons. These agents constitute the mobile gene pool of prokaryotes and together with bacteriophages are referred as mobile genetic elements (MGEs) or as “mobilome”. MGEs can facilitate the

mobilization of DNA fragments intracellular, within the genome, or intercellular, between different cells.[34, 35]

1.4.1 Plasmids

Plasmids, extrachromosomal double-stranded DNA molecules replicating autonomously, are widely distributed among prokaryotes and are also present in some eukaryotes such as fungi. They may be found as a single or multiple copy and can vary enormously in size from 1 kilobase (kb) up to several hundreds of kb. Unlike chromosomes, plasmids are not essential for the bacterial cell. However, plasmids frequently carry accessory cargo genes, including ARGs or virulence genes, that can provide a selective advantage for the bacterial cell.[34, 36]

Plasmids that share the same inheritance elements and in the absence of selective pressure are not able to stably coexist over a number of generations in the same cell are considered incompatible. This unique characteristic is used to classify plasmids into incompatibility groups also called Inc typing or replicon typing; plasmids belonging to the same Inc group cannot be propagated in the same cell. This typing method is based on a genetic module called replicon consisting of one or more origins of replication (*ori*), a Rep protein initiating the replication and a few other regulatory modules.[36, 37]

Besides replication, plasmids have some additional mechanisms, including partitioning systems, post-segregational killing, and multimer resolution, to ensure their inheritance into the daughter cell as the parent cell divides. Partitioning systems are ubiquitous in bacteria and can be encoded by chromosomes or plasmids and are of great importance particularly for low-copy-number plasmids. High-copy-number plasmids typically do not encode a partitioning system as random segregation is sufficient for stable inheritance. In partition the centromere-binding and motor proteins, known as ParA and ParB respectively, are responsible for transferring and positioning the plasmid inside the cell. Post-segregational killing, also known as plasmid addiction, is a widespread plasmid maintenance mechanism in prokaryotes. The later mechanism functions through the production of stable and long-lasting toxin and its cognate antitoxin neutralizing the toxin (this mechanism is also called toxin-antitoxin (TA) system. Finally, another surviving mechanism is the multimer resolution system converting multimeric plasmids to monomeric, thus ensuring that plasmids do not accumulate because of recombination between the identical copies of the plasmid.[35, 37]

1.4.2 Transposable elements

Transposable elements (TEs), known also as jumping genes, are DNA segments that can make copies of themselves or move from one position to another within a genome and within plasmids. These genetic elements were discovered by Barbara McClintock in the 1940s and since then it became apparent that TEs are abundant in prokaryotes and eukaryotes. In some cases, TEs can take up the vast majority of a genome, e.g., 50% of the human genome and 90% of maize is composed of TEs.[38, 39]

Insertion sequences (ISs) are the smallest and simplest TEs and are capable of self-transfer from one genetic location to another within the same or different DNA molecules. They typically retain one (and sometimes two) transposase, a specialized recombinase necessary for their own transposition. Classical ISs are flanked by 10 to 40 base pairs (bp) inverted repeats (IRs); the IR close to the transposase promoter is assigned as the left (IRL) and the remote IR as the right (IRR). ISs are bracketed by direct repeats (DR) generated during transposition and their length is specific for each IS.[34, 40] Beside their own transfer, IS elements can carry passenger genes as part of a composite transposon: a region bracketed by two copies of the same or related ISs that move as a discrete unit.[41] In *A. baumannii* the composite transposon Tn*125* flanked by two copies of IS*Aba125* orientated in the same direction are considered as the main vehicle of the carbapenemase $bla_{\text{NDM-1}}$.[42, 43] Based on the nature of the transposase and how breaking and re-joining of DNA occurs during transposition IS can be grouped into DDE (Asp, Asp, and Glu), DEDD (Asp, Glu, Asp, and Asp) and HUH (two His residues separated by a large hydrophobic amino acid) types. The latter catalytic domains are located towards the C-terminus of the transposase. Furthermore, ISs can be also divided based on their transposition mechanism: the conservative cut-and-paste mechanism whereby, the IS is excised from a genetic location and inserted into a new site, and the replicative copy-and-paste mechanism, where a copy of the IS is generated and inserted into a new genetic location.[34, 40, 44]

ISs can modify the genotype and phenotype of bacteria, e.g. by causing inactivation of a gene after insertion.[34] On the other hand, disruption of the *adeN*, encoding the repressor of the efflux pump AdeIJK in *A. baumannii*, has been associated with increased expression and tigecycline resistance.[45] But ISs can also encode a strong promoter that drives the overexpression of adjacent genes, e.g. insertion of IS*Aba1* upstream of the intrinsic $bla_{\text{OXA-51}}$ in *A. baumannii* results in a carbapenem-resistant phenotype.[18] This type of TEs are present in multiple copies

in the genome. *A. baumannii*, for instance, has a high IS element density with an average of ~33 copies per genome while, IS*Aba1* is the most abundant IS element in the species.[46]

Besides ISs and transposons, there are more natural genetic engineers undergoing HGT and playing a key role in the evolution of bacterial AMR, such as integrons, genomic islands (GIs) or ICE. Integrons, defined by the presence of an intergrase and a recombination site, are able to integrate, express and exchange exogenous genes, called gene cassettes. An integron can harbour more than one gene cassette in tandem and create an array.[47, 48] GIs are large genomic regions, between 10-200 kb, found in bacteria and based on the cluster of genes they harbour can be grouped, e.g. into metabolic islands, containing genes associated with metabolic properties or resistance islands (RIs), containing genes associated with antibiotic resistance.[49, 50] Finally, ICEs, self-transmissible chromosomally integrated MGEs, can insert themselves into the host chromosome and excise, circularize and move through conjugation to neighbouring cells.[51]

1.5 Genetic diversity

Bacteria reproduce asexually by binary fission and, pass a copy of the parent genome to their offspring. Binary fission reassures a clonal descent progeny, genetically identical copies of a bacterial cell. Conceptually, there are no opportunities for genetic diversity in bacteria in contrast to sexual reproduction. However, truly clonal bacteria do not often occur. Prokaryotes may not reproduce sexually but instead utilize HGT; the primary mechanism of diversification and the major force of bacterial evolution.[52, 53] MGEs, such as plasmids or ISs, carry a wide repertoire of accessory genes and can shape the bacterial genome of closely related isolates and cross even species boundaries. Besides MGEs, another source of diversity are *de novo* mutations or recombination, the exchange of DNA sequences between different regions within the chromosome or different chromosomes. Recombination is a means of DNA reshuffling and exchange of variants e.g. the outer membrane porin *carO* variants within the *A. baumannii* population or the mobilization of *ant(3")-II*, an aminoglycoside modifying enzyme, between different *Acinetobacter* spp.[54, 55] Taken together, HGT, MGEs, recombination and mutations are the major sources of genome plasticity, population diversity in bacteria and can alter not only the genotype but also phenotype of an organism.[56, 57]

1.6 Experimental approach for tracking clonal spread, genetic diversity and MGEs in bacterial populations

Phylogenetic analysis and typing of a bacterial population is a crucial step when studying bacterial genetics.[58] Moreover, HGT and MGEs are considered the main driving force in bacterial evolution and dissemination of antimicrobial resistance determinants.[35] Hence, the investigation of the genetics, distribution, and diversity of MGEs is the keystone to understand the diversity and plasticity in bacterial genomes. The methodological approach for tracing clonality, diversity and MGEs in bacterial isolates using currently available tools and methods will be described below.

1.6.1 Next-generation sequencing

Next-generation sequencing (NGS) is a catch all term for different technologies for massively parallel and deep sequencing which has revolutionized biosciences. NGS technologies are highly scalable compared to earlier sequencing methods, e.g., Sanger sequencing. The most important application of NGS is sequencing the complete genome at once, referred also as whole-genome sequencing (WGS), which is most commonly accomplished by the short-read sequencing technology at present.[59]

1.6.1.1 Short-read sequencing

Short-read sequencing platforms such as Illumina's MiSeq (https://emea.illumina.com/science/technology/next-generation-sequencing/sequencing-technology.html), which was used in the present study, generate highly accurate sequences of DNA, with 2 x 300 bp read length. More specifically, a DNA library is generated by random shearing of the genomic DNA into several million fragments (ideally between 200 bp to 1 kb) and, specialized adapters are ligated to both ends. These fragments are sequenced using the sequencing by synthesis technology; fluorescently labelled nucleotides serve as reversible terminators for polymerization. The fluorescent dye and therefore the corresponding nucleotide (base) is identified through laser excitation and imaging. Enzymatical cleavage enables the next cycle of incorporation.[60]

Short-read sequencing produces a huge number of raw reads which are subsequently used to downstream analysis including quality controls and genome assembly: a computational process taking raw read sequences and putting them back together based on overlapping regions to reconstruct the genome of an organism. A genome assembly can be accomplished either reference-based; by mapping the raw reads to a reference genome and assembling the reads to the right order, or *de novo*; without the aid of a reference genome and prior knowledge about the source of DNA.[61]

1.6.1.2 Long-read sequencing

Assembly of complete genomes is crucial for identifying novel genomic structures and gene mapping. Bacterial genomes are rich in repetitive elements, multi-copy genes or ISs complicating genome reconstruction. During short-read assembly multi-copies of a gene pile up, because of the short length of the reads, to a single copy breaking the genome into numerous contigs (sequence fragments) and leading to gaps and incomplete assembly. In contrast, long-read sequencing, also referred as third-generation sequencing, generates reads more than several tens of kb, which can bridge repetitive elements and resolve their complex structure (Figure 2). The use of long-reads can optimize *de novo* assembly, genome mapping and detection of indels (insertions and deletions) and structural variants. Hereafter, Oxford Nanopore Technologies' (ONT) sequencing using the MinION platform, established, and applied in the present work, will be further elucidated.[59, 62]

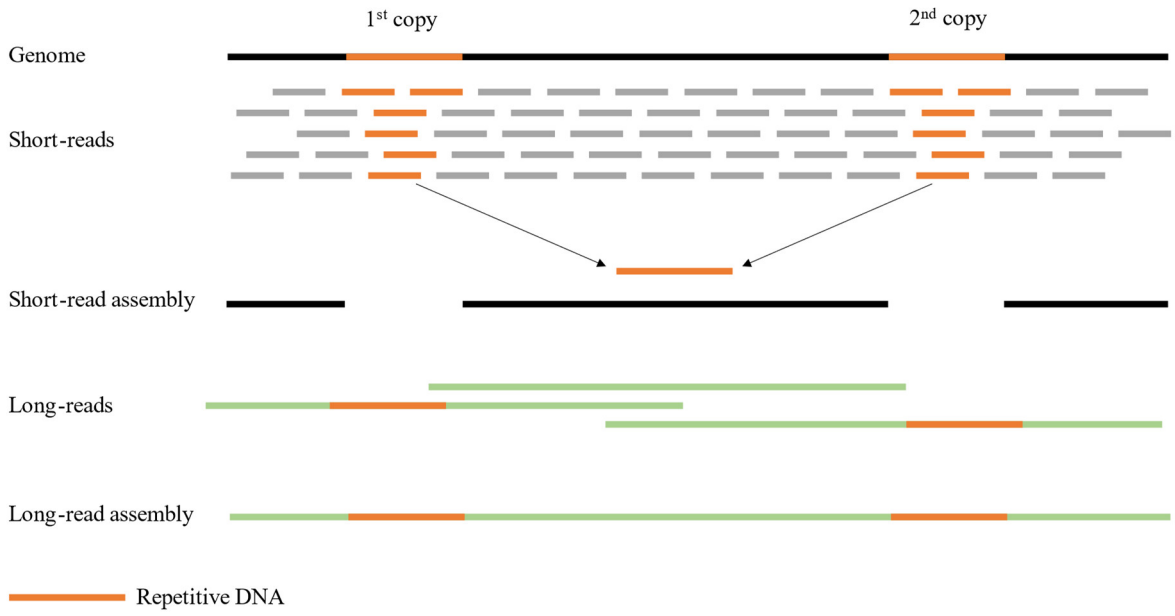

Figure 2: Comparison of short- and long-read assembly. Short-read sequencing produces reads 50-500 bp in length, which cannot span the length of the repetitive sequences leading to gaps and incomplete assembly. In contrast, long-read sequencing from Nanopore produces reads that can be more than 100 kb in length, spanning the repetitive regions and allowing a complete genome assembly.

MinION is a portable sequencer generating data in real-time. The DNA is sequenced while passing through a nanopore, a nano-scale hole on a flow cell, passed by ionic current. The DNA sequence is inferred from the changes in the current passing through the pore (https://nanoporetech.com). However, homopolymer stretches > 5 bp challenge this technology and result in low read accuracy. Consequently, the use of only long-read sequencing yields in higher genome-wide error rates compared to short-read sequencing. The method of choice for obtaining complete genomes with high accuracy and resolving complex structures, such as MGEs, is the combination of short- with long-reads in a hybrid assembly approach.[63]

1.6.2 Bacterial typing

The identification of clonal relationships or tracing the routes of isolate spread, can be accomplished by various bacterial typing techniques. The traditional multilocus sequence typing (MLST) scheme is based on seven loci, representing partial housekeeping genes, which define the unique allelic profile or ST of an isolate. The progeny of the MLST typing scheme

is the high discriminatory core genome MLST (cgMLST) method; a gene-by-gene approach for studying the clonal relationships in a bacterial population. cgMLST typing is based on WGS and uses a large number of loci to analyse the molecular epidemiology of bacterial isolates in higher resolution than MLST.[64]

In practice, the assembled genome is aligned (BLAST approach) to a species-specific scheme, a fixed set of core genes termed as alleles (e.g., the *A. baumannii* cgMLST scheme includes 2390 alleles). In this manner unique gene sequences which represent evolutionary events that may lead to polymorphisms, are detected. Allele calling results in a unique allelic profile; a set of allele numbers for each locus. The higher the number of shared alleles between two isolates the smaller is the genetic distance between them. The minimum spanning tree in Figure 3 describes the clonal relatedness of four ST85 *A. baumannii* isolates as a paradigm of cgMLST analysis. In detail, the isolates A and B are considered identical while they differ in only two alleles from isolate C and altogether form a cluster of closely related isolates while, for isolate D, no epidemiological relatedness is observed. Although the isolates were assigned to the same ST by MLST, not all were closely related highlighting the discriminatory power of cgMLST.[65, 66]

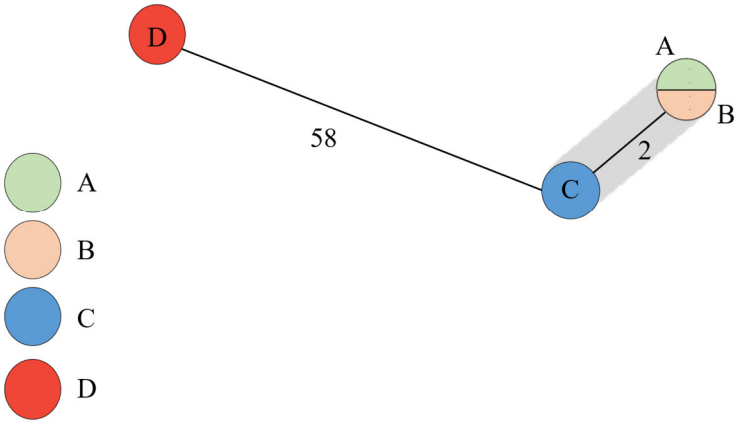

Figure 3: Minimum spanning tree generated using Ridom SeqSphere+ for four ST85 (Pasteur scheme) *A. baumannii* isolates grouped and coloured by isolate name, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 2390 cgMLST alleles. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

1.6.3 Pulsed-field gel electrophoresis

A bacterial cell can have from one up to numerous plasmids varying in size from 1 kb to more than 500 kb, even plasmids up to 1.7 megabases (Mb), in groundwater samples, in size have been reported.[67, 68] Plasmids from small to large size can be well separated using pulsed-field gel electrophoresis (PFGE) in order to elucidate their size and number.[69] PFGE resolves larger DNA fragments by introducing an alternating gradient voltage in contrast to the single and continuous electric field of the conventional gel electrophoresis. Every time the electric field changes the DNA is elongated and reorientates to the direction of the field and moves through the pores of the gel depending on its size.[70] PFGE was considered the 'gold-standard' molecular epidemiological method for bacterial typing prior to WGS era. Despite its more than 20-years history as a typing method, PFGE is still used as a fingerprinting method for plasmid profiling of bacteria.[71]

Plasmids occur in three conformations: supercoiled, the native conformation that migrates faster in an agarose gel; open-circular, resulting from single strand nicks which migrates slower in a gel than supercoiled; and linear, which occurs when the DNA helix has a double-strand break and migrates as predicted by its length. Therefore, the electrophoretic mobility of a DNA molecule in a gel is dependent upon its conformation and its length, so a crucial step to estimate accurately the size and profile of plasmids is their linearization by restriction enzymes. In PFGE for plasmid typing bacterial cells that are embedded in agarose plugs undergo S1-nuclease treatment to linearize plasmids. S1-nuclease is an endonuclease that cuts single-stranded nucleic acid but can also convert supercoiled plasmid DNA to linear double stranded DNA (dsDNA) primarily in inverted repeat regions. In these regions, the DNA structure adopts a cruciform structure that is recognized as single-stranded target by the S1-nuclease leading to a single and double-stranded break in the DNA molecule, whereas the chromosome molecule remains undigested and does not separate over a PFGE run. In contrast to the chromosome, linearized plasmids are sorted by size and give rise only to one band on the gel so that the total number of plasmids can be determined (Figure S2).[69, 72, 73]

1.6.4 Southern blot hybridization

The next crucial step after resolving the size and total number of plasmids, is the genetic location of the gene of interest. Genes encoding antimicrobial resistance determinants may be

encoded on the chromosome and/or on plasmids. In order to discriminate plasmid and chromosome encoding genes PFGE is followed by Southern blotting.[74]

Southern blot is a molecular method that allows the detection of specific DNA sequences such as plasmid replicons or ARGs. Thus, DNA fragments separated by S1-PFGE gel are transferred to a blotting membrane. A labelled DNA probe with a complementary sequence to the gene of interest is then applied, which will hybridize on the membrane and permit its detection among the different DNA fragments (Figure S2). As a confirmatory step, the chromosomal location can be shown by co-localization with a chromosomal marker, e.g., *rpoB*, enhancing this methodological approach.

1.6.5 *In silico* methods

NGS is a high-throughput molecular profiling method generating a huge amount of data. Hence, there is a high demand for *in silico* sequence analysis tools. Sequencing data analysis starts with quality controls of the sequencing output and continues with various applications such as assembly, alignment, or annotation. For this purpose, numerous online resources offer user-friendly web interface tools, such as the Centre for Genomic Epidemiology (http://www.genomicepidemiology.org/), or command-line tools (e.g. Canu assembly) distributed mainly for Linux-based systems (Table S1).[75]

1.7 Aim of the study

The aim of this study was to elucidate MGEs, clonal dynamics and genetic diversity in antibiotic-resistant hospital-acquired bacterial species commonly involved in nosocomial infections such as *A. baumannii*, *E. faecium* and *K. pneumoniae*.

2. Results

In the present thesis the clonal dissemination of VREfm and 3GCR *K. pneumoniae* colonising patients on hospital admission in Germany and the molecular epidemiology of CRAb isolates from two hospitals in Bolivia were investigated (2.1-2.3). Moreover, MGEs harbouring ARGs in *A. baumannii* (2.4 and 2.5) but also heterogeneity associated with MGEs among closely related isolates in *K. pneumoniae*, *A. baumannii* and *E. faecium* clinical isolates (2.6-2.8) were studied.

2.1 VREfm colonizing patients on hospital admission in Germany: prevalence and molecular epidemiology

In Germany the rates of VREfm causing invasive infections increased from 10.5% in 2015 to 23.8% in 2018.[21] An important factor contributing to this increase is the clonal expansion of VREfm healthcare-associated lineages.[76] Enterococci causing outbreaks and the increase of VREfm bloodstream infections have been reported in numerous hospital settings in Europe.[29, 76-79] One more crucial aspect for the nosocomial spread of enterococci, are patients colonized with VREfm on hospital admission, which could increase the risk of further transmission within the hospital.[80] However, no data about patients colonized with VREfm on hospital admission in Germany were available.

In the present manuscript we analysed the prevalence and the molecular epidemiology of VREfm obtained from patients upon admission to six German university hospitals. In a five-year study period, a prevalence of 1.6% was identified. Among these isolates 78.5% were *vanB*-positive and 20.2% were *vanA*-positive, while 1.2% were *vanAB*-positive. In the present study, ST117 (56.7%) was the predominant ST followed by ST80 (15%), ST203 (10.9%), ST78 (5.7%) and ST17 (3.2%). The vast majority of the ST117 isolates (92%) were *vanB*-positive. Over the study period, we identified an increase in the prevalence of ST117 (2014, 21.7%; 2015, 29.8%; 2016, 36.4%; 2017, 72.1%; 2018, 79%) consistent with an increase of *vanB* positive VREfm isolates (2014, 78.3%; 2015, 66%; 2016, 69.7%; 2017, 79.4%; 2018, 89.5%). Moreover, molecular typing revealed that about 70% of the ST117 *vanB*-positive VREfm isolates formed one large multi-centre cluster of closely related genotypes dispersed over six geographically separated study centres indicating inter-regional clonal relatedness. The majority these isolates were classified as cluster type (CT) 71. Our data indicate that the

epidemiological profile of VREfm in Germany has changed in the past five years and confirm the remarkable clonal expansion of the ST117/CT71/*vanB* clone.

My contribution to the present study included: phenotypic and genotypic characterization of the isolates, conception and design of the analysis, WGS and molecular epidemiology analysis, data interpretation and composing the manuscript.

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Vancomycin-resistant *Enterococcus faecium* colonizing patients on hospital admission in Germany: prevalence and molecular epidemiology

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Objectives: To analyse the rectal carriage rate and the molecular epidemiology of vancomycin-resistant *Enterococcus faecium* (VREfm) recovered from patients upon hospital admission.

Methods: Adult patients were screened at six German university hospitals from five different federal states upon hospital admission for rectal colonization with VREfm between 2014 and 2018. Molecular characterization of VREfm was performed by WGS followed by MLST and core-genome MLST analysis.

Results: Of 16350 patients recruited, 263 were colonized with VREfm, with increasing prevalence rates during the 5 year study period (from 0.8% to 2.6%). In total, 78.5% of the VREfm were *vanB* positive and 20.2% *vanA* positive, while 1.2% harboured both *vanA* and *vanB*. The predominant ST was ST117 (56.7%) followed by ST80 (15%), ST203 (10.9%), ST78 (5.7%) and ST17 (3.2%). ST117/*vanB* VREfm isolates formed a large cluster of 96 closely related isolates extending across all six study centres and four smaller clusters comprising 13, 5, 4 and 3 isolates each. In contrast, among the other STs inter-regional clonal relatedness was rarely observed.

Conclusions: To our knowledge, this is the largest admission prevalence and molecular epidemiology study of VREfm. These data provide insight into the epidemiology of VREfm at six German university hospitals and demonstrate the remarkable inter-regional clonal expansion of the ST117/*vanB* VREfm clone.

Introduction

Enterococci have emerged as an important cause of nosocomial infections. The vast majority of enterococcal healthcare-associated infections (HAIs) are caused by *Enterococcus faecium* and *Enterococcus faecalis*. Particularly worrisome is the increasing incidence of HAIs caused by VRE, and in particular vancomycin-resistant *E. faecium* (VREfm).[1,2] Vancomycin resistance is mainly

mediated by the *vanA* or *vanB* operon, which can be encoded chromosomally or extra-chromosomally on plasmids. Furthermore, these genes are often associated with transposons facilitating their horizontal gene transfer.[3,4]

The European Antimicrobial Resistance Network (EARS-Net) reported an increase in the EU and European Economic Area (EEA) population-weighted mean percentage of resistance to vancomycin among *E. faecium* isolates causing invasive infections from 10.5% in 2015 to 17.3% in 2018.[5] The increasing VREfm prevalence was observed in southern, eastern and northern Europe. In Germany, this study found an increase in vancomycin resistance rates among *E. faecium* causing invasive infections from 10.5% in 2015 to 23.8% in 2018, constituting a serious concern for patients and the healthcare system.[5] The WHO published in 2018 a list of priority bacterial pathogens for which new treatments are urgently needed, and VRE is listed in the high-priority category.[6] In addition, the CDC classified VRE as a 'serious antibiotic resistance threat'.[7]

Intestinal colonization is a prerequisite for VREfm infections and for patient-to-patient transmission of VREfm, highlighting the need for surveillance.[1] The prevalence of VREfm has been assessed in several reports focusing mainly on invasive infections occurring in ICUs.[8,9] An increase in VRE among enterococcal bloodstream infections in ICUs from 5.9% in 2007 to 16.7% in 2016 was reported in Germany.[10] A retrospective analysis of the German Antimicrobial Resistance Surveillance (ARS) database between 2012 and 2017 reported an increased prevalence of VREfm among *E. faecium* isolates, from 11.2% in 2014 to 26.1% in 2017.[11] However, the contribution of VREfm colonization in the community setting to the evolution of VREfm in the hospital remains unclear and no data on the colonization of patients with VREfm on hospital admission in Germany are currently available.

A surveillance study was conducted as part of the multicentre Resistance-Network study (R-Net) and the Antibiotic Therapy Optimisation Study (ATHOS) of the German Centre for Infection Research (DZIF). We aimed to analyse the prevalence and the molecular epidemiology of VREfm recovered from patients upon admission to German university hospitals between 2014 and 2018.

Material and methods

Study participants and settings

This prospective epidemiological survey of the prevalence of VREfm colonization on hospital admission was conducted at six German tertiary care university hospitals in the eastern (Centre A), western (Centre B), southwestern (Centres C and E), northern (Centre D) and central (Centre F) parts of the country. Patients aged ≥18 years and admitted to the hospital between April 2014 and December 2018 were included in the present study. Centre F did not participate in the survey in the years 2014–16. The number of patients included in each department corresponded to the average number of occupied beds. Patients hospitalized in the departments of ophthalmology, paediatrics and psychiatry and in ICUs were excluded.

Ethics

The R-Net and ATHOS studies were approved by the respective institutional review boards at each study site (approval numbers 16-309 and 14-170, respectively). Surveillance samples were obtained with patients' informed consent.

Bacterial isolate collection and antimicrobial susceptibility testing

Patients were screened on admission for rectal colonization with VRE using rectal swabs or stool samples. Specimens were plated on selective medium (ChromID® VRE agar, bioMérieux, Nürtingen, Germany) and incubated for 48 h. Species identification of isolates growing on selective media was performed using MALDI-TOF MS (Bruker Daltonic GmbH, Bremen, Germany). Susceptibility testing for ampicillin, linezolid, teicoplanin and vancomycin was carried out using VITEK®2 AST P592 cards (bioMérieux) and Etest (bioMérieux). MICs were interpreted using the EUCAST breakpoints for *Enterococcus* spp. (Version 9.0, January 2019, http://www.eucast.org/).

WGS

Total DNA was extracted from the bacterial isolates using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing libraries were prepared using a Nextera XT Library Prep Kit (Illumina GmbH, Munich, Germany) for a 250 bp paired-end sequencing run on an Illumina MiSeq platform. The obtained reads were *de novo* assembled with the Velvet assembler.[12] The assembled genomes were used to analyse the resistome by ResFinder v.3.1.[13] The raw sequencing reads generated in this project were submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/ena/) under the study accession number PRJEB28593.

Molecular epidemiology

The *E. faecium* MLST scheme was used to assign the ST (https://pubmlst.org/). The molecular epidemiology was investigated with a validated core genome MLST (cgMLST) scheme, including 1453 target alleles, using the Ridom SeqSphere+ v.6.0.7 software (Ridom GmbH, Münster, Germany).[14] Isolates differing in ≤20 alleles were considered as clonally related.

Results

Species distribution and antimicrobial susceptibility

Between 2014 and 2018, a total of 16350 patients were screened for rectal colonization with VRE within 3 days of hospital admission. Of these, 263 patients were found to be colonized with VREfm, accounting for a prevalence of 1.6%, while 6 patients harboured vancomycin-resistant *E. faecalis* (VREfc), corresponding to a prevalence of 0.04% (Table 1). A steady increase in the prevalence of VREfm colonizing patients upon hospital admission (2014, 0.8%; 2015, 1.2%; 2016, 1.3%; 2017, 1.5%; 2018, 2.6%) was observed in the present study (Figure 1 and Table S1, available as Supplementary data at *JAC* Online). In almost every study centre, increased rates of VREfm were detected over the study period, with the highest increase observed in Centre A (2014, 0.8%; 2015, 1.5%; 2016, 1%; 2017, 1.9%; 2018, 3.1%) (Figure 2). A total of 247 VREfm isolates were available for molecular characterization and antimicrobial susceptibility testing. Among VREfm isolates, 99.6% ($n=246$) were resistant to ampicillin, 1.6% ($n=4$) were resistant to linezolid and 21.5% ($n=53$) were resistant to teicoplanin (Table S2).

MLST and van-type distribution of VREfm

Using seven-locus MLST, all VREfm isolates were grouped into 12 STs. The distribution of STs over the study period is depicted in Table 2 and Figure 3. The predominant ST among the 247 isolates was ST117 ($n=140$, 56.7%), with a steady increase (2014, 21.7%;

Table 1. Total number of VREfm isolates recovered between 2014 and 2018 at the six study centres

	No. of isolates by year of study					
Centre	2014	2015	2016	2017	2018	total
A	15 (13)	30 (29)	5 (5)	10 (9)	16 (16)	76 (72)
B	4 (0)	6 (6)	5 (5)	7 (7)	15 (12)	37 (30)
C	10 (9)	4 (4)	10 (10)	10 (10)	15 (15)	49 (48)
D	2 (1)	3 (3)	4 (4)	9 (9)	13 (13)	31 (30)
E	0 (0)	5 (5)	9 (9)	3 (3)	6 (5)	23 (22)
F	nd	nd	nd	32 (30)	15 (15)	47 (45)
Total	31 (23)	48 (47)	33 (33)	71 (68)	80 (76)	263 (247)

Numbers in parentheses represent available VREfm isolates subjected to WGS.
nd, no data available as Centre F participated only in 2017 and 2018.

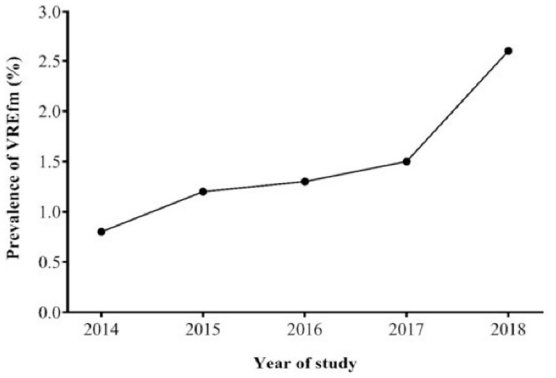

Figure 1. Increasing prevalence of VREfm carriage of patients on hospital admission between 2014 and 2018. Study Centre F did not participate in the survey between 2014 and 2016 and is not included in the figure.

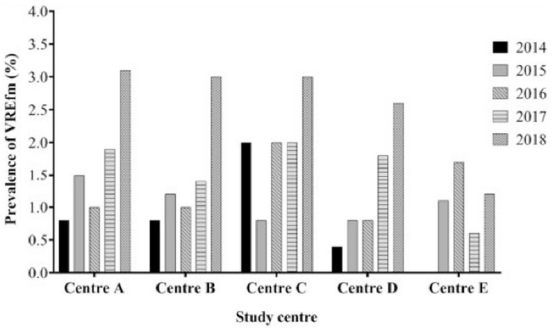

Figure 2. Increasing prevalence of VREfm carriage of patients on admission at five university hospitals between 2014 and 2018. Study Centre F did not participate in the survey between 2014 and 2016 and is not included in the figure.

2015, 29.8%; 2016, 36.4%; 2017, 72.1%; 2018, 79%) observed in the 5 year study period. ST80 ($n = 37$, 15%) was the second most predominant ST; its prevalence increased from 4.3% in 2014 to 11.8% in 2018. Next in frequency was ST203 ($n = 27$, 11%), but with decreasing prevalence from 30.4% in 2014 to 2.6% in 2018. In addition, 5.7% of VREfm isolates were assigned ST78 ($n = 14$) and 3.2% were ST17 ($n = 8$). ST18 and ST192 accounted for six isolates (2.4%) each, while five isolates (2%) were designated ST1595. Finally, four isolates were singletons belonging to ST89, ST262, ST280 and ST535, respectively (Figure 3 and Table 2). More than 90% of the MLST profiles of the investigated VREfm isolates were linked to the globally reported hospital-associated lineage clade A1.[15,16]

WGS analysis revealed that the predominant gene cluster conferring vancomycin resistance among the tested VREfm isolates was the *vanB* cluster, present in 78.5% of isolates ($n = 194$), while 20.2% of isolates ($n = 50$) harboured the *vanA* operon and 3 isolates (1.2%) harboured both *vanA* and *vanB* (Table 2). Moreover, in Centres C, E and F the *vanA* gene cluster was nearly absent (Table S3). There was a substantial increase in *vanB*-positive VREfm isolates over the study period (2014, 78.3%; 2015, 66%; 2016, 69.7%; 2017, 79.4%; 2018, 89.5%; Table S4), which was consistent with the increase in the prevalence of ST117 (2014, 21.7%; 2015, 29.8%; 2016, 36.4%; 2017, 72.1%; 2018, 79%; Table 2). Among ST117 VREfm isolates, 92.1% ($n = 129$) were *vanB* positive (Table 2). Among ST80 VREfm isolates, 28 (75.7%) harboured the *vanB* operon, whereas all ST17 isolates tested were *vanB* positive ($n = 8$). Among ST203 VREfm isolates, the prevalences of *vanA* and *vanB* were almost equal, at 51.9% ($n = 14$) and 48.1% ($n = 13$), respectively. Finally, 92.1% ($n = 13$) of ST78 isolates harboured the *vanA* operon and all three *vanAB*-positive VREfm isolates were ST117 (Table 2). Only one (2%) *vanA*-positive VREfm isolate was susceptible to teicoplanin. Finally, analysis of the resistome did not reveal any association between acquired resistance genes and *van* type among VREfm isolates (Table S5).

Core genome MLST

In the present study, cgMLST analysis was used to further characterize the epidemiology of VREfm in German university hospitals and to identify potential links between isolates.

In Centre A, the ST117 isolates ($n = 28$) formed three distinct clusters with ≤20 allelic differences, followed by ST203 ($n = 20$), which formed five small clusters. In addition, three smaller clusters of VREfm isolates representing ST18, ST78 and ST80 were observed (Figure S1 and Table 3). In Centre B (30 VREfm isolates) ST117 ($n = 18$) formed one large cluster, and two small clusters representing ST80 and ST203 were also observed (Figure S2 and Table 3). Similarly, in Centre C with a total of 48 VREfm isolates, ST117 isolates ($n = 30$) formed one large cluster, while two small clusters of VREfm isolates representing ST17 and ST80 were also detected. In addition, four ST1595 VREfm isolates formed a cluster that also included a single ST192 isolate (Figure 4 and Table 3). Also, in Centre D, ST117 was the most frequent ST, accounting for 16 among 30 VREfm isolates, with 12 isolates forming one larger cluster while smaller clusters of VREfm isolates representing ST17, ST78 and ST80 were also detected (Figure 5 and Table 3). In contrast, ST80 was the predominant ST ($n = 15$) among 22 VREfm in Centre E, which made two clusters, with a smaller ST117 cluster

Table 2. Overview of the ST types and *van* types among 247 VREfm isolates

	No. (%) of isolates representing different ST types per year						*van* type		
ST	2014	2015	2016	2017	2018	total	*vanA*	*vanB*	*vanAB*
ST117	5 (21.7)	14 (29.8)	12 (36.4)	49 (72.1)	60 (79)	140 (56.7)	8	129	3
ST80	1 (4.3)	5 (10.6)	10 (30.3)	12 (17.6)	9 (11.8)	37 (15)	9	28	0
ST203	7 (30.4)	14 (29.8)	3 (9.1)	1 (1.5)	2 (2.6)	27 (11)	14	13	0
ST78	1 (4.3)	0 (0)	4 (12.1)	6 (8.8)	3 (4)	14 (5.7)	13	1	0
ST17	0 (0)	5 (10.6)	3 (9.1)	0 (0)	0 (0)	8 (3.2)	0	8	0
ST18	3 (13)	3 (6.4)	0 (0)	0 (0)	0 (0)	6 (2.4)	0	6	0
ST192	1 (4.3)	3 (6.4)	1 (3)	0 (0)	1 (1.3)	6 (2.4)	3	3	0
ST1595	4 (17.4)	1 (2.1)	0 (0)	0 (0)	0 (0)	5 (2)	0	5	0
ST89	0 (0)	1 (2.1)	0 (0)	0 (0)	0 (0)	1 (0.4)	1	0	0
ST262	1 (4.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.4)	1	0	0
ST280	0 (0)	1 (2.1)	0 (0)	0 (0)	0 (0)	1 (0.4)	1	0	0
ST535	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.3)	1 (0.4)	0	1	0
Total	23 (100)	47 (100)	33 (100)	68 (100)	76 (100)	247 (100)	50	194	3

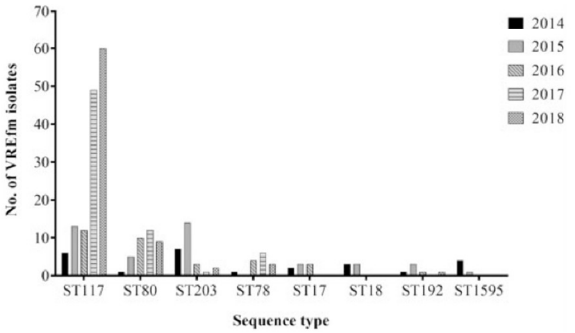

Figure 3. Temporal distribution of VREfm ST types during the 5 year study period. ST types recovered in one study year only were excluded.

also observed (Figure S3 and Table 3). Centre F comprised 45 isolates, 42 of which were ST117, and the majority formed a large transmission cluster (Figure S3 and Table 3).

We also analysed all VREfm isolates together by cgMLST to investigate whether there were national VREfm clusters expanding across the study sites. This revealed that ST117 *vanB*-positive VREfm isolates formed one large complex of 96 closely related isolates differing in fewer than 20 alleles that was found extending across all six study centres (Figures 6, S4 and S5). Of these, 85 isolates (88.5%) were assigned to the cgMLST cluster type (CT) 71 (SeqSphere nomenclature) and 11 isolates (11.5%) to CT469. In addition, ST117 formed four smaller clusters comprising 13, 5, 4 and 3 isolates. In one cluster only isolates from one study centre (Centre A) were represented, while the other three clusters also extended across multiple study sites (Centres A and C, Centres A and F, and Centres B, C, D and E).

In contrast to the inter-regional clustering of VREfm ST117 isolates, this was rarely observed for the other MLST types detected in the present study. For example, separate clusters of ST17 were found in Centres C and D, and separate clusters of ST78 were detected in Centres A and D, while isolates representing ST18 were limited to Centre A (Figures 6 and S6). ST203 VREfm isolates formed four different clusters limited to Centre A, while a fifth cluster comprised isolates shared between Centres A and B (Figures 6 and S6). Finally, independent ST80 clusters were found in Centres B, C and E, but there were a few exceptions within this ST, indicating inter-regional clustering, with two smaller clusters sharing isolates between Centres A and D as well as between Centres E and F (Figures 6 and S7).

Figures 4 and 5, representing the results of cgMLST analysis of VREfm isolates for Centres C and D, are included as examples in the main body of the present article, while the results of cgMLST analysis of isolates from Centres A, B, E and F are included as Supplementary data.

Discussion

Increasing rates of VREfm among enterococcal bloodstream infections have been observed in many countries in Europe, including Germany and its neighbouring countries, such as Belgium, Czech Republic, Denmark and Poland.[5] Many studies have reported on the transmission of VRE in the hospital setting and numerous outbreaks of VRE infections have been observed.[17,18] However, there are few available data about the import of VRE from the community into the hospital. The VRE admission prevalence was 3.6% in the surgical ICU of a German university hospital and 13% in the general ICU of two Greek hospitals.[8,19] In the present study, we found a prevalence of VREfm of 1.6% among patients admitted to general wards at six German university hospitals over a 5 year period. In our study, the admission prevalence of VREfc, the second clinically most important enterococcal species, was rather low (0.04%) in the participating study centres, confirming that vancomycin resistance among *E. faecalis* isolates is currently exceedingly rare in Germany.[20]

Resistance to ampicillin was almost universally present in VREfm colonizing isolates, confirming previous studies that have analysed hospital-adapted *E. faecium* isolates.[1,20,21] In 2018,

Table 3. Overview of the ST types grouped by study centre

	No. (%) of isolates representing different ST types for Centres A–F						
ST	A	B	C	D	E	F	total
ST117	28 (38.9)	18 (60)	30 (62.5)	16 (53.3)	6 (27.3)	42 (93.3)	140 (56.7)
ST80	6 (8.3)	4 (13.3)	6 (12.5)	4 (13.3)	15 (68.2)	2 (4.4)	37 (15)
ST203	20 (27.8)	6 (20)	0 (0)	1 (3.3)	0 (0)	0 (0)	27 (10.9)
ST78	8 (11.1)	0 (0)	1 (2.1)	5 (16.7)	0 (0)	0 (0)	14 (5.7)
ST17	1 (1.4)	0 (0)	5 (10.4)	2 (6.7)	0 (0)	0 (0)	8 (3.2)
ST18	6 (8.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6 (2.4)
ST192	1 (1.4)	2 (6.7)	1 (2.1)	2 (6.7)	0 (0)	0 (0)	6 (2.4)
ST1595	0 (0)	0 (0)	5 (10.4)	0 (0)	0 (0)	0 (0)	5 (2)
ST89	0 (0)	0 (0)	0 (0)	0 (0)	1 (4.5)	0 (0)	1 (0.4)
ST262	1 (1.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.4)
ST280	1 (1.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.4)
ST535	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.2)	1 (0.4)
Total	72 (100)	30 (100)	48 (100)	30 (100)	22 (100)	45 (100)	247 (100)

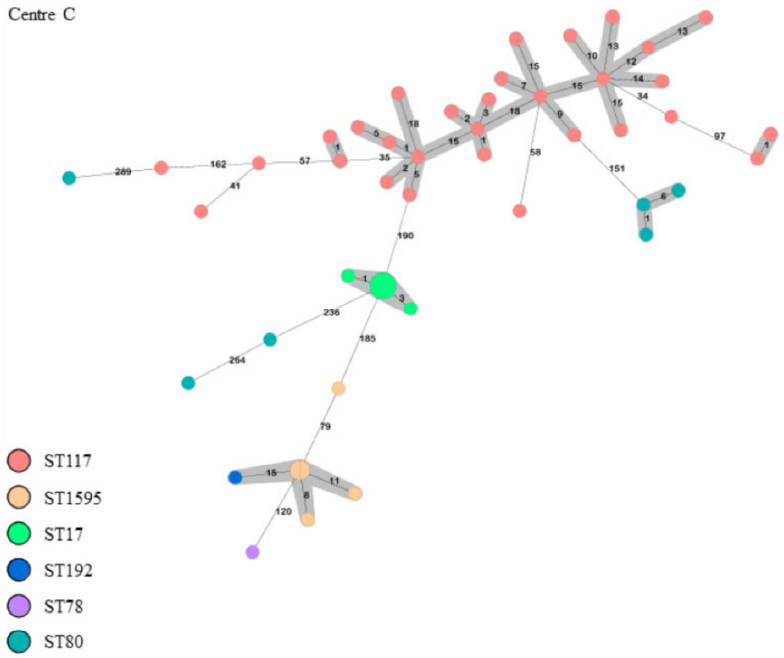

Figure 4. Minimum spanning tree generated using Ridom SeqSphere+ for Study Centre C grouped and coloured by ST type, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 1453 cgMLST target genes. The size of the circles represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

the German National Reference Centre for Staphylococci and Enterococci (NRC) received 1540 VREfm isolates from German laboratories for further analysis, of which 77.2% were *vanB* positive and 21.4% *vanA* positive.[22] In concordance with these findings, in the present study the *vanB* type was the predominant glycopeptide resistance determinant, accounting for 78.5% of VREfm

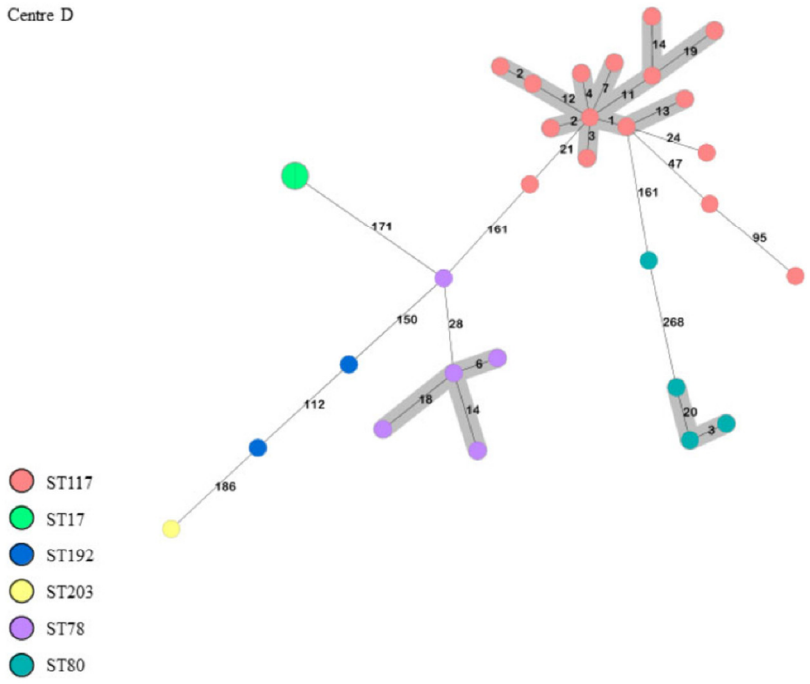

Figure 5. Minimum spanning tree generated using Ridom SeqSphere+ for Study Centre D grouped and coloured by ST type, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 1453 cgMLST target genes. The size of the circles represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

isolates detected in patients on admission, while the *vanA* type was rarely encountered in Centres C, E and F. Resistance to linezolid was still low (1.6%) among colonizing VREfm isolates, comparable to results of earlier studies.[23] In contrast, the German NRC reported an increase in linezolid resistance among *E. faecium* from <1% in 2008 to >9% in 2014 in Germany.[24] However, it has to be noted that the German NRC frequently receives isolates because they exhibit unusual resistance phenotypes, and therefore linezolid resistance may be overestimated and not be representative of unselected isolates.

The dominant MLST type among the VREfm colonizing isolates obtained by rectal admission screening was ST117 (56.7%), and of these more than 90% were *vanB*-type VREfm. The continuous increase in the prevalence of ST117 observed at all our study sites over the 5 year study period is consistent with the national trend reported by the German NRC with ST117 representing the most frequent ST, accounting for 50% of *E. faecium* blood isolates in 2017 and 58% in 2018.[22] An increasing prevalence of ST117 has also been observed in a Spanish tertiary care hospital where the ST117 clone has become endemic since 2009.[25] Similarly, in a hospital in Switzerland ST117 (36/156), along with ST80 (77/156) and ST17 (30/156), represented the predominant STs causing sequential outbreaks between 2014 and 2017.[26]

Between 2011 and 2014, the second most predominant ST type among bloodstream isolates in Germany was ST203, followed by ST192.[27] In our survey, the second most frequent ST type, ST80, showed increasing prevalence, from 4.3% in 2014 to 11.8% in 2018, and replaced ST203, which experienced a reverse trend and decreased in prevalence from 30.4% in 2014 to 2.6% in 2018. These data indicate the dynamic evolution of hospital-associated VREfm isolates. In addition, more than 90% of the ST types found in this study, which includes ST117, represent the successful hospital-adapted lineage clade A1.[15,16]

In the present study, ST117 was the predominant clonal lineage at five of the six study sites. Almost 70% of the ST117 *vanB*-positive VREfm isolates formed one large multi-centre cluster of closely related genotypes, differing in ≤20 cgMLST alleles that were dispersed over six geographically separated study centres, indicating inter-regional spread. In addition, 88.5% of these isolates were classified as CT71. This result mirrors recent findings suggesting the near-ubiquitous presence of an ST117/CT71/*vanB* clone within the Frankfurt metropolitan area in Germany,[28] as well as data from the German NRC indicating that the ST117/CT71/*vanB* clone accounted for 39% (69/176) of VREfm bloodstream isolates recovered from patients hospitalized in various regions in Germany in 2018.[22] Recently, ST117/CT71/*vanB* was also identified as one of the main clonal lineages causing nosocomial outbreaks in different hospitals in Bavaria, Germany.[29]

Among the other ST types we identified there were significant regional differences. While ST117 had a nationwide distribution, the other ST types primarily formed centre-specific clusters. ST80 is a single-locus variant (SLV) of the widely disseminated VREfm

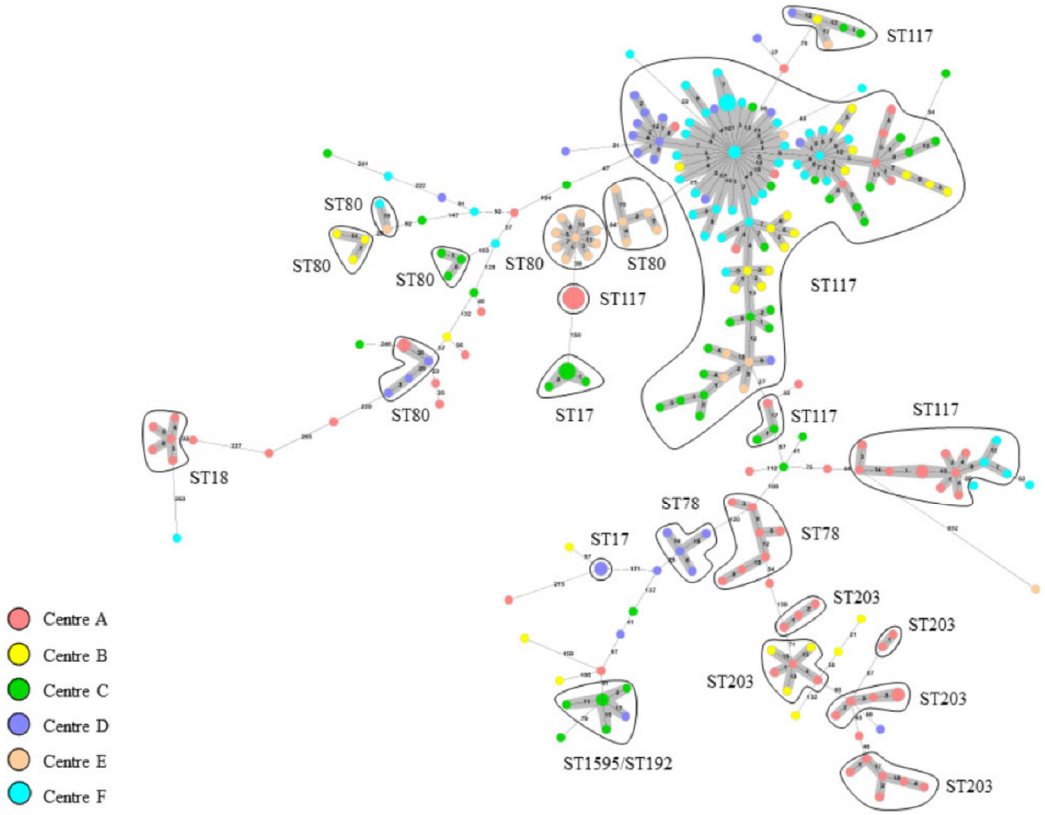

Figure 6. Minimum spanning tree generated using Ridom SeqSphere+ for 247 samples grouped and coloured by study centre, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 1453 cgMLST target genes. The size of the circles represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

clone ST117 and also belongs to the hospital-adapted lineage clade A1.[15,16] In a study centre in the south-west of Germany, a sharp increase in VREfm has been observed since 2015 resulting from local transmission of ST80 VREfm, indicating that this ST may be a challenge for the regional healthcare system.[17] ST80 was the second most prevalent ST type in the present study, forming mostly local clusters in five of the six study centres; however, we identified only two small clusters of closely related ST80 VREfm isolates that were recovered at two different study sites, indicating inter-regional spread. Similarly, centre-specific clusters were detected for ST17 and ST78 in Centres C and D and Centres A and D, respectively. In addition, a cluster of isolates representing ST18 and four clusters representing ST203 were limited to Centre A.

Our study has several limitations. The centres involved in the study are tertiary care hospitals, and many patients have frequent hospital contacts, which could limit the transferability of our results to the patient population seeking healthcare in community hospitals. In fact, our data do not reflect community acquisition of VREfm but also include healthcare-associated acquisition during previous healthcare contacts. Thus, our data are not representative of the general population. Also, since our screening window was 72 h, we cannot exclude that some patients might in fact have acquired VREfm after admission. Furthermore, the design of the present study does not allow the prevalence of vancomycin-susceptible *E. faecium* on hospital admission to be determined. In addition, no data about the clinical course of the patients colonized with VREfm were collected and no data were obtained regarding the molecular evolution of VREfm clinical infections potentially caused by the strain recovered during admission screening. Another limitation of the study design could be the screening sensitivity, as these prevalence data are based on a single rectal swab obtained upon hospital admission, which could lead to an underestimation because of low-density rectal VREfm carriage.

In conclusion, our data indicate that the VREfm epidemiological profile in Germany has changed in the past 5 years and confirm the remarkable clonal expansion of the ST117/*vanB* clone. Import of VREfm into the hospital through patients already colonized

on admission is a critical issue that could increase the risk of further transmission within the hospital setting and should prompt consideration of infection control interventions, at least in high-risk settings.[1,30] To our knowledge, the present work constitutes the largest study assessing the prevalence and molecular epidemiology of VREfm isolates recovered from patients at hospital admission and may contribute to further elucidation of the complex epidemiology of VREfm in the hospital setting.

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Transparency declarations

None to declare.

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Supplementary data

Tables S1–S5 and Figures S1–S7 are available as Supplementary data at *JAC* Online.

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Supplementary data

Table S1: Number of patients screened per study centre over the study period.

	Year of study					
	2014	2015	2016	2017	2018	Total
Centre A	1994	2019	487	515	514	5529
Centre B	500	499	499	500	499	2497
Centre C	503	502	495	501	499	2500
Centre D	473	387	498	500	500	2358
Centre E	406	471	520	505	515	2417
Centre F	0	0	0	763	286	1049
Total	3876	3878	2499	3284	2813	16350

Table S2: Antimicrobial resistance phenotype among 247 VREfm isolates.

		No. (%) of resistant isolates per study centre		
	No. of isolates	AMP	LZD	TEC
Centre A	72	72 (100)	1 (1.4)	23 (31.9)
Centre B	30	30 (100)	2 (6.7)	13 (43.3)
Centre C	48	48 (100)	0 (0)	4 (8.3)
Centre D	30	30 (100)	0 (0)	9 (30.0)
Centre E	22	21 (95.5)	1 (4.5)	3 (13.6)
Centre F	45	45 (100)	0 (0)	1 (2.2)
Total	247	246 (99.5)	4 (1.6)	53 (21.5)

Abbreviations: AMP, ampicillin; LZD, linezolid; TEC, teicoplanin.

Table S3: Distribution of VREfm *van* types per study centre.

	No. (%) of isolates representing different *van* type			
	vanA	*vanB*	*vanAB*	Total
Centre A	21 (29.2)	49 (68)	2 (2.8)	72 (100)
Centre B	13 (43.3)	17 (56.7)	0 (0)	30 (100)
Centre C	3 (6.2)	45 (93.7)	0 (0)	48 (100)
Centre D	11 (36.7)	19 (63.3)	0 (0)	30 (100)
Centre E	2 (9.1)	20 (90.9)	0 (0)	22 (100)
Centre F	0 (0)	44 (97.8)	1 (2.2)	45 (100)
Total	50 (20.2)	194 (78.5)	3 (1.2)	247 (100)

Table S4: Distribution of *van* genotypes in VREfm over the study period.

	No. (%) of isolates representing different *van* types per year					
	2014	2015	2016	2017	2018	Total
vanA	5 (21.7)	16 (34)	9 (27.3)	13 (19.1)	7 (9.2)	50 (20.2)
vanB	18 (78.3)	31 (66)	23 (67.9)	54 (79.4)	68 (89.5)	194 (78.5)
vanAB	0 (0)	0 (0)	1 (3)	1 (1.5)	1 (1.3)	3 (1.2)
Total	23 (100)	47 (100)	33 (100)	68 (100)	76 (100)	247 (100)

Table S5: Antibiotic resistance genes detected in 247 VREfm isolates grouped by *van* type.

Antibiotic class	Resistance gene	No. of isolates representing different *van* types			
		vanA (n=50)	*vanB* (n=194)	*vanAB* (n=3)	Total (n=247)
Aminoglycosides	*ant(6)-Ia*-like, *aph(3')-III*-like	11	58	2	71
	ant(6)-Ia	16	1	0	17
	aph(3')-III	1	7	0	8
	aac(6')-aph(2'')-like, *ant(6)-Ia*, *aph(3')-III*	1	4	0	5
	ant(6)-Ia, *spc*	3	0	0	3
	spc-like	0	1	0	1
	aac(6')-aph(2'')-like, *ant(6)-Ia*	2	0	0	2
Macrolides,	*erm*(B)-like	42	176	3	221
lincosamides and	*erm*(T)-like	1	5	0	6
streptogramin B	*erm*(A), *erm*(B)-like	3	1	0	4
	erm(B), *erm*(T)	2	0	0	2
	msr(C)-like	50	193	3	246
Phenicols	*cat*(pC221)-like	21	1	1	23
	cat-like	0	3	0	3
	cat(pC221)-like, *cat*-like	1	0	0	1
Tetracyclines	*tet*(L)-like, *tet*(M)-like	3	25	0	28
	tet(M)-like	14	2	0	16
	tet(M), *tet*(U)-like	4	5	0	9
	tet(U)-like	8	4	1	13
	tet(L)	1	1	0	2
	tet(L)-like, *tet*(M)-like, *tet*(U)-like	0	1	0	1
Trimethoprim	*dfrG*-like	27	141	3	171

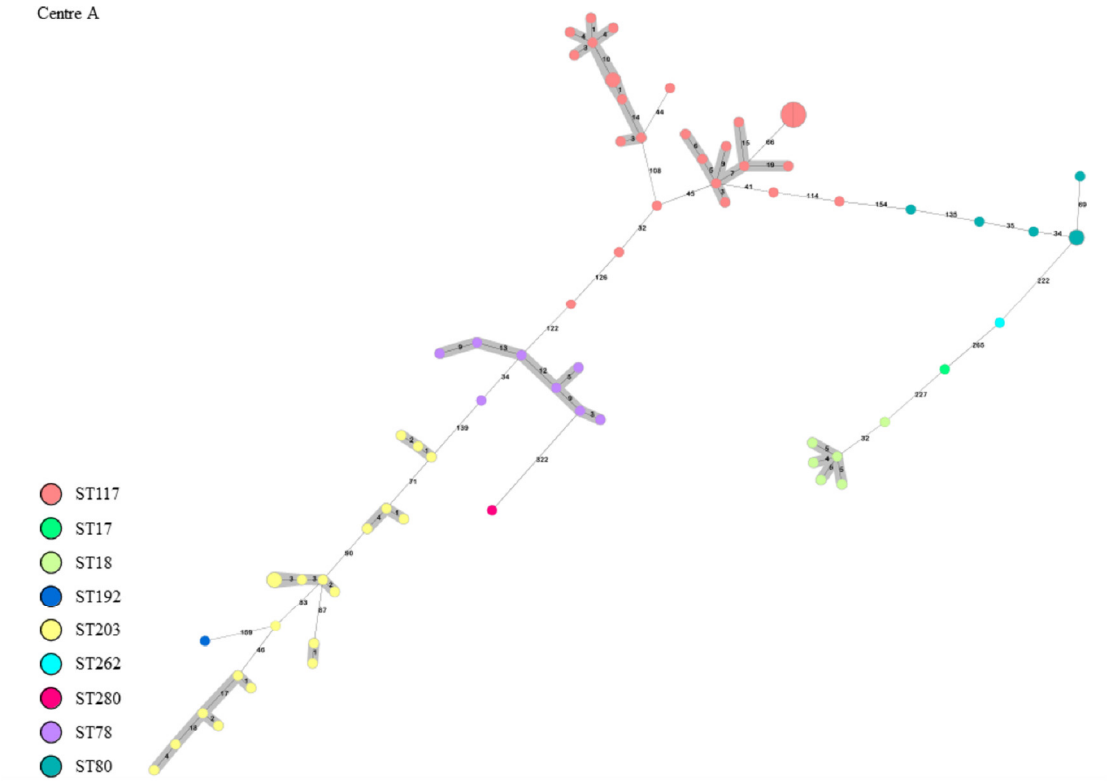

Figure S1. Minimum spanning tree generated using Ridom SeqSphere+ for study centre A grouped and coloured by ST type, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 1453 cgMLST target genes. The size of the circle represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

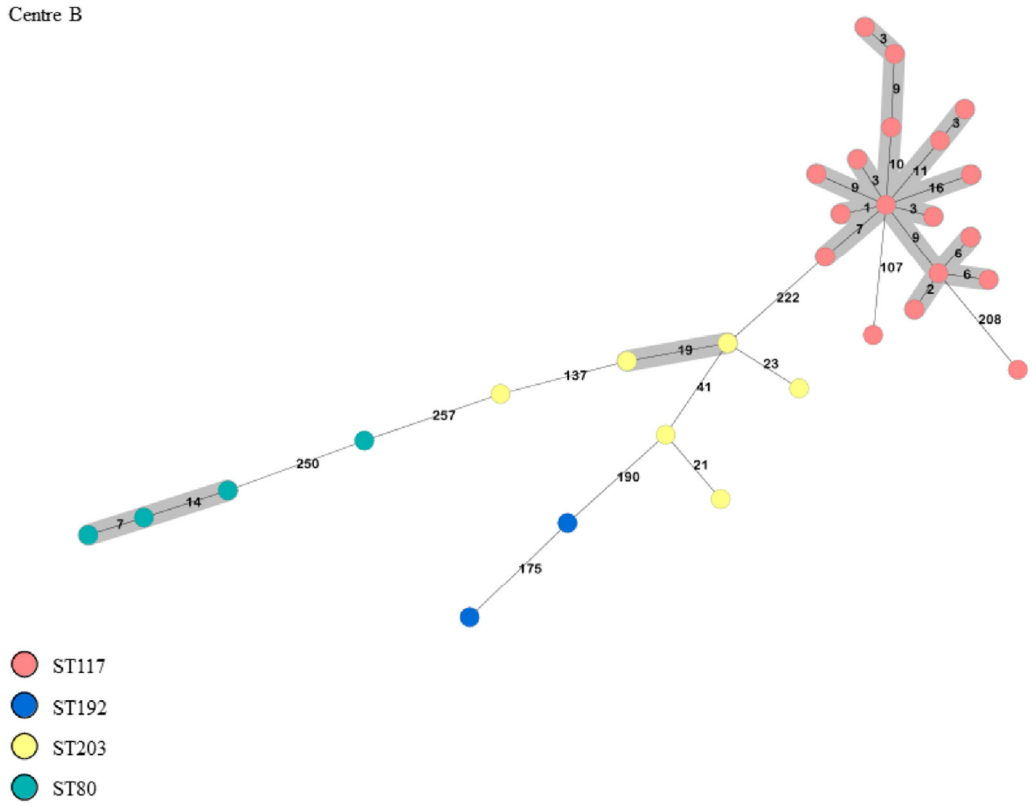

Figure S2. Minimum spanning tree generated using Ridom SeqSphere+ for study centre B grouped and coloured by ST type, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 1453 cgMLST target genes. The size of the circle represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

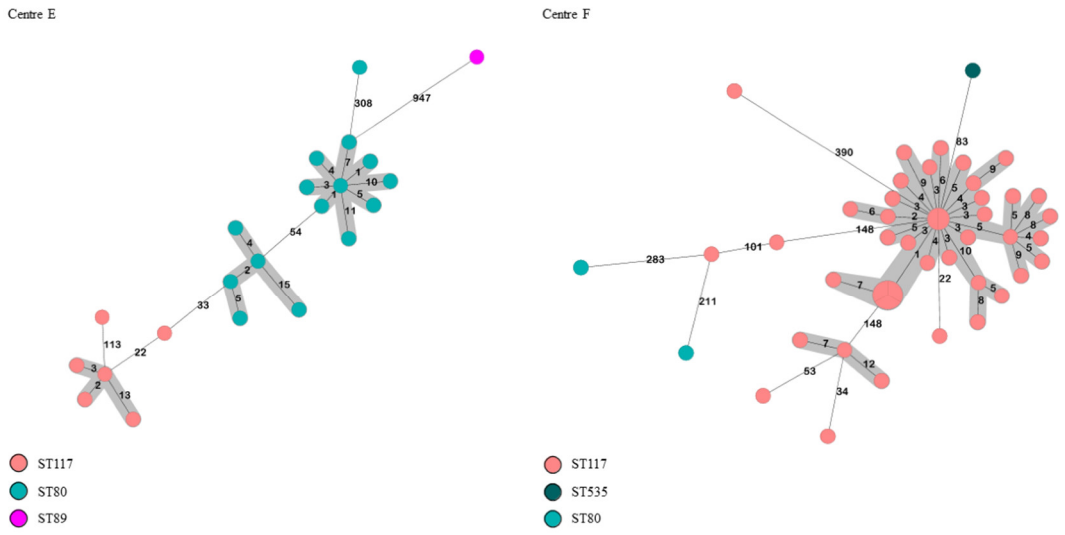

Figure S3. Minimum spanning tree generated using Ridom SeqSphere+ for study centre D and F grouped and coloured by ST type, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 1453 cgMLST target genes. The size of the circle represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

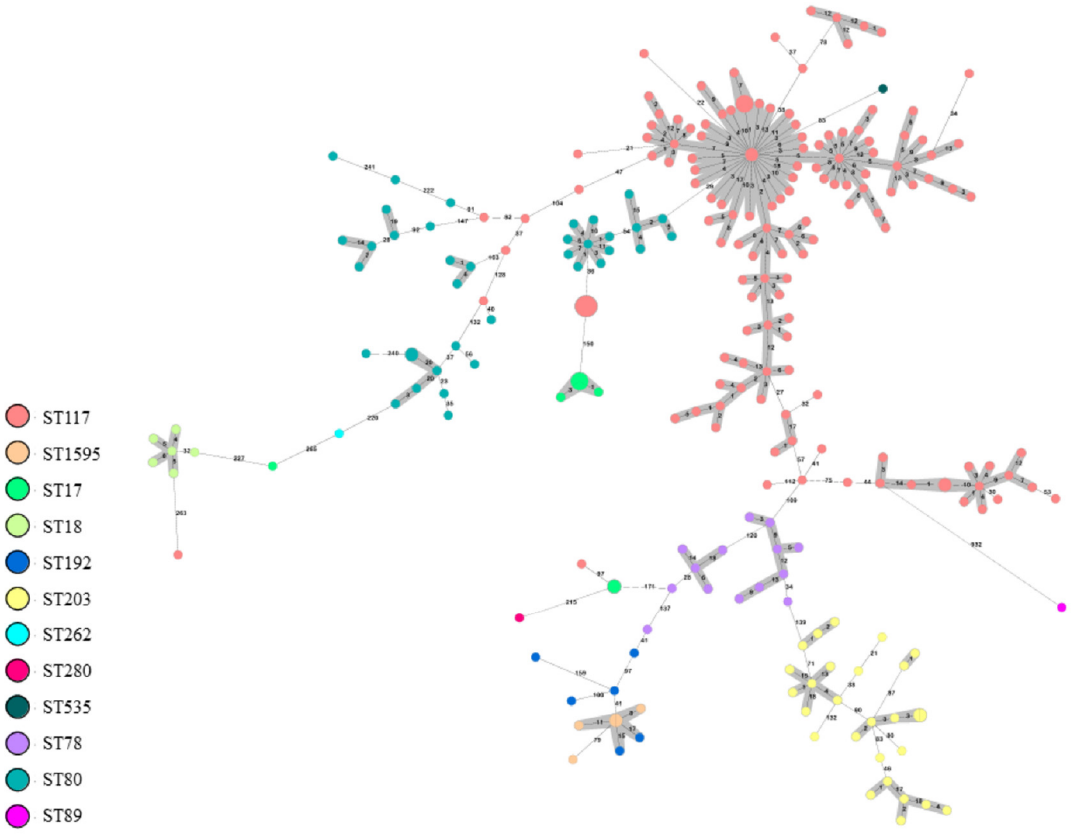

Figure S4. Minimum spanning tree generated using Ridom SeqSphere+ for 247 samples grouped and coloured by ST type, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 1453 cgMLST target genes. The size of the circle represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

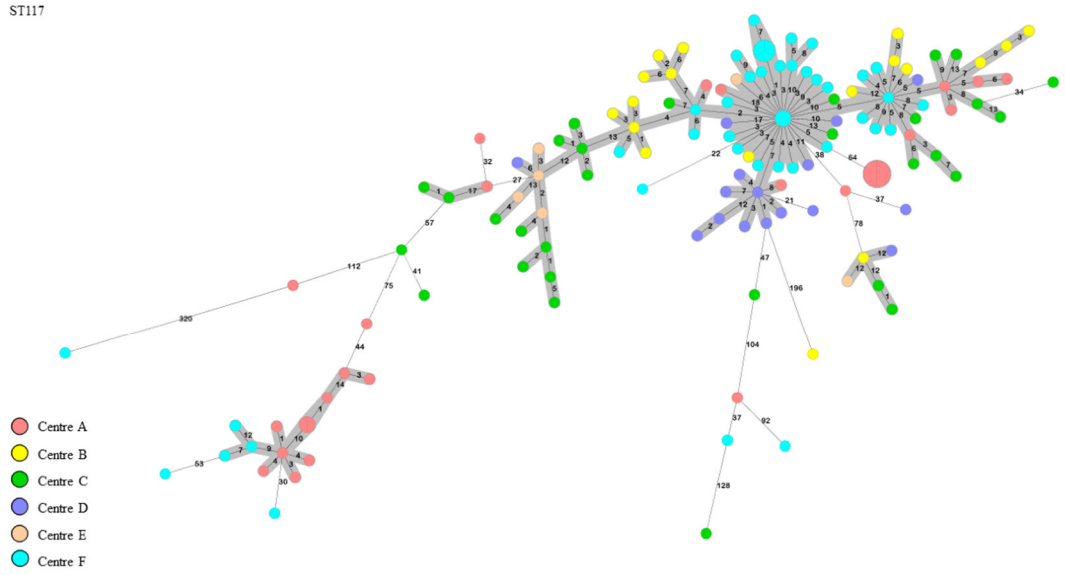

Figure S5. Minimum spanning tree generated using Ridom SeqSphere+ for ST117 VREfm isolates (n=140) grouped and coloured by study centre, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 1453 cgMLST target genes. The size of the circle represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

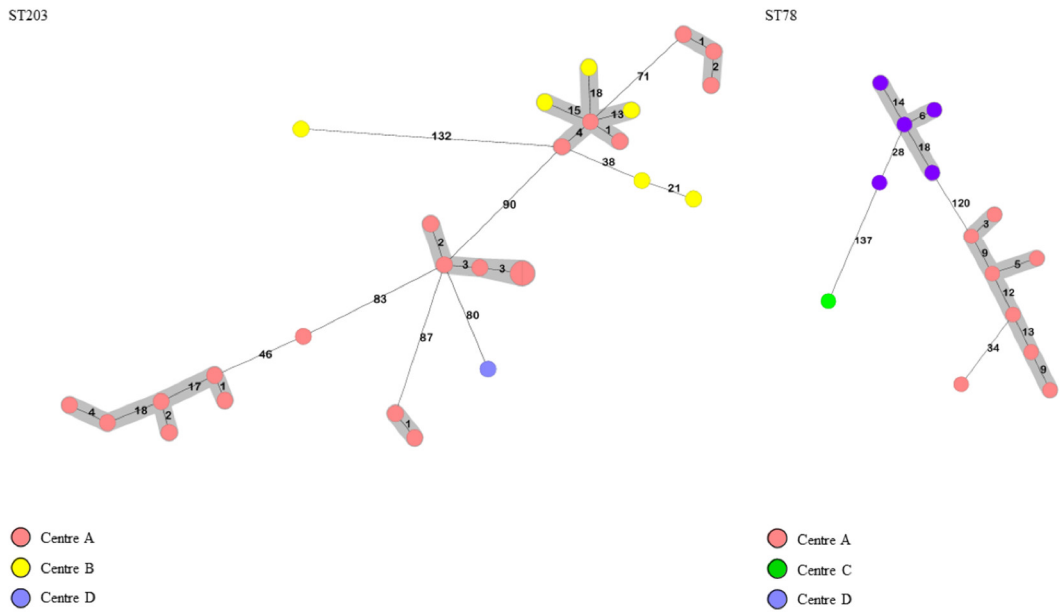

Figure S6. Minimum spanning tree generated using Ridom SeqSphere+ for ST203 VREfm isolates (n=27) and ST78 VREfm isolates (n=14) grouped and coloured by study centre, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 1453 cgMLST target genes. The size of the circle represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

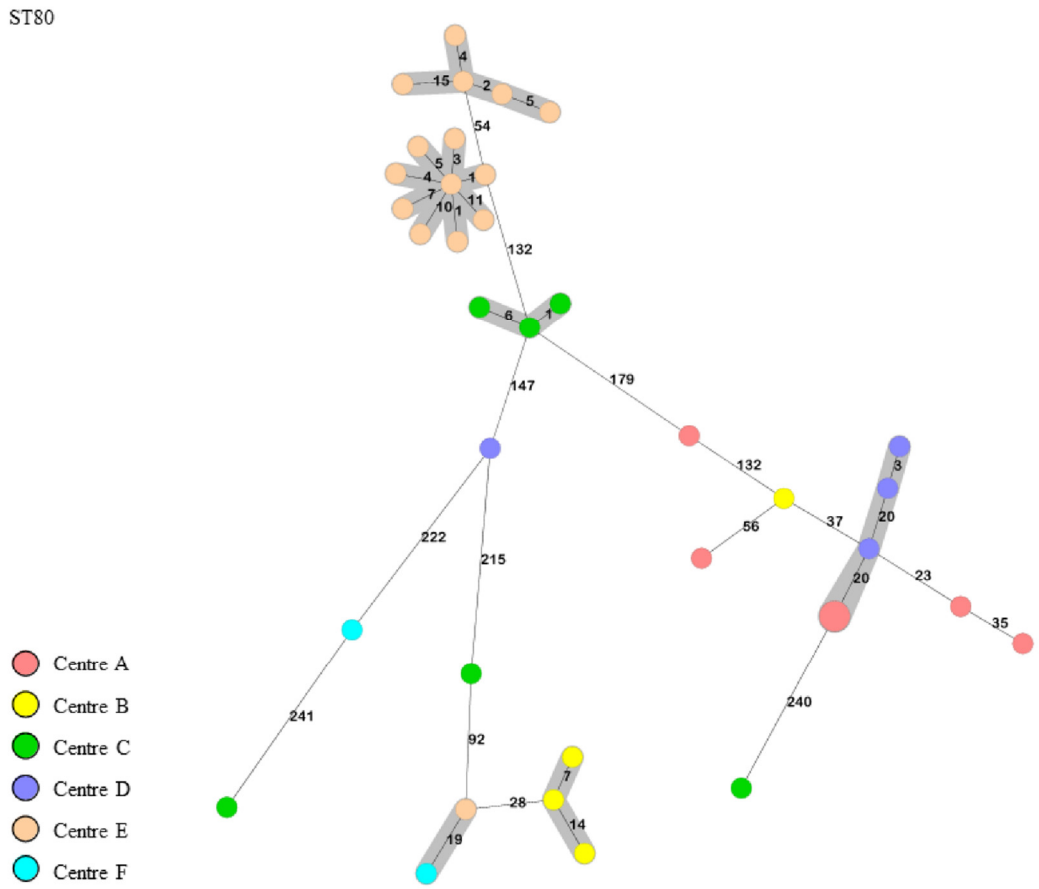

Figure S7. Minimum spanning tree generated using Ridom SeqSphere+ for ST80 VREfm isolates (n=37) grouped and coloured by study centre, ignoring missing values. Each circle represents one isolate from an individual patient based on sequence analysis of 1453 cgMLST target genes. The size of the circle represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences. Closely related genotypes (≤20 alleles different) are shaded grey.

2.2 Population structure of third-generation cephalosporin-resistant and carbapenem-resistant *K. pneumoniae* in Germany

With the advent of NGS and large number of genomes being sequenced, the taxonomy of *K. pneumoniae* has been recently updated and expanded to the *K. pneumoniae* complex which contains the species *K. pneumoniae, Klebsiella quasipneumoniae* subsp. *quasipneumoniae*, *Klebsiella quasipneumoniae* subsp. *similipneumoniae*, *Klebsiella variicola* subsp. v*ariicola*, *Klebsiella variicola* subsp. *tropicalensis, Klebsiella quasivariicola* and *Klebsiella africanensis.* Among these species, *K. pneumoniae* has the greatest clinical relevance. Nevertheless, other members have been also involved in clinical infections or nosocomial outbreaks.[81-85] Of particular concern are the increasing rates of third-generation cephalosporin-resistant (3GCR) and carbapenem-resistant *K. pneumoniae*. In Europe in 2018 31.7% and 7.5% of *K. pneumoniae* isolates causing invasive infections were 3GCR and carbapenem-resistant, respectively.[21]

The prevalence and molecular epidemiology of 3GCR and carbapenem-resistant *K. pneumoniae* complex isolates colonizing patients on hospital admission to six German tertiary care university hospitals, in the eastern (Centre A), western (Centre B), southwestern (Centre C), central (Centre E), northern (Centre D) and southwestern (Centre F) parts of the country were investigated. The number of patients included in each department corresponded to the average number of occupied beds. Patients aged ≥18 years and admitted to the hospital between January 2016 and December 2018 were included in the present study. 3GCR was defined as minimum inhibitory concentration (MIC) ≥2 mg/L for cefotaxime or ceftazidime while carbapenem resistance was defined as MIC ≥4 mg/L for imipenem or meropenem. The resistance phenotype was assessed by the automated VITEK®2 system and Etest (bioMérieux, Nürtingen, Germany). Furthermore, the recovered samples were subjected to WGS and downstream analysis including MLST (https://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html), cgMLST typing (Ridom® SeqSphere+) and resistome analysis (https://cge.cbs.dtu.dk/services/ResFinder/).[86] Identification to species level was confirmed by the JSpeciesWS webserver (http://jspecies.ribohost.com/jspeciesws/) by determining the average nucleotide identity based on BLAST+ (ANIb).

In total 8596 patients were screened upon hospital admission and 80 3GCR *Klebsiella* spp. were recovered corresponding to a prevalence of 0.9%. A steady increase in the prevalence of

3GCR *K. pneumoniae* complex (2016, 0.8%; 2017, 0.9%; 2018, 1.1%) was observed in the present study. Of these 3GCR isolates, 76 were available for molecular characterization. Among these isolates 71 were identified as *K. pneumoniae* and harboured the species-intrinsic SHV-like, three as *K. variicola* subsp. *variicola* and two as *K. quasipneumoniae* subsp. *quasipneumoniae* encoding the LEN-type and OKP-type intrinsic β-lactamases, respectively. Furthermore, in the present study the most prevalent β-lactam resistance determinants in *K. pneumoniae* isolates were the CTX-M-type ESBLs followed by TEM-1B. Lastly, only one *K. variicola* isolate exhibited a carbapenem non-susceptible phenotype and harboured the $bla_{\text{OXA-181}}$, an OXA-48 variant (Table 1).

Table 1. Acquired β-lactamase-encoding genes detected in 76 3GCR *K. pneumoniae* complex isolates grouped by species.

Antibiotic class	Resistance gene	No. of isolates harbouring β-lactamases		
		K. pneumoniae (n=71)	*K. variicola* (n=3)	*K. quasipneumoniae* (n=2)
β-lactams	$bla_{\text{CTX-M-15}}$	43	0	2
	$bla_{\text{CTX-M-14}}$	9	0	0
	$bla_{\text{CTX-M-1}}$	1	0	0
	$bla_{\text{CTX-M-3}}$	1	0	0
	$bla_{\text{CTX-M-27}}$	1	0	0
	$bla_{\text{CTX-M-55}}$	1	0	0
	$bla_{\text{TEM-1B}}$	35	0	1
	$bla_{\text{OXA-1}}$	17	0	0
	$bla_{\text{OXA-181}}$	0	1	0

MLST typing revealed that the most prevalent ST in *K. pneumoniae* was ST307, followed by ST45, ST17 and ST219 while, the *K. variicola* and *K. quasipneumoniae* isolates were singletons (Table 2). The molecular epidemiology of the isolates was studied using cgMLST. Clonal clusters were rare in the present study and included a ST307 cluster of closely related *K. pneumoniae* isolates (n=5) recovered from two different study centres (A and D). In centre A, two clusters of closely related isolates of ST20 and ST3191 isolates were detected. Another

cluster of two ST14 *K. pneumoniae* isolates were observed in centre C and two ST219 colonizing isolates from two different centres were closely related. Finally, although there was no clonal relatedness observed between the three *K. variicola* isolates, these isolates clustered together and had more than 2000 alleles difference from the *K. pneumoniae* isolates. The same was observed for the two *K. quasipneumoniae* isolates (Figure 4).

Table 2. Distribution of ST types during the three-year study period. In total 34 *K. pneumoniae* isolates were singletons and three had an unknown ST and were excluded from the present table.

ST	No. of isolates		
	K. pneumoniae (n=71)	*K. variicola* (n=3)	*K. quasipneumoniae* (n=2)
307	7	0	0
45	7	0	0
17	4	0	0
219	4	0	0
14	3	0	0
405	3	0	0
20	2	0	0
29	2	0	0
3191	2	0	0
208	0	1	0
1563	0	1	0
1599	0	1	0
1040	0	0	1
2010	0	0	1

Here we could demonstrate that among 3GCR *K. pneumoniae* isolates, CTX-M-15 was the dominant β-lactamase followed by TEM-1B. Based on the cgMLST results of the present study, it becomes evident that there was no dominant clone among K. *pneumoniae* isolates colonizing patients on hospital admission in the six tertiary care hospitals in Germany. Only small clusters of closely related isolates could be identified indicating that a diverse *K.*

pneumoniae population is circulating in the country. Finally, these data demonstrate that carriage of 3GCR and carbapenem-resistant in *K. pneumoniae* complex in hospitalized patients on admission is still rare in Germany.

The isolates of the present study were collected as part of the multicentre Resistance-Network (R-Net) study of the German Centre for Infection Research (DZIF). Preliminary data of this study have been presented as a poster at the German Society of Infectious Diseases and DZIF Joint Annual Meeting in Bad Nauheim, Germany, in November 2019.[87] Finally, preliminary data was accepted as a poster presentation at the 30th European Congress of Clinical Microbiology and Infectious Diseases in Paris, France in April 2020. The conference was cancelled due to the SARS-CoV-2 pandemic.

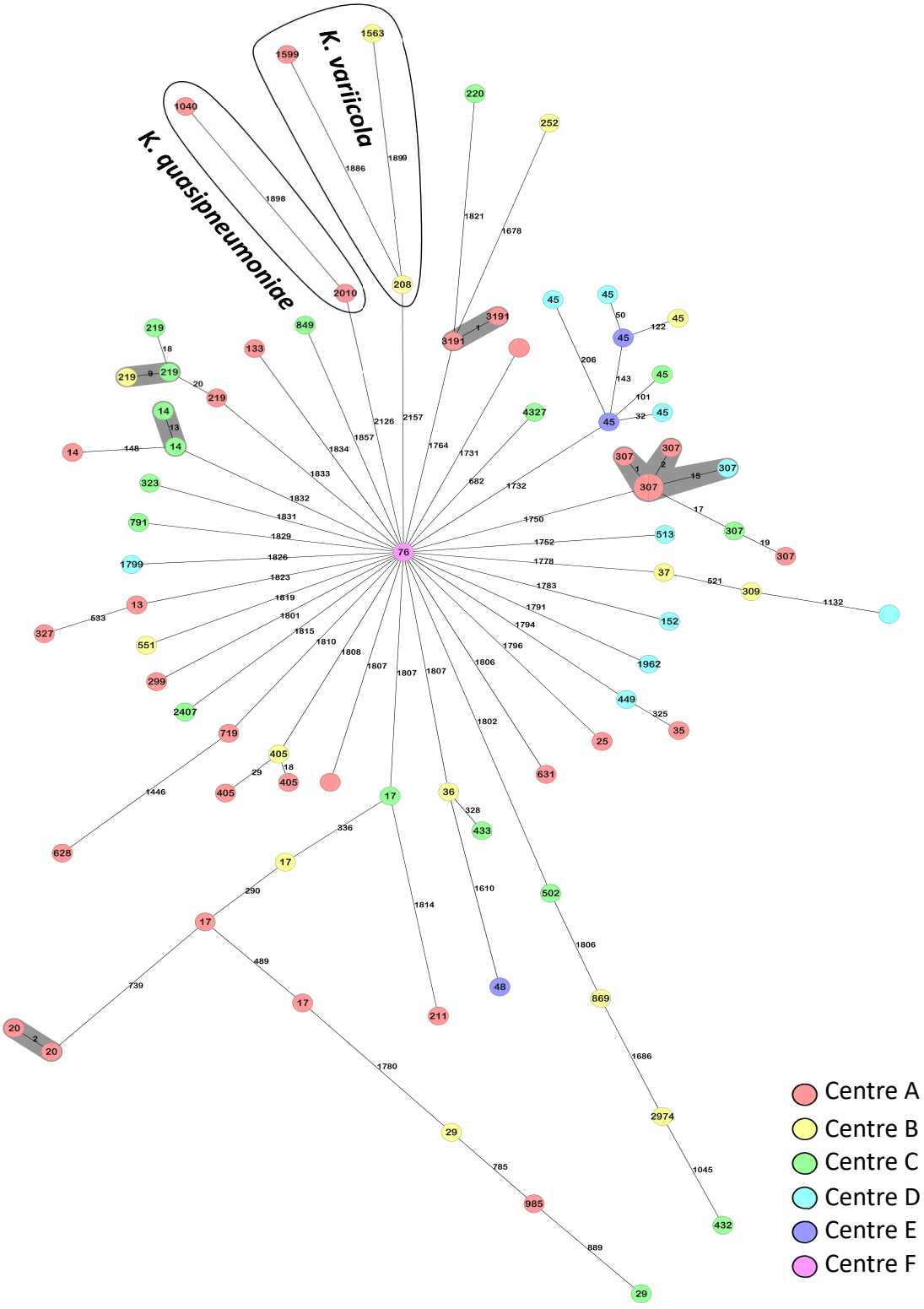

Figure 4. Minimum spanning tree generated using Ridom SeqSphere+ for the 76 3GCR *Klebsiella* spp. isolates coloured by study centre and labelled by ST. For three isolates, no ST could be assigned. Each coloured circle represents one individual isolate based on sequence

analysis of 2358 *sensu lato* cgMLST target genes and ignoring missing values. Closely related genotypes (up to 15 alleles difference) are shaded grey.

2.3 *A. baumannii* analysis by cgMLST in two hospitals in Bolivia: endemicity of international clone 7 isolates

Of major concern are the increasing rates of MDR *A. baumannii* infections worldwide and in particular resistance to carbapenems, the last resort antibiotics.[11, 12] In the following publication, we have investigated 95 *A. baumannii* isolates recovered from two hospitals in Cochabamba, Bolivia. Out of these, 51 isolates were CRAb and along with four carbapenem-susceptible isolates were further investigated by WGS and cgMLST. The vast majority (91%) of the CRAb isolates were identified as ST25 or ST991, a single locus variant (SLV) of ST25, according to the Pasteur scheme [clonal complex 25 (CC25)]. Molecular typing revealed that 50 out of 51 CRAb belonged to international clone (IC) 7 and formed five transmission clusters while one isolate was assigned to IC4. Furthermore, all CRAb isolates harboured the carbapenemase OXA-23 encoded on a Tn*2008*. Among the four carbapenem-susceptible isolates one belonged to IC5 while the remaining three did not cluster to a known IC. These data demonstrate the molecular epidemiology and endemicity of several IC7 OXA-23-positive CRAb clones in two hospitals in Cochambamba, Bolivia.

In the present study, I was involved in the phenotypic and genotypic characterization and data analysis of the investigated isolates as also in reviewing and editing the manuscript.

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Short Communication

Acinetobacter baumannii analysis by core genome multi-locus sequence typing in two hospitals in Bolivia: endemicity of international clone 7 isolates (CC25)

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ABSTRACT

In total, 95 *Acinetobacter baumannii* isolates recovered from patients from two hospitals in Cochabamba, Bolivia were studied. The presence of class D and B β-lactamases was investigated using polymerase chain reaction, and antimicrobial susceptibility testing was performed by agar dilution and broth microdilution. The resistance rate to carbapenems was 53.7%. All carbapenem-resistant *A. baumannii* (CRAb, n=51) and four carbapenem-susceptible isolates were further analysed by whole-genome sequencing. The resulting genome assemblies were used to identify the acquired resistome, and core genome multi-locus sequence typing (cgMLST) was used to determine their molecular epidemiology. All but one of the CRAb isolates (n=50) belonged to international clone (IC) 7 and they clustered into five sequence types; on cgMLST, they were found to be separated by ≥40 alleles. All CRAb isolates carried bla_{OXA-23} on transposon Tn*2008*. Metallo-β-lactamases were not detected. These data show that dissemination of several IC7 *A. baumannii* clones harbouring the carbapenem resistance determinant bla_{OXA-23} is occurring in these two hospitals in Cochambamba.

1. Introduction

Acinetobacter baumannii is an important nosocomial pathogen, and its prevalence in compromised patient groups and intensive care units (ICU) is a health challenge worldwide. It is responsible for a variety of infections including bloodstream infections, meningitis, ventilator-associated pneumonia, wound infections and urinary tract infections [1]. The World Health Organization published a priority list for research and development of new antimicrobials in 2017, and *A. baumannii* was set as the number one priority. The presence of different international clones (ICs), such as IC5 (Pan-American clone), IC4 and IC7, has been described in several Latin American countries [2–4]. Furthermore, in Latin America, there has been an increase in carbapenem-resistant *A. baumannii* (CRAb) isolates from 27% in 2006 to 76% in 2009, which mirrors the high prevalence of this pathogen [5]. Moreover, its unique ability to survive for long periods in dry environments contributes to its spread and persistance in the hospital setting [1].

The dissemination of these ICs is often associated with antimicrobial resistance, especially resistance to carbapenems. The spread of these ICs mirrors the increase in circulating carbapenemase encoding genes such as bla_{OXA-23}, which has been widely reported worldwide [5–7]. The most prevalent ICs in South America are IC5 (CC79^{Pas}) and IC7 (CC25^{Pas}), and this differs from the situation in North America and Europe where IC2 (CC2^{Pas}) is the predominant lineage. In South America and other regions, the number of CRAb isolates is also increasing [3,5–7].

As an additional problem, mobile genetic elements carrying antimicrobial resistance genes are also being disseminated among *A. baumannii* isolates. Carbapenemase encoding genes, particularly the most common in *A. baumannii*, the oxacillinases (OXAs), are often associated with insertion elements (IS) forming transposons [1,8] and with plasmids harbouring resistance genes [1].

The objective of this study was to determine the molecular epidemiology and to analyse the antimicrobial resistance rates of carbapenem-resistant *A. baumannii* isolates recovered from

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patients in two Bolivian hospitals between September 2015 and December 2016.

2. Materials and methods

2.1. Bacterial isolates

In total, 95 *A. baumannii* isolates recovered between September 2015 and December 2016 from patients in two hospitals in close geographic proximity (Hospital Materno Infantil and Hospital Viedma) in the city of Cochabamba, Bolivia were included in this study. These two hospitals have a combined total of 408 beds and 21 ICU beds.

The isolates were initially identified as *Acinetobacter* spp. by biochemical methods in the hospital laboratories, and were confirmed as *A. baumannii* by *gyrB* multiplex polymerase chain reaction (PCR) [9] and the presence of $bla_{\text{OXA-51-like}}$ [10].

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the agar dilution method according to EUCAST guidelines (http://www.eucast.org/clinical_breakpoints/). Minimum inhibitory concentrations (MICs) were determined for ciprofloxacin, gentamicin, imipenem, meropenem and tigecycline. For tigecycline, the EUCAST clinical MIC breakpoint for Enterobacteriaceae was used as no breakpoints are available for *A. baumannii*. The reference strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC 29213 were used as control strains. Susceptibility testing was repeated three times for all isolates.

MICs for colistin in those carbapenem-resistant isolates with colistin MICs ≥2 mg/L by agar dilution were further tested by microbroth dilution using Micronaut-S MHK colistin plates (Merlin Diagnostika GmbH, Bornheim, Germany), with *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 used as control strains.

2.3. Class D and B β-lactamases

A multiplex PCR was performed to detect the presence of genes encoding bla_{OXA} carbapenemases (51-like, 23-like, 58-like, 40-like, 143-like and 235-like) [11]. Additionally, two in-house multiplex PCRs were performed. The first PCR included the following genes: VIM, KPC, $bla_{\text{OXA-40-like}}$, NDM, $bla_{\text{OXA-48-like}}$ and $bla_{\text{OXA-23}}$-IMI, $bla_{\text{OXA-58-like}}$, GES, GIM, IMP and IS*Aba1*-$bla_{\text{OXA-51-like}}$ were screened by the second PCR [12].

2.4. DNA extraction and whole-genome sequencing

All CRAb isolates, CRAb (n=51) and a subset of carbapenem-susceptible (n=4) isolates were further investigated by whole-genome sequencing (WGS). Total DNA was prepared using the MagAttract HMW DNA kit (Qiagen, Hilden, Germany). Sequencing libraries were prepared using the Nextera XT library prep kit (Illumina GmbH, Munich, Germany) for a 250-bp paired-end sequencing run on an Illumina MiSeq sequencer. The FASTQ files containing paired reads were assembled de novo with the Velvet assembler using Ridom SeqSphere+ v.3.0 and SPAdes 3.9 (https://cge.cbs.dtu.dk/services/SPAdes/).

2.5. Molecular epidemiology and whole-genome sequencing analysis

A core genome multi-locus sequence typing (cgMLST) scheme was defined using Ridom SeqSphere+ v.3.0, with *A. baumannii* ACICU used as the reference genome. The resulting core genome of 2390 alleles was used to investigate the molecular epidemiology of the isolates [13]. A minimum spanning tree based on the core genome of 2390 alleles was generated using Ridom SeqSphere+, ignoring the missing values.

The assembled genomes were used to identify the acquired resistome using ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/). Sequence types were determined using the traditional seven loci MLST schemes of Oxford and Pasteur (https://pubmlst.org/abaumannii/). The $bla_{\text{OXA-51}}$ variant, sequence type (ST) as determined by the Pasteur scheme and cgMLST were also used to assign the isolates to an IC [13].

2.6. Determination of the gene location

S1 nuclease-pulsed field gel electrophoresis (S1-PFGE) and Southern blot hybridization were perfomed to determine the plasmid size and the plasmid/chromosomal location of $bla_{\text{OXA-23}}$ and *strA*, a gene conferring resistance to aminoglycosides, in a selection of isolates representing all the ICs and unique Oxford STs (n=10). Total bacterial DNA embedded in agarose plugs was digested with 50 U of S1 nuclease (Thermo Fisher Scientific, Waltham, MA, USA), incubated at 37°C for 45 min and separated using a CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA, USA). The PFGE conditions were 17 h at 6 V/cm and 14°C. Initial and final pulses were conducted at 4 and 16 s, respectively.

DNA was transferred to a Hybond-N membrane by capillary transfer followed by hybridization with digoxigenin-labelled specific probes (Roche, Mannheim, Germany) for $bla_{\text{OXA-23-like}}$ and *strA*. Chromosomal location was shown by co-localization with the $bla_{\text{OXA-51-like}}$ probe. Signal detection was performed according to the manufacturer's instructions using CDP-Star ready-to-use chemiluminescent substrate (Roche) by autoradiography on an X-ray film.

2.7. Plasmid analysis

Plasmid analysis was performed by combining the acquired resistome with S1-PFGE and Southern blot hybridization with WGS data. Overlapping of putative plasmid contigs carrying resistance genes from assembled genomes was examined. Plasmid assemblies were further confirmed using PCR-based gap closure [14].

3. Results

3.1. Bacterial isolates: antimicrobial susceptibility and polymerase chain reaction experiments

All isolates were confirmed as *A. baumannii* by *gyrB* multiplex PCR and presence of the intrinsic $bla_{\text{OXA-51-like}}$ carbapenemase gene. The most prevalent source of the isolates was the respiratory tract (n=34; 35.8%), followed by wound secretions (n=17; 17.9%), ulcers (n=9, 9.5%) and urine culture (n=8; 8.4%). The rest of the isolates were recovered from diverse sources such as blood cultures, catheters, abscesses and exudates.

MICs for the tested antibiotics are summarized in Table 1, and resistance rates to ciprofloxacin, gentamicin, imipenem, meropenem and tigecycline are shown in Table 2. All of the isolates except one, MC96 ST1489$^{\text{Ox}}$-ST25$^{\text{Pas}}$ (MIC = 8 mg/L), were susceptible to colistin (MICs = 1–2 mg/L). MC96 was resistant to all the tested antimicrobial agents and is considered to be pan-drug resistant. The carbapenem resistance rate in Hospital Materno Infantil was higher than that in Hospital Viedma (66.7% vs. 48.7%, respectively).

By using multiplex PCR, the $bla_{\text{OXA-23-like}}$ gene was detected in the 51 CRAb isolates. No other acquired class D β-lactamases and no metallo-β-lactamases were detected in any of the isolates.

Table 1
Epidemiological data of the 55 sequenced isolates, minimum inhibitory concentrations of the tested antibiotics, results of S1 nuclease-pulsed field gel electrophoresis (S1-PFGE) and Southern blot hybridization, and the resistome.

			Isolate	Hospital	COL	IPM	MEM	CIP	TGC	GEN	S1-PFGE and Southern blot	Resistome
CRAb	IC7	$ST991_{pas}$/$ST1518_{ox}$	MC1	HMI	0.5 S	32 R	64 R	>128 R	16 R	32 R	bla_{OXA-23} chrom$^{\alpha}$/ *strA* ~180 Kb p$^{\#}$	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC2	HMI	1 S	32 R	32 R	32 R	1 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC3	HMI	1 S	32 R	32 R	128 R	4 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC5	HMI	1 S	32 R	32 R	128 R	4 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC6	HMI	1 S	32 R	32 R	64 R	1 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC8	HMI	1 S	32 R	64 R	32 R	1 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC12	HMI	1 S	32 R	32 R	>128 R	8 R	32 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC19	HMI	1 S	32 R	32 R	64 R	1 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC21	HMI	1 S	32 R	32 R	64 R	0.5 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC22	HMI	1 S	32 R	32 R	64 R	1 S	1 S	N.P.	bla_{OXA-23} *ISAba1-ampC*
			MC33	HV	0.5 S	32 R	32 R	32 R	1 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC35	HV	1 S	32 R	64 R	32 R	1 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC44	HV	0.25 S	32 R	64 R	>128 R	4 R	>128 R	N.P.	bla_{OXA-23} *ISAba1-ampC*
			MC50	HV	0.25 S	32 R	64 R	32 R	1 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC53	HV	1 S	32 R	32 R	32 R	0.5 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC60	HV	0.5 S	32 R	32 R	64 R	1 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC62	HV	0.25 S	32 R	32 R	32 R	0.5 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC105	HV	1 S	32 R	32 R	32 R	1 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
	IC7	$ST25_{pas}$/$ST1489_{ox}$	MC31	HV	1 S	32 R	32 R	128 R	8 R	>128 R	bla_{OXA-23} chrom$^{\alpha}$/ *strA* ~180 Kb p$^{\#}$	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC32	HV	1 S	32 R	32 R	128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC87	HV	1 S	32 R	64 R	128 R	8 R	>128 R	bla_{OXA-23} chrom$^{\alpha}$/ *strA* ~180 Kb p$^{\#}$	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC89	HV	1 S	32 R	64 R	64 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC90	HV	1 S	32 R	64 R	64 R	4 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC91	HV	1 S	32 R	64 R	128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC93	HV	1 S	32 R	64 R	64 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* $_{64}$ bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC94	HV	1 S	32 R	64 R	64 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC95	HV	1 S	32 R	64 R	128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC96	HV	8 R	32 R	64 R	128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC98	HV	1 S	32 R	32 R	128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC100	HV	1 S	32 R	32 R	64 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC101	HV	1 S	32 R	32 R	64 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC102	HV	2 S	32 R	32 R	64 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC103	HV	1 S	32 R	64 R	64 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC104	HV	1 S	32 R	32 R	128 R	4 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
CRAb	IC7	$ST25_{pas}$/$ST1519_{ox}$	MC14	HMI	1 S	32 R	32 R	>128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC18	HMI	0.5 S	32 R	64 R	128 R	8 R	>128 R	bla_{OXA-23} chrom$^{\alpha}$/ *strA* ~180 Kb p$^{\#}$	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC29	HV	0.5 S	32 R	64 R	>128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC34	HV	0.5 S	32 R	64 R	128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC48	HV	0.5 S	32 R	32 R	128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC59	HV	0.25 S	32 R	32 R	128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC63	HV	0.25 S	32 R	32 R	>128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC64	HV	0.5 S	32 R	32 R	128 R	4 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC69	HV	1 S	32 R	32 R	>128 R	4 R	>128 R	bla_{OXA-23} chrom$^{\alpha}$/ *strA* ~180 Kb p$^{\#}$	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC78	HV	1 S	32 R	32 R	128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
	IC7	$ST25_{pas}$/$ST1529_{ox}$	MC39	HV	1 S	32 R	64 R	>128 R	8 R	4 S	N.P.	bla_{OXA-23} *ISAba1-ampC*
			MC51	HV	0.5 S	32 R	32 R	128 R	8 R	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC57	HV	0.5 S	32 R	32 R	128 R	4 R	>128 R	bla_{OXA-23} chrom$^{\alpha}$/ *strA* ~180 Kb p$^{\#}$	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
	IC7	$ST25_{pas}$/$ST1528_{ox}$	MC27	HV	1 S	32 R	32 R	32 R	0.5 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC71	HV	1 S	32 R	64 R	64 R	0.5 S	>128 R	bla_{OXA-23} chrom$^{\alpha}$/ *strA* ~180 Kb p$^{\#}$	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
			MC77	HV	1 S	32 R	32 R	32 R	0.5 S	>128 R	N.P.	*strA strB aac(3)-IIa* bla_{OXA-23} *ISAba1-ampC sul2 tet(B)*
	IC4	$ST15_{pas}$/$ST236_{ox}$	MC75	HV	1 S	32 R	64 R	128 R	2 S	>128 R	bla_{OXA-23} chrom$^{\alpha}$/ *strA* ~150 Kb p$^{\#}$	*strA strB aac(3)-IIa aph(3')-VIa* bla_{OXA-23} *ISAba1-ampC* bla_{TEM-1B} *sul2*
Non-CRAb	IC5	$ST79_{pas}$/$ST1520_{ox}$	MC23	HV	1 S	1 S	2 S	>128 R	4 R	>128 R	*strA* chrom$^{\alpha}$	*strA strB aadA1 aadB aph(3')-VIa ISAba1-ampC* bla_{TEM-1A} *sul2 floR dfrA1*
		$ST79_{pas}$/$ST233_{nx}$	MC17	HMI	1 S	1 S	2 S	>128 R	4 R	>128 R	N.P.	*strA strB aadA1 aadB aph(3')-VIa ISAba1-ampC* bla_{TEM-1A} *sul2 floR dfrA1*
			MC38	HV	1 S	0.5 S	1 S	>128 R	2 S	>128 R	N.P.	*strA strB aadA1 aadB aph(3')-VIa ISAba1-ampC* bla_{TEM-1A} *sul2 floR dfrA1*
	Sg^{β}	$ST267_{pas}$/$ST942_{nx}$	MC47	HV	1 S	0.5 S	2 S	128 R	0.5 S	>128 R	*strA* ~180 Kb p$^{\#}$	*strA strB aac(3)-IIa aph(3')-VIa ampC* bla_{TEM-1B} *sul2 tet(B)*

COL, colistin; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; TGC, tigecycline, GEN, gentamicin; S, susceptible; R, resistant. Sg^{β}, singleton; HV, Hospital Viedma; HMI, Hospital Materno Infantil. chrom$^{\alpha}$, chromosome; p$^{\#}$, plasmid; N.P., S1-PFGE+Southern blot not performed in these isolates.

Table 2
Minimum inhibitory concentrations (MICs) as determined by agar dilution for ciprofloxacin, gentamicin, imipenem, meropenem and tigecycline in all 95 isolates.

		Total (n=95)		CRAb (n=51)		Non-CRAb (n=44)	
		Susceptible n (%)	Resistant n (%)	Susceptible n (%)	Resistant n (%)	Susceptible n (%)	Resistant n (%)
Ciprofloxacin (n=95)		9 (9.47%)	86 (90.53%)	0 (0%)	51 (100%)	9 (20.45%)	35 (79.54%)
Colistin	BMD (n=39)	38 (97.44%)	1 (2.56%)	34 (66.67%)	1 (1.96%)	4 (9.10%)	0 (0%)
	Agar dilution (n=56)	56 (100%)	0 (0%)	16 (31.37%)	0 (0%)	40 (90.90%)	0 (0%)
Gentamicin (n=95)		12 (12.63%)	83 (87.37%)	2 (3.92%)	49 (96.08%)	10 (22.73%)	34 (77.27%)
Imipenem (n=95)		44 (46.32%)	51 (53.68%)	0 (0%)	51 (100%)	44 (100%)	0 (0%)
Meropenem (n=95)		44 (46.32%)	51 (53.68%)	0 (0%)	51 (100%)	44 (100%)	0 (0%)
Tigecycline (n=95)		37 (38.95%)	58 (61.05%)	17 (33.33%)	34 (66.67%)	20 (45.45%)	24 (54.54%)

CRAb, carbapenem-resistant *Acinetobacter baumannii*; BMD, broth microdilution method.
Colistin MICs were tested using the BMD in 39 isolates and the agar dilution method in 56 isolates.

Furthermore, IS*Aba1* was not detected upstream of the intrinsic $bla_{\text{OXA-51-like}}$ gene.

3.2. Molecular epidemiology and whole-genome sequencing analysis

Seven loci MLST revealed that the majority of the isolates [90.9% (n=50)] were ST25 or its single locus variant (SLV) ST991 according to the Pasteur scheme [clonal complex 25 (CC25)] which is associated with IC7. The majority of the isolates from Hospital Materno Infantil (77%) were IC7 (ST991$^{\text{Pas}}$), two were ST25$^{\text{Pas}}$ and one was IC5; the remaining ST25$^{\text{Pas}}$ isolates were found in Hospital Viedma. The IC7 isolates had $bla_{\text{OXA-64}}$, the characteristic $bla_{\text{OXA-51}}$ variant from this lineage. Furthermore, these isolates clustered with IC7 control strains using cgMLST (data not shown). These three pieces of evidence showed that IC7 was predominant among these *A. baumannii* isolates. Following the Oxford scheme, the 50 IC7 isolates were further delineated into five different groups belonging to CC110 [SLV: ST1489, ST1519, ST1529 and ST1518; double locus variant (DLV): ST1528].

The higher resolution of cgMLST showed that most isolates were not from the same clone (Fig. 1). There were five potential transmission clusters using a cut-off of 0–10 allelic differences. The other CC25 isolates differed by up to 110 alleles.

Five unrelated isolates differed from the IC7 group by over 1950 alleles. The three isolates carrying $bla_{\text{OXA-65}}$ were ST79 (IC5) according to the Pasteur scheme, and ST233 and DLV ST1520 according to the Oxford scheme, respectively, and clustered with the IC5 control strain by cgMLST. One isolate clustered with the IC4 control strains, carried $bla_{\text{OXA-51}}$, and was ST15 according to the Pasteur scheme and ST236 according to the Oxford scheme. One isolate was a singleton, carrying $bla_{\text{OXA-180}}$; this was ST942$^{\text{Ox}}$/ST267$^{\text{Pas}}$ and was not considered to represent one of the ICs.

WGS analysis confirmed that all the 51 CRAb isolates carried the $bla_{\text{OXA-23}}$ gene on *Tn2008*. Other genes conferring resistance to antibiotics such as aminoglycosides (*strA*, n=49; *strB*, n=49; *aac(3')-IIa*, n=49; *aac(3')-VIa*, n=5; *aadA1*, n=3; *aadB*, n=3), β-lactams (*ampC*, n=55; $bla_{\text{TEM-1A}}$, n=4; $bla_{\text{TEM-1B}}$, n=2), sulphonamides (*sul2*, n=52), tetracyclines (*tet(B)*, n=48), trimethoprim (*dfrA*, n=3) and phenicols (*floR*, n=3) were also present. Additionally, the three isolates belonging to IC5, the IC4 isolate and the singleton carried the *aac(3')-VIa* gene located on a Tn*aphA6* transposon.

Results of the molecular epidemiology and acquired resistance genes of the isolates are shown in Table 1.

3.3. Location of resistance genes

Southern blot analysis revealed that $bla_{\text{OXA-23}}$ was located on the chromosome in all tested isolates (n=8). However, the *strA* gene was encoded on a ~180-Kb plasmid in all the IC7 isolates as well as in the singleton; the IC4 isolate carried the *strA* gene on a ~150-Kb plasmid. Genome assemblies and PCR-based gap closure revealed that *strA* was always linked to *strB*, *sul 2*, *aac(3)-IIa* and *tetB* in the IC7 isolates. No *tetB* gene was detected in the IC4 isolate. In contrast, Southern blot analysis revealed that *strA* was located on the chromosome in the IC5 isolates, and was associated with *strB*, *sul2* and *floR*. Additionally, the IC5 isolates harboured *aadB* on a ~6-Kb plasmid, already described and named 'pRAY' [15].

4. Discussion

IC7 isolates have been described previously in some Latin American countries, such as Paraguay and Argentina, but were usually sporadic isolates [2]. However, IC7 was the most prevalent group in studies performed in Bolivia or Uruguay [7], which is in concordance with the present findings. This study found only one carbapenem-resistant IC4 isolate and three carbapenem-susceptible IC5 isolates, although IC5 is the prevalent clonal lineage found in Latin America, the so-called 'Pan-American clone', followed by IC4 [6,7,16,17]. The prevalence of IC7 isolates suggests a change in the epidemiology of carbapenem-resistant *A. baumannii* isolates in Bolivia, particularly in the city of Cochabamba, when comparing these results with previous studies [17].

According to the Pan American Health Organization (PAHO) annual study, in Bolivia, 19% of *Acinetobacter* spp. were resistant to imipenem in 2010; in 2014, 51% of *A. baumannii* isolates were resistant to imipenem and 57% were resistant to meropenem [18,19]. This study found similar results to those obtained by PAHO in 2014, and the resistance rates were similar to those in Colombia, where the presence of ST229 (Oxford) isolates (with $bla_{\text{OXA-64}}$) and others that belong to IC7 have also been reported [20]. High rates of carbapenem resistance in Hospital Materno Infantil have been described in a previous study [21]. In addition, as can be seen in Table 2, the CRAb isolates present higher resistance rates to other antimicrobials, such as ciprofloxacin and gentamicin, compared with the non-CRAb isolates, thus complicating antimicrobial treatment options. When analysing the population within both hospitals, it can be seen that different clusters are associated with each of them; almost all the isolates from Hospital Materno Infantil were ST991$^{\text{Pas}}$, while ST25$^{\text{Pas}}$ isolates were mainly found in Hospital Viedma. Just two ST25$^{\text{Pas}}$ isolates were found in Hospital Materno Infantil. Some ST991$^{\text{Pas}}$ isolates were also isolated in Hospital Viedma (n=8), which may suggest that there is cross-transmission of ST991$^{\text{Pas}}$ from Hospital Materno Infantil to Hospital Viedma (Fig. 1).

Diverse $bla_{\text{OXA-51-like}}$ variants such as $bla_{\text{OXA-65}}$, $bla_{\text{OXA-64}}$, $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-66}}$ have been reported in Latin America, but until now, no $bla_{\text{OXA-180}}$ carbapenemase had been found [6]. Moreover, Sennati et al. described the presence of Tn*2008* in an ST25 (Pasteur) *A. baumannii* isolate in Bolivia with the intrinsic $bla_{\text{OXA-64}}$ [22]. In the Tn*2008* transposon, IS*Aba1* is not only serving as a

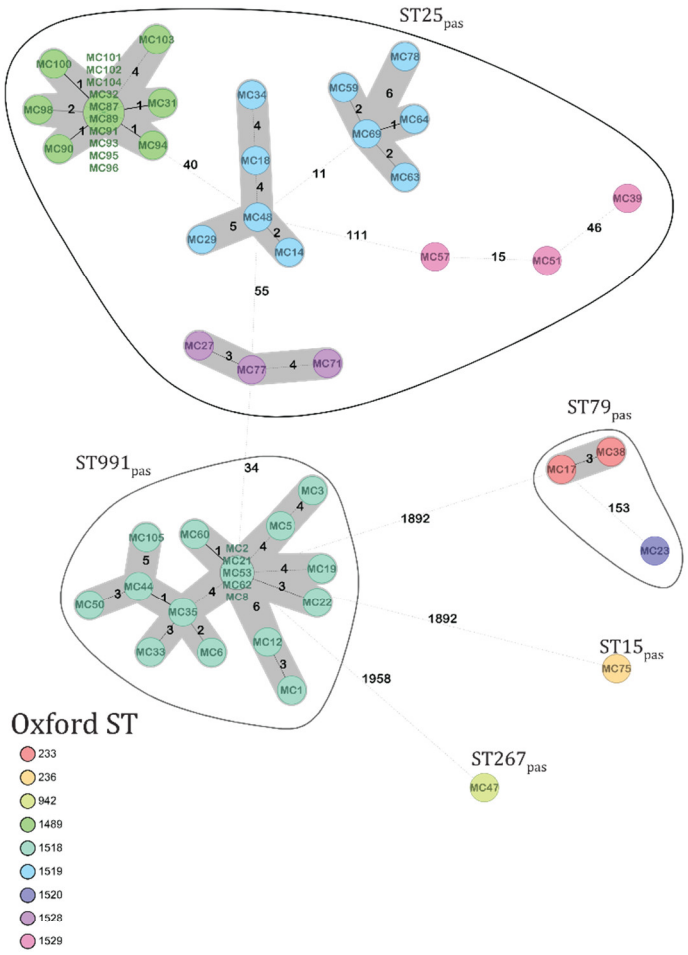

Fig. 1. Minimum spanning tree generated using Ridom SeqSphere+ for 55 samples, ignoring missing values. Distance based on columns from *Acinetobacter baumannii* core genome multi-locus sequence typing 2390 targets ACICU. Numbers between the nodes indicate the number of allelic differences. Shaded nodes represent transmission clusters. MC17, MC23, MC38 and MC47 are the carbapenem-susceptible *A. baumannii* isolates. There may be cross-transmission of ST991^{Pas} between Hospital Materno Infantil and Hospital Viedma.

promoter for the carbapenemase encoding gene, but is also involved in mobilization of the gene [8]. This carbapenemase encoding vehicle has spread worldwide [8] and, in common with Sennati et al., the present study found it has been acquired by all the isolates belonging to ST25 (IC7) and the ST15 isolate (IC4). The increasing resistance rates to carbapenems were mediated by the mobilization of Tn*2008* among diverse ICs [8].

Furthermore, these isolates carried resistance genes encoded on different structures such as transposons or plasmids that can also spread and confer antimicrobial resistance to other groups of drugs such as aminoglycosides. In combination with carbapenem resistance, this further reduces the remaining therapeutic options [5].

Despite a number of published studies from South America [2,5,7,16,20], the epidemiology of *Acinetobacter* spp. is not well known for Bolivia. This study of CRAb isolates shed some light on the population dynamics of *A. baumannii* between these two Bolivian hospitals, and demonstrated the endemicity and dissemination of several strains of CC25 (IC7) within both hospitals. Evidence of cross-transmission is important in order to implement effective infection control strategies in the hospital setting. Finally, the high rates of antimicrobial resistance, especially to the carbapenems – mediated by the resistance determinant Tn*2008* and the presence of a pan-drug-resistant *A. baumannii* isolate – is of great concern due to the endemicity and the potential for epidemic spread in these hospitals.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.03.019.

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Appendix A.

GenBank/EMBL/DDBJ/PIR accession numbers for the whole genome shotgun projects of *Acinetobacter baumannii* isolates

Strain	WGS	BioSample
MC1	QXPV00000000	SAMN09951315
MC2	QXPU00000000	SAMN09951316
MC3	QXPT00000000	SAMN09951317
MC5	QXPS00000000	SAMN09951318
MC6	QXPR00000000	SAMN09951319
MC8	QXPQ00000000	SAMN09951320
MC12	QXPP00000000	SAMN09951321
MC14	QXPO00000000	SAMN09951322
MC17	QXPN00000000	SAMN09951323
MC18	QXPM00000000	SAMN09951324
MC19	QXPL00000000	SAMN09951325
MC21	QXPK00000000	SAMN09951326
MC22	QYCX00000000	SAMN09951327
MC23	QXPJ00000000	SAMN09951328
MC27	QXPI00000000	SAMN09951329
MC29	QYCW00000000	SAMN09951330
MC31	QXPH00000000	SAMN09951331
MC32	QXPG00000000	SAMN09951332
MC33	QYCV00000000	SAMN09951333
MC34	QXPF00000000	SAMN09951334
MC35	QXPE00000000	SAMN09951335
MC38	QXPD00000000	SAMN09951336
MC39	QXPC00000000	SAMN09951337
MC44	QXPB00000000	SAMN09951338
MC47	QXPA00000000	SAMN09951339
MC48	QXOZ00000000	SAMN09951340
MC50	QYCU00000000	SAMN09951341
MC51	QXOY00000000	SAMN09951342

Strain	WGS	BioSample
MC53	QXOX00000000	SAMN09951343
MC57	QXOW00000000	SAMN09951344
MC59	QXOV00000000	SAMN09951345
MC60	QXOU00000000	SAMN09951346
MC62	QXOT00000000	SAMN09951347
MC63	QXOS00000000	SAMN09951348
MC64	QXOR00000000	SAMN09951349
MC69	QXOQ00000000	SAMN09951350
MC71	QXOP00000000	SAMN09951351
MC75	QXOL00000000	SAMN09951355
MC77	QXOJ00000000	SAMN09951357
MC78	QXOK00000000	SAMN09951356
MC87	QXON00000000	SAMN09951353
MC89	QXOM00000000	SAMN09951354
MC90	QXOO00000000	SAMN09951352
MC91	QXOI00000000	SAMN09951358
MC93	QYCT00000000	SAMN09951359
MC94	QXOH00000000	SAMN09951360
MC95	QXOG00000000	SAMN09951361
MC96	QXOF00000000	SAMN09951362
MC98	QXOE00000000	SAMN09951363
MC100	QYCS00000000	SAMN09951364
MC101	QXOD00000000	SAMN09951365
MC102	QXOC00000000	SAMN09951366
MC103	QXOB00000000	SAMN09951367
MC104	QXOA00000000	SAMN09951368
MC105	QXNZ00000000	SAMN09951369

2.4 First report of New Delhi Metallo-β-lactamase-6 in a clinical *A. baumannii* isolate from Northern Spain.

Class B metallo-β-lactamases (MBLs), requiring divalent cations (usually zinc) as metal cofactors for enzyme activity, have been frequently reported in Gram-negative bacteria but have been rarely described among CRAb isolates.[19, 88] The New Delhi metallo-β-lactamase (NDM) has been described the first time in a *K. pneumoniae* isolate recovered in Sweden from a patient of Indian origin who had travelled to New Delhi in 2008.[89]

In the following study we phenotypically and genotypically characterized a CRAb isolate recovered in 2019 in Northern Spain. The isolate was identified as ST85 (Pasteur scheme), harboured the intrinsic β-lactamase OXA-94, an OXA-51-like, with an IS*Aba1* upstream of it, and clustered with recently described IC9 isolates. Phenotypic tests and WGS revealed that the isolate harboured the MBL *bla*$_{\text{NDM-6}}$. NDM-6 differs from NDM-1 in one amino acid substitution (A233V) and has been mainly reported in Enterobacterales species. S1-PFGE and hybridization experiments showed that the MBL was chromosomally encoded. The adjacent genetic environment of *bla*$_{\text{NDM-6}}$ included IS*Aba14*, *aphA6*, IS*Aba125*, *bla*$_{\text{NDM-6}}$, *ble*$_{\text{MBL}}$, *trpF*, *dsbC*, *cutA* and IS*Aba14*. While a 10,462 bp duplication could be detected further downstream including another copy of *bla*$_{\text{NDM-6}}$ in the following order: IS*Aba125*, *bla*$_{\text{NDM-6}}$, *ble*$_{\text{MBL}}$, *trpF*, *dsbC, cutA* and IS*Aba14*. The present study describes the first report of *bla*$_{\text{NDM-6}}$ in *A. baumannii* which was present in two copies in a novel genetic environment which included multiple copies of IS elements and underlines the impact of MGEs in the dissemination of ARGs.

My contribution to the following manuscript included WGS, *in silico* analysis, experiments about the genetic localization of *bla*$_{\text{NDM-6}}$, data interpretation and composing the manuscript.

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First Report of New Delhi Metallo-β-Lactamase-6 (NDM-6) in a Clinical *Acinetobacter baumannii* Isolate From Northern Spain

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The objective of this study was the phenotypic and genotypic characterization of a carbapenem resistant *Acinetobacter baumannii* (CRAB) isolate. The isolate, recovered in Northern Spain in 2019, was identified by MALDI-TOF to the species level. Antimicrobial susceptibility testing was performed using the Phoenix BD NMIC-502 Panel, E-test, and broth microdilution methods. The presence of a metallo-β-lactamase (MBL) was verified by PCR and immunochromatographic assays. The genetic location of the MBL was confirmed using S1-pulsed-field gel electrophoresis (S1-PFGE) followed by Southern blot hybridization. Whole genome sequencing (WGS) was completed using the Miseq and MinION platforms, followed by core-genome MLST (cgMLST) and seven-locus MLST analysis. The CRAB was assigned ST85 (Pasteur scheme) and ST957 (Oxford scheme) representing international clone (IC) 9 and harbored the intrinsic β-lactamase OXA-94 with IS*Aba1* upstream of it, and the MBL bla_{NDM-6}. Hybridization experiments revealed that the bla_{NDM-6} was encoded on the chromosome. Using WGS the bla_{NDM-6} environment could be identified arranged in the following order: IS*Aba14*, *aphA6*, IS*Aba125*, bla_{NDM-6}, ble_{MBL}, *trpF*, *dsbC*, *cutA*, and IS*Aba14*. Downstream, a 10,462 bp duplication was identified, including a second copy of bla_{NDM-6} in the following genetic composition: IS*Aba125*, bla_{NDM-6}, ble_{MBL}, *trpF*, *dsbC*, *cutA*, and IS*Aba14*. To our knowledge, this is the first description of bla_{NDM-6} in *A. baumannii*. The MBL was present in two copies in the chromosome in a new genetic environment associated with IS elements highlighting the contribution of mobile genetic elements in the dissemination of this gene.

Keywords: carbapenemase, whole genome sequencing, long reads, NDM-6, *Acinetobacter baumannii*

INTRODUCTION

Infections caused by multidrug-resistant *Acinetobacter baumannii* have become a health care challenge worldwide (Peleg et al., 2008; Higgins et al., 2010). Carbapenems are often the antimicrobials of choice of treatment of *A. baumannii* infections; however, their use has led to the development of carbapenem resistance front-line antimicrobial agents (Tal-Jasper et al., 2016). In 2019, the World Health Organization (WHO) classified carbapenem resistant *A. baumannii* (CRAB) as one of the "Priority 1: Critical group" organisms for which new

antimicrobials are urgently needed.[1] Carbapenem resistance in *A. baumannii* is mainly mediated through acquired carbapenem-hydrolyzing class D ß-lactamases (oxacillinases), encoded by $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-143-like}}$, and $bla_{\text{OXA-235-like}}$ (Higgins et al., 2013; Evans and Amyes, 2014). There are two pillars of CRAB prevailing; the widespread international clone 2 (IC2) isolates, and the most prevalent carbapenemase in the species, OXA-23 (Higgins et al., 2010; Tomaschek et al., 2016; Müller et al., 2019). Class B metallo-β-lactamases (MBLs), such as IMP, VIM, SIM, and NDM are less frequently reported in CRAB isolates (Müller et al., 2019). Nevertheless, MBL-positive *A. baumannii* are increasingly reported worldwide (Kaase et al., 2011; Pfeifer et al., 2011; Berrazeg et al., 2014; Karampatakis et al., 2017; Adams et al., 2020; Ramirez et al., 2020).

The emergence of NDM-type carbapenemases, hampering the efficacy of almost all β-lactams, including carbapenems, is of great medical concern. The main reservoir of NDM-like producers is the Indian subcontinent, the Balkans region, and the Middle East (Dortet et al., 2014; Khan et al., 2017). Since the first description of NDM-1, 29 variants have been reported, mainly in members of the Enterobacterales family, such as *Escherichia coli* and *Klebsiella pneumoniae*, but also in *A. baumannii* (e.g., NDM-1, −2, −3, −4, −5, and −7) and *Acinetobacter lwoffii*[2] (e.g., NDM-14; Elbrolosy et al., 2019).

The aim of the present study was the phenotypic and genotypic characterization of a CRAB isolate harboring $bla_{\text{NDM-6}}$ recovered from a patient in Northern Spain.

MATERIALS AND METHODS

Patient and Bacterial Isolate Data, Species Identification, and Antimicrobial Susceptibility Testing

A 70–74 year-old patient, from Maghreb (Northwest Africa) presented to the Hospital de Basurto (Bilbao, Northern Spain) with dysuria in September 2019. The patient had a positive urine culture with >100.000 CFU/ml of a Gram-negative bacillus and reported a previous hospitalization in his home country due to a prostatectomy. Unfortunately, no further data were available about the country of origin.

Species identification of the isolate AbBAS-1 was performed by MALDI-TOF mass spectrometry (Bruker Daltonics, Madrid, Spain) and biochemically with the Phoenix BD UNMIC/ID-409 Panel (Becton Dickinson, Madrid, Spain). Antimicrobial susceptibility testing was performed using the Phoenix BD NMIC-502 Panel, while susceptibility to colistin was tested using the microdilution UMIC kit (Biocentric, Bandol, France). Susceptibility to tigecycline (Molekula, Newcastle upon Tyne, United Kingdom) was also determined using broth microdilution following CLSI guidelines (CLSI, 2019). Finally, susceptibility to imipenem and meropenem was determined by Etest (bioMérieux, Nürtingen, Germany). Minimal inhibitory concentration (MIC) were interpreted using the resistance breakpoints for *Acinetobacter* spp. from EUCAST (Version 10.0, January 2020, http://www.eucast.org/clinical_breakpoints/). For tigecycline, the EUCAST PK-PD (Non-species related) breakpoint of 0.5 mg/L was used.

Detection of Carbapenemase-Encoding Genes

The presence of the carbapenemase-encoding genes $bla_{\text{OXA-51-like, -23-like, -58-like, -40-like, -143-like, and -235-like}}$ was investigated by PCR (Woodford et al., 2006; Higgins et al., 2010, 2013). MBLs genes were investigated by in-house PCRs targeting the genes: bla_{VIM}, bla_{IMP}, and bla_{NDM}. Positive PCR products were purified by mi-PCR purification kit (Metabion, Planegg, Germany) and allelic variants were determined by Sanger sequencing followed by NCBI BLAST analysis. The presence of an MBL was phenotypically confirmed using the Total Metallo-beta-lactamase Confirm Kit (Rosco Diagnostica A/S, Taastrup, Denmark) and the Phoenix BD NMIC-502 Panel followed by an immunochromatographic assay NG-test Carba 5a (NG Biotech, Guipry, France).

S1-Pulsed-Field Gel Electrophoresis and Southern Blot Hybridization

Bacterial DNA embedded in agarose plugs was digested using 50 units S1-nuclease (Thermo Fisher Scientific, Waltham, MA, United States) per plug slice and followed by pulsed-field gel electrophoresis (PFGE). Samples were run on a CHEF-DR II system (Bio-Rad, Munich, Germany) for 17 h at 6 V/cm and 14°C, while initial and final pulses of 4 and 16 s, respectively, were applied. The Lambda PFG and λ DNA-Mono Cut Mix (New England Biolabs, Frankfurt, Germany) were used as markers. Southern blot hybridization was performed to determine the plasmid/chromosomal location by hybridization with digoxigenin-labeled probes (Roche, Mannheim, Germany). A $bla_{\text{NDM-6}}$ specific probe was generated and the chromosomal location was shown by colocalization with a $bla_{\text{OXA-51-like}}$ probe. Signal detection was performed using CDP-Star® ready-to-use (Roche) chemiluminescent substrate by autoradiography on X-ray film (GE Healthcare, Buckinghamshire, United Kingdom).

Electroporation Experiments

To determine the transferability of $bla_{\text{NDM-like}}$ variants, plasmid DNA isolated from AbBAS-1 using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and electroporated into the reference strain *A. baumannii* ATCC 17978. Selection of *A. baumannii* transformants was performed on Luria-Bertani agar (Oxoid, Wesel, Germany) supplemented with ticarcillin (150 mg/L). The presence of $bla_{\text{NDM like}}$ in the obtained transformants was confirmed by PCR.

Whole Genome Sequencing

Total DNA was extracted using the MagAttract HMW DNA Kit (Qiagen) according to manufacturer's instructions and used for short-read sequencing. Sequencing libraries were prepared

[1]https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf
[2]http://www.bldb.eu/BLDB.php?prot=B1#NDM

using a Nextera XT library prep kit (Illumina GmbH, Munich, Germany) for a 250 bp paired-end sequencing run on an Illumina MiSeq platform. The obtained reads were *de novo* assembled with the Velvet assembler integrated in the Ridom SeqSphere+ v. 7.0.4 software (Ridom GmbH, Münster, Germany).

DNA extraction for long-read sequencing was performed using the Genomic-Tips 100/G kit and Genomic DNA Buffers kit (Qiagen) according to the manufacturer's instructions. Libraries were prepared using the 1D Ligation Sequencing Kit (SQK-LSK109) in combination with Native Barcoding Kit (EXP-NBD104; Oxford Nanopore Technologies, Oxford, United Kingdom) and were loaded onto a R9.4 flow cell (Oxford Nanopore Technologies). The run was performed on a MinION MK1b device. Collection of raw electronic signal data and live base-calling was performed using the MinKNOW software and the Guppy basecaller (Oxford Nanopore Technologies). The long-reads were assembled using ONT assembly and Illumina polishing pipeline (Oxford Nanopore Technologies), performing Canu assembly followed by polishing steps, including pilon and BWA mem mapping using the Illumina reads.[3]

Molecular Typing, Genome Annotation, Analysis and Visualization

Multi-locus sequence typing (MLST) was performed using the Oxford and Pasteur typing schemes[4] to assign the sequence type (ST). Clonal complexes (CCs) were assigned using the BURST function available at pubmlst.org. The $bla_{\text{OXA-51-like}}$ variant combined with the CCs derived from both schemes and core-genome MLST (cgMLST) analysis, using the Ridom SeqSphere+ v. 7.0.4 software, were used to assign the isolate to an IC (Higgins et al., 2017).

The resistome of the bacterial isolate was identified using ResFinder v.3.2.0 (Zankari et al., 2012). Capsular polysaccharide-type (KL-type) and the outer core of the lipooligopolisacharide (OCL-type) were assigned using Kaptive Web (Wick et al., 2018). The motility phenotype was analyzed on 0.5% agarose plates, supplemented with 5 g/l tryptone, 2.5 g/l NaCl, and pH 7.4, inoculated on the surface and incubated overnight at 37°C (Skiebe et al., 2012). Prophage-related sequences were screened using the PHASTER tool and virulence factors using virulence factor database (VFDB; Arndt et al., 2016; Liu et al., 2019). The genome was annotated using Prokka integrated in the Galaxy web platform[5] and partially manually edited. SnapGene and SnapGene Viewer (from Insightful Science; available at snapgene.com) were used to predict open reading frames (ORF) and for genome visualization.

RESULTS AND DISCUSSION

The bacterial isolate AbBAS-1 was identified by MALDI-TOF and WGS as *A. baumannii*. Antimicrobial susceptibility testing revealed that the isolate was resistant to all tested antimicrobial agents except for amikacin (MIC <=4 mg/L), colistin (MIC <=0.5 mg/L), and tigecycline (MIC 0.5 mg/L; **Table 1**). Using phenotypical tests, a halo difference of 10 mm between the meropenem disk and both the dipicolinic acid and EDTA disks was observed, suggesting the presence of a MBL. The UNMIC/ID-409 signaled the presence of a carbapenemase and the NMIC-502 and lateral flow immunochromatography identified it as a class B carbapenemase, while, by lateral flow immunochromatography the MBL was identified as part of the NDM-β-lactamase complex.

Sequencing identified the carbapenemase as $bla_{\text{NDM-6}}$, which differs from $bla_{\text{NDM-1}}$ in one amino acid substitution (A233V). New Delhi Metallo-β-Lactamase-6 (NDM-6) has a similar hydrolyzing activity as NDM-1 and has been mainly reported in members of the Enterobacterales family to date (Williamson et al., 2012; Rahman et al., 2014; Bahramian et al., 2019). NDM-1-like derivative enzymes have been reported in carbapenem resistant Gram-negative organisms from multiple countries worldwide (Berrazeg et al., 2014; Dortet et al., 2014) including European countries, e.g., Germany, Switzerland, Slovenia, France, Belgium, Czech Republic, and very recently also in Southern Spain (Pfeifer et al., 2011; Bonnin et al., 2012; Fernandez-Cuenca et al., 2020). However, in the region of the Basque Country (Northern Spain), no NDM-like-enzymes have been previously reported. Our isolate AbBAS-1 was resistant to all β-lactams tested but also to fluoroquinolones and aminoglycosides, these findings are consistent with other NDM-producing isolates limiting the therapeutic options to amikacin, colistin, and tigecycline (Bonnin et al., 2012) or new promising molecules such as cefiderocol (Delgado-Valverde et al., 2020).

The patient had a radical prostatectomy performed in a North African country, where numerous NDM-positive *A. baumannii* isolates have been reported (Berrazeg et al., 2014; Ramoul et al., 2016; Jaidane et al., 2018; Al-Hassan et al., 2019). Unfortunately, there is a lack of a clinical follow up of the patient, who after empiric treatment with cefixime 400 mg/24 h

TABLE 1 | Antimicrobial susceptibility profile of the AbBAS-1 isolate.

Antimicrobial class	Antimicrobial agent	MIC (mg/L)
β-lactam	Amoxicillin-clavulanic acid[a]	>32/2
	Ertapenem[a]	>1
	Imipenem[a]	>32
	Meropenem[a]	>32
Aminoglycoside	Gentamicin	>4
	Tobramycin	>4
	Amikacin	<=4
Fluoroquinolone	Ciprofloxacin	>1
	Levofloxacin	>2
	Norfloxacin[a]	>2
Polymyxin	Colistin[b]	<=0.5
Tetracycline	Tigecycline[a,b]	0.5
Other	Fosfomycin[a]	>128
	Nitrofurantoin[a]	>64
	Trimethoprim-sulfamethoxazole	>4/76

[a]*No breakpoint available.*
[a]*Tested by E-test.*
[b]*Tested by broth microdilution method.*

[3]https://github.com/nanoporetech/ont-assembly-polish
[4]https://pubmlst.org/abaumannii/
[5]https://usegalaxy.org/

never consulted again the Basque Public Health System (Osakidetza). Because up until now NDM-6 has not been identified in Spain, we speculate that the patient acquired the NDM-6-positive CRAB isolate during his previous hospitalization in Northern Africa.

The AbBAS-1 isolate has been assigned as ST85^{Pas} (Pasteur scheme) and ST957^{Ox} (Oxford scheme) and harbored the $bla_{OXA-51\ like}$ variant bla_{OXA-94}. Furthermore, AbBAS-1 belonged to the clonal complex CC464^{Pas} and CC1078^{Ox} and could be assigned to the recently described IC9 (Müller et al., 2019). The novel IC9 was previously identified in *A. baumannii* isolates recovered between 2012 and 2016 in Belgium (bla_{NDM-1}-positive), Egypt (bla_{OXA-23}-positive), Italy (bla_{NDM-1}-positive), and Pakistan (bla_{OXA-23}-positive; Müller et al., 2019). ST85 *A. baumannii*, carrying bla_{OXA-91} and bla_{NDM-1}, recovered from Syrian civil war victims were first reported from Lebanon (Rafei et al., 2014). Furthermore, isolates encoding bla_{OXA-91} and bla_{NDM-1} were also reported from Southern Spain, Saudi Arabia, and Tunisia, or harboring bla_{VIM-1} in Egypt, indicating that the novel IC9 clonal lineage has a widespread distribution and was found repeatedly harboring MBLs (Jaidane et al., 2018; Al-Hassan et al., 2019; Al-Hamad et al., 2020; Fernandez-Cuenca et al., 2020). cgMLST analysis using currently available complete genomes of ST85^{Pas} *A. baumannii* isolates revealed that the AbBAS-1 isolate is closely related (81 alleles difference) to an NDM-1-positive CRAB isolated in Southern Spain in 2017 (**Supplementary Figure 1**).

Attempts to transfer the bla_{NDM-6} by electroporation experiments were not successful, suggesting that the MBL was encoded on the chromosome. S1-PFGE and Southern blot experiments confirmed that bla_{NDM-6} was encoded on the chromosome. Using WGS the bla_{NDM-6} environment could be identified arranged in the following order: IS*Aba14*, *aphA6*, IS*Aba125*, bla_{NDM-6}, ble_{MBL} (resistance to bleomycin), *trpF* (phosphoribosylanthranilate isomerase), *dsbC* (tat twin-arginine translocation pathway signal sequence domain protein), *cutA* (periplasmic divalent cation tolerance protein), and IS*Aba14* (**Figure 1**). By BLASTn, the genetic environment of NMD-6 showed 99% similarity to an ST1089^{Ox} *A. baumannii* isolated in India in 2018 (Acc. No. CP038644), a single locus variant of ST957^{Ox} with bla_{OXA-94}, and which harbored a single copy of NDM-1. Further downstream, a 10,462 bp duplication was identified including a second copy of bla_{NDM-6} in the following genetic composition: IS*Aba125*, bla_{NDM-6}, ble_{MBL}, *trpF*, *dsbC*, *cutA*, and IS*Aba14* (**Figure 1**). The genetic environment of the second MBL was missing the type VI aminoglycoside phosphotransferase. The IS*Aba125* located directly upstream of both copies of bla_{NDM-6} could indicate that the two copies of the MBL were the result of duplication mediated through the mobile genetic element IS*Aba125* (IS30 family). IS element members of the family IS*30* are known to transpose by a copy-and-paste mechanism (Szabo et al., 2010). Upstream of the *aphA6* and bla_{NDM-6} a gene (colored green in **Figure 1**) truncated in three fragments was identified and was identical to an ATP-binding protein from an *A. baumannii* (Acc. No. CP038644.1) with the locus tag E5D09_10165. Particularly, 480 bp of the 5′ end of the ATP-binding protein containing the start codon were located upstream of the IS*Aba33*, which is followed directly by the next 44 bp of the ATP-binding protein. The 711 bp of the 3′ end were found directly downstream of the second copy of IS*Aba14*. Another copy of the 711 bp of the 3′ end was also present 9,7 kb downstream supporting the finding of the duplication of the genetic environment of the bla_{NDM-6}.

The AbBAs-1 isolate harbored in addition to bla_{NDM-6} the intrinsic, ADC-158-like (with no insertion element upstream), *ant(2'')-Ia*-like, *aph(3')-VI*, *mph(E)*, *msr(E)* antibiotic resistance determinants, while upstream of the bla_{OXA-91} IS*Aba1* was located. The isolate AbBAS-1 also presents an S81L substitution in DNA gyrase subunit A and S84L substitution in ParC, which are known to be associated with fluoroquinolone resistance. By typing the capsular polysaccharide (K and/or O-antigen), which is a critical determinant of virulence, the AbBAS-1 isolate was assigned as KL77, a KL type previously reported in ST2 and ST10 isolates, and OCL6 the third most common OCL type in *A. baumannii*. In accordance to recent studies that have found K & O-antigen diversity within members belonging to the same clone, capsule typing is a promising epidemiological marker in combination with MLST (Wyres et al., 2020). Of note is the mucoviscous phenotype of the AbBAS-1 colonies when grown on agar plates generating a viscous string >5 mm in length between a colony and a inoculation loop (string test), a phenotype that has been associated with hypervirulent *K. pneumoniae* strains (Fang et al., 2004). In addition, the *A. baumannii* isolate exhibited a nonmotile phenotype. Phage analysis identified in the AbBAS-1 two questionable phage regions both similar to *Acinetobacter* phage YMC/09/02/B1251_ABA_BP (Acc. No. NC_019541.1) and an incomplete phage region similar to *Pseudomonas* phage nickie (Acc. No. NC_042091.1). Virulence factors known to be associated with *A. baumannii* have been identified using VFDB and included genes linked with adherence (*ompA*); biofilm formation (*csuE*, *csuC*, *csuB*, *csuA*, *csuA/B*, *pgaD*, *pgaC*, *pgaB*, and *pgaA*); regulation (*bfmR*, *bfmS*, *abaI*, and *abaR*); phospholipases (*plc*, *plcD*); and iron uptake (*basJ*, *basI*, *basH*, *barB*, *barA*, *basG*, *basF*, *entE*, *basD*, *basC*, *bauB*, *bauE*, *bauC*, *basB*, *basA*, and *bauF*).

In conclusion, to the best of our knowledge, this is the first report of bla_{NDM-6} in an *A. baumannii* isolate. The CRAB isolate encoded two copies of bla_{NDM-6} in close proximity with IS*Aba125*. The carbapenemase NDM-6 has been detected in a ST85^{Pas} multidrug-resistant isolate belonging to the recently described IC9. The present study highlights the complexity and diversity of the genetic environment of NDM-1-like enzymes contributing to its dissemination. The emergence of NDM-6 in an *A. baumannii* clinical isolate highlights the need of surveillance studies and exhaustive control to prevent its spread in the clinical setting. The implementation of infection control measures should also be a priority to fight against multidrug-resistant isolates in the nosocomial environment.

DATA AVAILABILITY STATEMENT

The assembled genome generated in this project has been deposited in the NCBI and we are awaiting for processing to include the accession number in the manuscript.

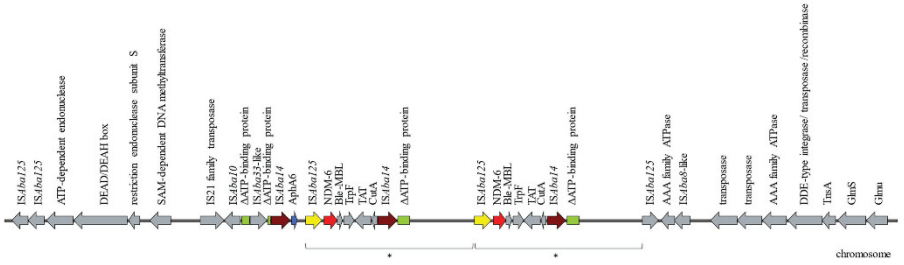

FIGURE 1 | Schematic diagram of the genetic environment of bla_{NDM-6} in the *Acinetobacter baumannii* AbBAS-1 isolate. Arrows indicate the deduced open reading frames (ORFs) and their orientations. The region marked with the asterisk represents a 10,462 bp duplication. Hypothetical proteins are not shown.

ETHICS STATEMENT

Ethical approval for this study was obtained from the clinical research ethics committee of the Hospital Universitario de Basurto-OSI Bilbao Basurto, Northern Spain.

AUTHOR CONTRIBUTIONS

KX, MU-G, PGH, and LG contributed to the design of the experiments. KX, JW, MU-G, and SS-U performed the experiments. KX, JW, HS, PGH, and LG analyzed and interpreted the data. KX, PGH, and LG wrote the manuscript. MU-G, MV-G, and J-LD were responsible for the clinical follow up of the patient, and identification of the isolate and the resistance profile. MU-G also contributed to the preliminary sequence analysis and capsule sequence typing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.589253/full#supplementary-material

Supplementary Figure 1 | Minimum spanning tree generated using Ridom SeqSphere+ for the six ST85Pas *A. baumannii* NDM-positive isolates. Each colored circle represents one individual isolate based on sequence analysis of 2390 cgMLST target genes. Information about the five publicly available isolates: Ab-NDM-1 (Acc. No. NZ_QBBY00000000) recovered in Spain in 2017, AB0964 (available at the https://pubmlst.org under the id 5019) recovered in Singapore, ACMH-6200 (Acc. No. LKMA00000000) recovered in Lebanon in 2012, ACN21 (Acc. No. CP038644) recovered in India in 2018 and MBL_M1 (Acc. No. MWTR00000000) recovered in Tunisia in 2013.

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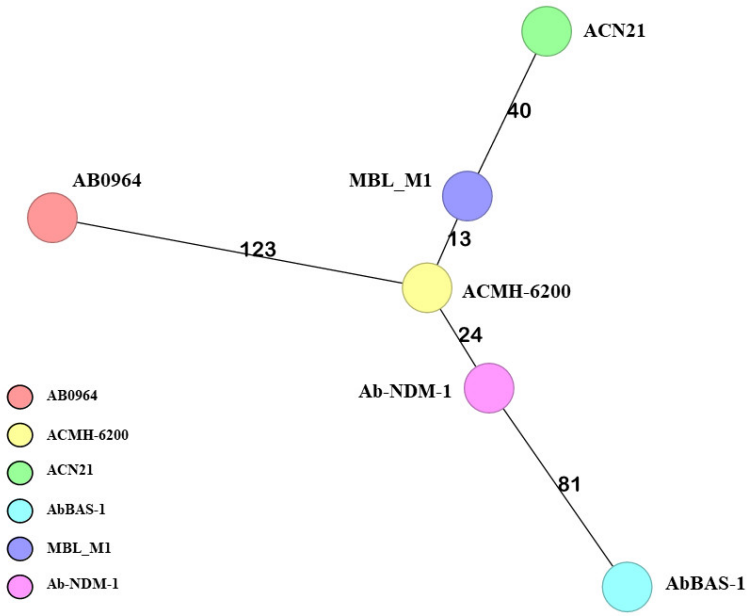

Supplementary Figure 1 | Minimum spanning tree generated using Ridom SeqSphere+ for the six ST85Pas *A. baumannii* NDM-positive isolates. Each colored circle represents one individual isolate based on sequence analysis of 2390 cgMLST target genes. Information about the five publicly available isolates: Ab-NDM-1 (Acc. No. NZ_QBBY00000000) recovered in Spain in 2017, AB0964 (available at the https://pubmlst.org under the id 5019) recovered in Singapore, ACMH-6200 (Acc. No. LKMA00000000) recovered in Lebanon in 2012, ACN21 (Acc. No. CP038644) recovered in India in 2018 and MBL_M1 (Acc. No. MWTR00000000) recovered in Tunisia in 2013.

2.5 MGEs harbouring antibiotic resistance determinants in *A. baumannii* isolates from Bolivia

The open pan genome and the ability to gain new genetic material have contributed to the clinical success of *A. baumannii*.[90] CRAb isolates are especially armed with a plethora of MGEs, which contribute to the dissemination of resistance determinants.[34] In *A. baumannii*, IS*Aba1* is a widely distributed IS element, with a median number of 19 and a maximum number of 34 IS*Aba*1 sites per genome, has been linked to the mobilization of ARGs, such as $bla_{\text{OXA-23-like}}$.[46, 91] Furthermore, carbapenem resistance in *A. baumannii* isolates can be acquired by plasmid-encoded ARGs, such as $bla_{\text{OXA-143-like}}$.[92] Plasmids found in *Acinetobacter* spp. represent distinct plasmid lineages that rarely cross the genus boundaries.[93]

MGEs harbouring ARGs were further investigated in a selection of isolates originating from the previous study about the molecular epidemiology of CRAb and non CRAb from Bolivia. Three MDR *A. baumannii* representatives of the clonal lineages IC4, IC5 and IC7 (MC75, MC23 and MC1 respectively) were analysed. We identified various MGE structures including transposons and plasmids, e.g. $bla_{\text{OXA-23}}$ embedded in Tn*2008* on the chromosome in the isolates MC1 and MC75, or a 6 kb pMC23.3 harbouring the aminoglycoside modifying enzyme *aadB* in the isolate MC23. In the latter isolate, *strA* and *strB* were encoded on a RI embedded in the chromosome (RI1.MC23) including the *sul2* and *floR* resistance genes, responsible for streptomycin, sulphonamide, and chloramphenicol resistance, respectively. In contrast, in the isolate MC75 *strA*, *strB* and *sul2* were encoded on a Tn*6172* on a 150 kb plasmid (pMC75.1). A 8.7 kb plasmid (pMC1.2/pMC23.2) was found to cross the clonal lineage boundaries and was present in both IC7 and IC5 isolates. These results mirror the prevalence of MGEs and their contribution to the dissemination of ARGs in Bolivia.

In the following research paper, my contribution included WGS, plasmid profiling and analysis, data interpretation, editing and reviewing the manuscript.

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Mobile Genetic Elements Harboring Antibiotic Resistance Determinants in *Acinetobacter baumannii* Isolates From Bolivia

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Using a combination of short- and long-read DNA sequencing, we have investigated the location of antibiotic resistance genes and characterized mobile genetic elements (MGEs) in three clinical multi-drug resistant *Acinetobacter baumannii*. The isolates, collected in Bolivia, clustered separately with three different international clonal lineages. We found a diverse array of transposons, plasmids and resistance islands related to different insertion sequence (IS) elements, which were located in both the chromosome and in plasmids, which conferred resistance to multiple antimicrobials, including carbapenems. Carbapenem resistance might be caused by a *Tn2008* carrying the bla_{OXA-23} gene. Some plasmids were shared between the isolates. Larger plasmids were less conserved than smaller ones and they shared some homologous regions, while others were more diverse, suggesting that these big plasmids are more plastic than the smaller ones. The genetic basis of antimicrobial resistance in Bolivia has not been deeply studied until now, and the mobilome of these *A. baumannii* isolates, combined with their multi-drug resistant phenotype, mirror the transfer and prevalence of MGEs contributing to the spread of antibiotic resistance worldwide and require special attention. These findings could be useful to understand the antimicrobial resistance genetics of *A. baumannii* in Bolivia and the difficulty in tackling these infections.

Keywords: *A. baumannii*, plasmids, mobile genetic elements, antimicrobial resistance, carbapenemase

INTRODUCTION

Acinetobacter baumannii is a non-fermenting Gram-negative bacilli and it is the second most common species after *Pseudomonas aeruginosa* in this group causing bacterial infections (Gonzalez-Villoria and Valverde-Garduno, 2016). While *A. baumannii* has been isolated from the wider environment such as water, soil, and animals, most studied isolates come from clinical samples, where *A. baumannii* has become a serious health problem, particularly in the intensive care unit, where it can cause serious and prolonged outbreaks (Gonzalez-Villoria and Valverde-Garduno, 2016). *A. baumannii* is often multidrug resistant (Peleg et al., 2008; Gonzalez-Villoria and Valverde-Garduno, 2016) making antimicrobial therapy of *A. baumannii* infections difficult.

In some cases, with the advent of resistance to last line antibiotics such as colistin, there are few therapeutic options left (Higgins et al., 2010; Manchanda et al., 2010; Göttig et al., 2014; Cayô et al., 2016).

Acinetobacter baumannii is known to have a great genome plasticity, which is the capacity to acquire and disseminate genes, especially those related to antimicrobial resistance which are commonly associated with insertion sequence (IS) elements in transposons and plasmids; this dynamism in the genome of *A. baumannii* contributed to the rapid evolution of drug resistance (Adams et al., 2010) as has been demonstrated for IS*Aba1* mobilizing antimicrobial resistance genes (Mugnier et al., 2009). These processes are achieved thanks to mobile genetic elements (MGEs) harboring resistance genes. The simplest MGEs are ISs, that can also form transposons (Tn), and there are more complex structures such as integrons, resistance islands (RI), and plasmids. Antimicrobial resistance genes are often integrated into resistance cassettes related to translocation elements, causing cumulative resistance to multiple drugs (Roca et al., 2012).

A diverse range of MGEs have been described in *A. baumannii*, for example transposons such as *Tn2008*, *Tn2008B*, *Tn2006*, *Tn2009*, or *Tn2007*, which represent different transposon configurations carrying the bla_{OXA-23} gene together with IS*Aba1* or IS*Aba4*, and additional genes (Nigro and Hall, 2016). Great variability in antimicrobial resistance platforms, including MGEs, have been recorded even within the same international clone (IC), illustrating their contribution to the evolution of drug resistance (Adams et al., 2010). Plasmids in *Acinetobacter* spp. are unique and unrelated to those from other genera, although they often share the same resistance determinants, such as *strA*, *strB*, *tet(B)* or *sul2*. In *A. baumannii*, a diverse array of plasmids have been found, ranging in size from 2 Kb to more than 150 Kb. The larger plasmids normally encode for more than one resistance gene, but up to now little is known about these plasmids (Carattoli, 2013; Hamidian et al., 2016).

The aim of this study was to characterize the MGEs such as plasmids and RI of three different *A. baumannii* clinical isolates, representing different clonal lineages.

MATERIALS AND METHODS

Bacterial Isolates

Three *A. baumannii* isolates recovered from two hospitals in Cochabamba, Bolivia, in September 2015, January 2016, and October 2016 (**Table 1**) representing three different ICs (IC4, IC5. and IC7) were selected for this study. We previously reported their carbapenem resistance mechanisms and molecular epidemiology (Cerezales et al., 2019).

Antimicrobial Susceptibility Testing

In addition to previously reported carbapenem susceptibility testing results, in the present study we investigated the following antimicrobials by agar dilution: amikacin, azithromycin, chloramphenicol, trimethoprim-sulfamethoxazole, erythromycin, levofloxacin, minocycline, kanamycin, and tetracycline.

TABLE 1 | *Acinetobacter baumannii* isolates data.

Isolate	Molecular epidemiology					MIC (mg/L) for various antimicrobials												
	STs Ox/Pas	IC	$bla_{OXA-51-like}$	Sample origin	GenBank accession number	AMK	AZI*	CHL	CIP	SXT*	CST	ERY*	GEN	IPM	KAN*	LVX	MEM	MIN*	TET*	TGC*
MC1	1518/991	IC7	bla_{OXA-64}	Catheter	NZ_QXPV00000000.1	>128 R	64	>128 R	>128 R	128	1 S	64	32 R	32 R	>256	32 R	64 R	64	>128	16
MC23	1520/79	IC5	bla_{OXA-65}	Urine	NZ_QXPJ00000000.1	>128 R	64	>128 R	128 R	128	1 S	64	>128 R	1 S	>256	32 R	2 S	1	16	4
MC75	236/15	IC4	bla_{OXA-51}	Ulcer	NZ_QXOL00000000.1	>128 R	32	128 R	>128 R	128	1 S	32	>128 R	32 R	>256	8 R	64 R	0,5	16	2

*AMK, amikacin; AZI, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; CST, colistin; ERY, erythromycin; GEN, gentamicin; IPM, imipenem; KAN, kanamycin; LVX, levofloxacin; MEM, meropenem; MIN, minocycline; TET, tetracycline; TGC, tigecycline. *No ELCAST breakpoints are available for these antimicrobials.*

TABLE 2 | Plasmid content, size, location resistance genes as determined by WGS, and accession numbers.

Isolate	Plasmids	Accession number	Size	Location of resistance genes	
				Plasmid	Chromosome
MC1	pMC1.1	MK531536	184 Kb	*strA* *strB* *aac(3)-IIa* *aac(6')-Ian* *tet(B)* *sul2*	bla_{OXA-23}
	pMC1.2	MK531537	8.7 Kb		
MC23	pMC23.1	MK531538	67 Kb		*strA* *strB* *sul2* *floR* *aadA1* *sat2* *dfrA1*
	pMC23.2	MK531537	8.7 Kb		
	pMC23.3	MK531539	6 Kb	*aadB*	
MC75	pMC75.1	MK531540	149 Kb	*strA* *strB* *sul2*	bla_{OXA-23} *aphA6*
	pMC75.2	MK531541	13.9 Kb	bla_{TEM-1} *aac(3)-IIa*	

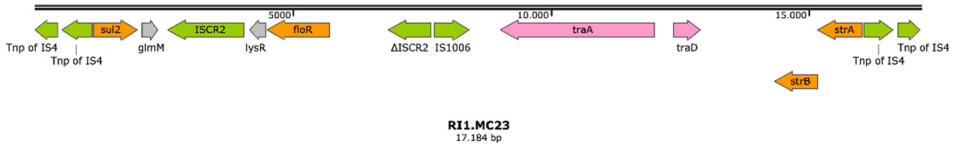

FIGURE 1 | Resistance island RI1.MC23 in isolate MC23. Arrows represent predicted ORFs and the direction of the arrow represents the direction of transcription. Resistance genes are shown by orange arrows and transposon-related genes, recombinases, and insertion sequences are indicated by green arrows. Genes involved in plasmid mobility are shown in pink. Other genes are indicated by gray arrows. Hypothetical proteins are not shown.

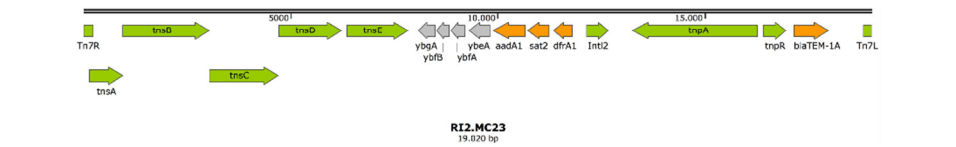

FIGURE 2 | Resistance island RI2.MC23 in isolate MC23. Arrows represent predicted ORFs and the direction of the arrow represents the direction of transcription. Resistance genes are shown by orange arrows and transposon-related genes, recombinases, and insertion sequences are indicated by green arrows. Other genes are indicated by gray arrows. Hypothetical proteins are not shown.

MICs were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints[1].

MinION Long-Read Sequencing and Assembly

The Oxford Nanopore Technologies (Oxford, United Kingdom) MinION sequencer was used to obtain long reads to span repetitive elements and close genomes and plasmids. DNA extraction was performed using the Genomic-tip 100/G kit (Qiagen, Hilden, Germany). Library preparation was carried out according to manufacturer's indications using a combination of Native Barcoding Kit 1D and Ligation Sequencing Kit 1D; EXP-NBD103 and SQK-LSK108 (Oxford Nanopore Technologies, Oxford, United Kingdom), respectively.

The tool Albacore (Oxford Nanopore Technologies, Oxford, United Kingdom) was used for demultiplexing the reads which were later used to perform the Canu assembly (Koren et al., 2017). A hybrid assembly combining previous MiSeq short reads with MinION-generated long reads was performed using a hybridSpades (Antipov et al., 2016).

[1]http://www.eucast.org/clinical_breakpoints/

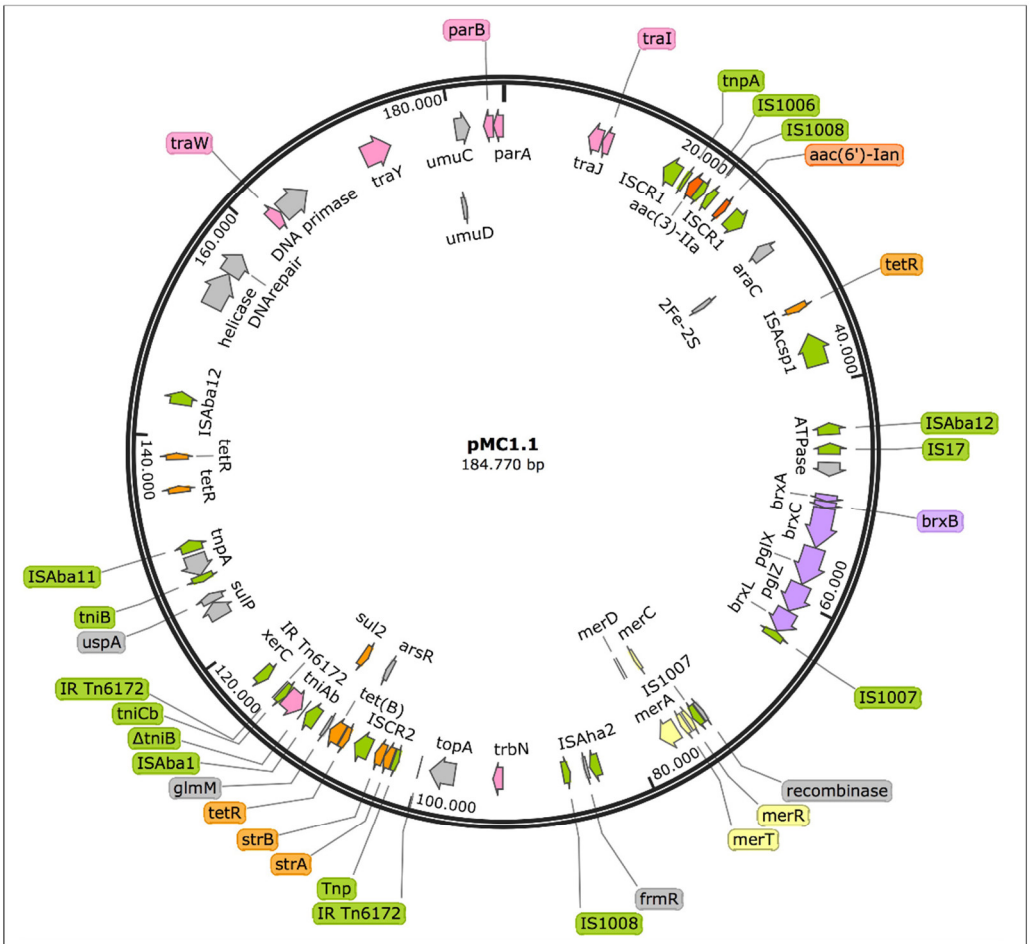

FIGURE 3 | Plasmid pMC1.1 in isolate MC1. Arrows represent predicted ORFs and the direction of the arrow represents the direction of transcription. Resistance genes are shown by orange arrows and transposon-related genes, recombinases and insertion sequences are indicated by green arrows. Transfer protein encoding genes, conjugal transfer protein encoding genes, and genes involved in plasmid partition and replication are shown in pink. The mercury resistance operon genes are indicated by yellow arrows and the BREX type 1 system is shown in purple. Other genes are indicated by gray arrows. Hypothetical proteins are not shown.

Plasmid Annotation and Visualization

ORFfinder (NCBI)[2] was used to predict the open reading frames (ORF) of the plasmids. A second functional annotation of the genomes was performed using the online tool Rapid Annotation Subsystem Technology (RAST)[3] (Genomics et al., 2008). Subsequently, the tool SnapGene Viewer (GSL Biotech)[4] was used to obtain a circular diagram of the plasmids. Graphic comparisons between similar plasmids, pMC1.1 and pA297-3, as well as pMC23.1 and pAC30c, were carried out with the tool Kablammo (Wintersinger and Wasmuth, 2015).

Conjugation Experiments

Broth mate conjugation experiments were performed to determine the location of antimicrobial resistance genes using the sodium azide-resistant *Escherichia coli* J53 and the rifampicin-resistant *A. baumannii* BM4547 as recipient

[2]https://www.ncbi.nlm.nih.gov/orffinder/
[3]https://rast.nmpdr.org/
[4]https://www.snapgene.com

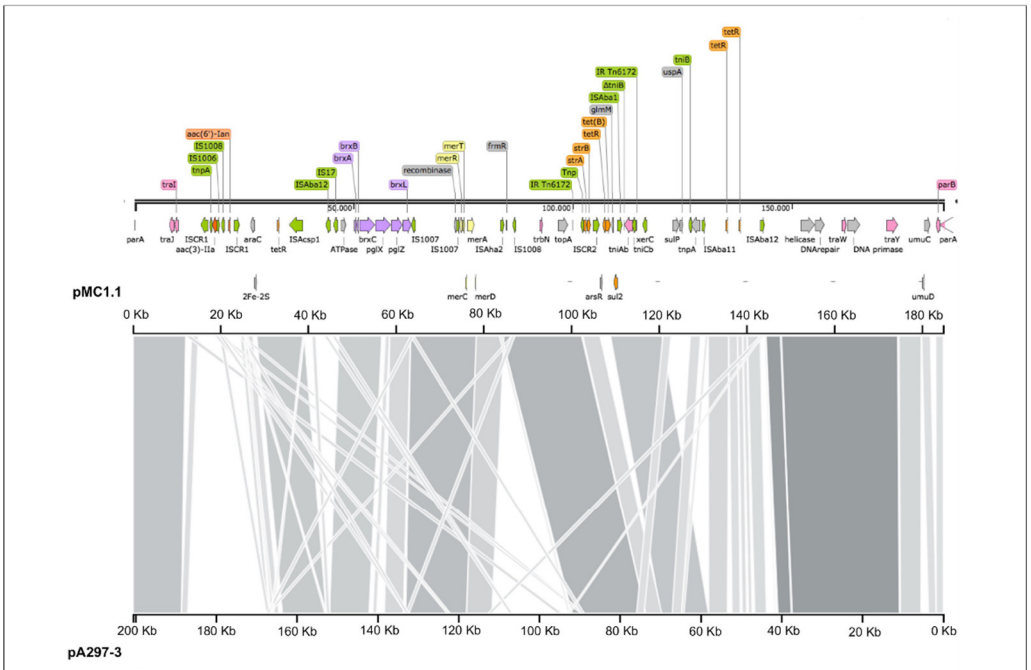

FIGURE 4 | Comparison of plasmids pMC1.1 and pA297-3. The top axis represents the pMC1.1, the bottom axis represents pA297-3. Gray shaded regions show the homologous regions between the two plasmids.

strains. Selection of *E. coli* J53 transconjugants was performed using sodium azide (200 mg/L) combined either with amikacin (30 mg/L), streptomycin (30 mg/L), kanamycin (30 mg/L), gentamicin (30 mg/L), or ticarcillin (100 mg/L), and selection for *A. baumannii* BM4547 was performed using rifampicin (60 mg/L) combined with gentamicin (30 mg/L) or ticarcillin (100 mg/L. Transconjugants were selected with the antimicrobials to select for the plasmids encoding their respective resistance genes. Strain MC1 was resistant to rifampicin, therefore conjugation with *A. baumannii* BM4547 could not be performed. The transconjugants were tested by PCR for the bla_{TEM} gene.

RESULTS AND DISCUSSION

MC1 and MC75 were previously tested as carbapenem-resistant and carried the carbapenemase encoding $bla_{\mathrm{OXA-23-like}}$ gene (Cerezales et al., 2019). Further testing revealed that MC1 and MC75 were also resistant to amikacin, chloramphenicol, ciprofloxacin, gentamicin, and levofloxacin. MC23 was resistant to amikacin, chloramphenicol, ciprofloxacin, gentamicin, and levofloxacin but was susceptible to carbapenems. All three isolates were susceptible to colistin (**Table 1**).

The $bla_{\mathrm{OXA-23}}$ encoding gene was located on the chromosome in a *Tn2008* vehicle in the isolates MC1 and MC75 (**Table 2**). In *A. baumannii*, the $bla_{\mathrm{OXA-23-like}}$ gene is associated with IS*Aba1*, which contributes to its overexpression as well as its mobilization (Nigro and Hall, 2016). *Tn2008* has previously been described in Bolivian *A. baumannii* isolates and this mirrors the spread of this structure among different ICs leading to a carbapenem-resistant phenotype (Nigro and Hall, 2016; Sennati et al., 2016; Chen et al., 2017; Ewers et al., 2017; Cerezales et al., 2018).

Resistance Islands

In the isolate MC23, the gene *strA* was located on a resistance island in the chromosome (RI1.MC23) (accession number MK531542), together with other antimicrobial resistance genes such as *sul2*, *floR*, and *strB*. Diverse IS elements were found, with the resistance island bracketed by two copies of a transposase from the IS4 family in reverse orientation (**Figure 1**). Two genes involved in conjugation were also present in this structure, suggesting a plasmid origin.

In addition, a second chromosomal resistance island was also found in this isolate (RI2.MC23) (accession number MK531543), that carried a typical structure from class 2 integrons,

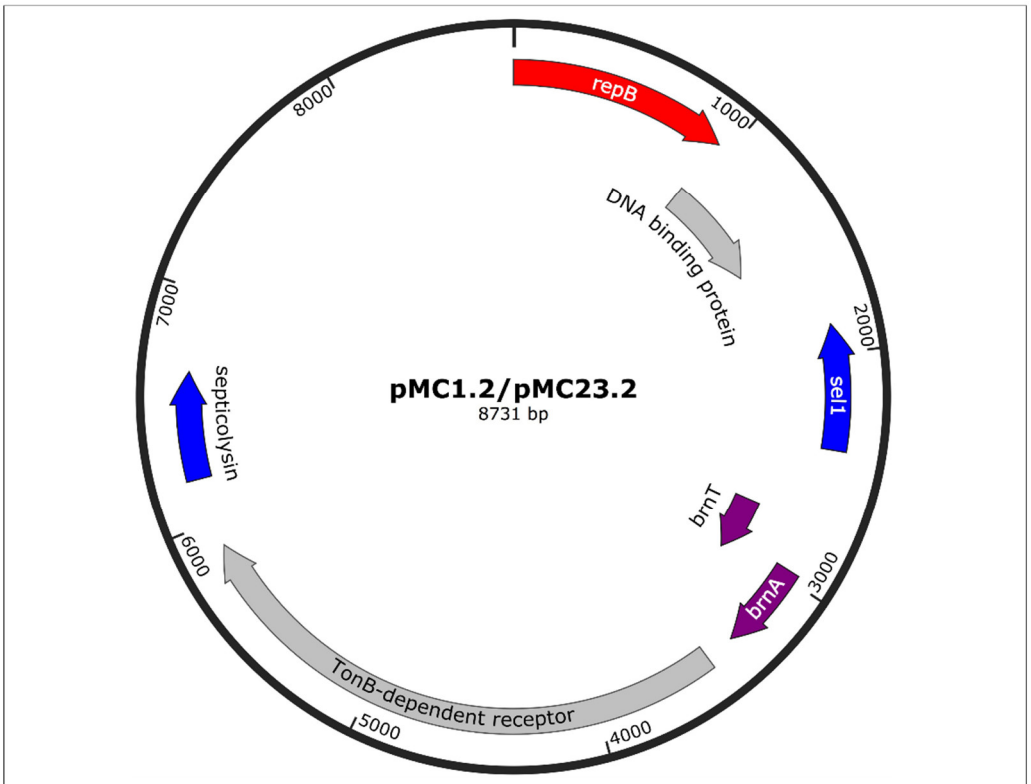

FIGURE 5 | Plasmids pMC1.2 and pMC23.2 in isolates MC1 and MC23, respectively. Arrows represent predicted ORFs and the direction of the arrow represents the direction of transcription. Red arrow is used for the replicon. The toxin-antitoxin system is indicated by violet arrows and blue arrows represent virulence genes. Other genes are shown in gray.

dfrA-sat2-aadA1-ybeA-ybfAybfB-ybgA, located between the Tn7 transposition module *tns*ABCDE and a non-functional IntI2 integrase. Additionally, a Tn3 transposon was found inserted in the Tn7 transposon, carrying three genes, *tnpA*, encoding for a Tn3 transposase; *tnpR* encoding a Tn3 resolvase; and the antimicrobial resistance gene $bla_{\text{TEM-1A}}$ (**Figure 2**).

The gene encoding Apha6 was found on the chromosome of MC75 bracketed by two IS*Aba125* that is a composite transposon known as Tn*aphA6* (Matos et al., 2019).

Plasmids

pMC1.1

Annotation of pMC1.1 (accession number MK531536), 39% GC content, revealed many different IS elements such as IS*1006*, IS*1007*, IS*1008*, IS*Acsp1*, IS91 family, IS*Aba2*, IS*Aba11*, IS*Aba12*, and IS*17*. This plasmid carried a mercuric resistance operon, similar to an already described mercuric Tn in a 200 Kb plasmid (pA297-3) from an IC1 *A. baumannii* isolate, but it lacks the *merP* open reading frame (Hamidian et al., 2016). Different antimicrobial resistance determinants such as *strA, strB, aac(3)-IIa,* and *aac(6′)-Ian,* conferring resistance to aminoglycosides, *sul2* conferring resistance to sulphonamides, and *tet(B)* conferring resistance to tetracycline were also present. The region of the plasmid carrying *strA, strB, and sul2* shared high homology with *Tn6172,* located in pA297-3 as well (**Figure 3**), however, in pMC1.1 *arsR, tetR,* and *tet(B)* genes were also located within *Tn6172* with an IS*CR2* transposable element (IS91 family). This IS*CR* element has been described associated with different antimicrobial resistance genes in *A. baumannii*, especially with *sul2*, contributing to their mobilization thanks to a rolling circle transposition mechanism (Toleman et al., 2006), and was similar to other plasmids from Argentina (Vilacoba et al., 2013) and to plasmids found in an ST25 isolate from Australia (Hamidian and Hall, 2016). However, the location of *tetR-tetB* genes was different; they were located between *glmM* and *arsR*, suggesting a possible later insertion of these genes in

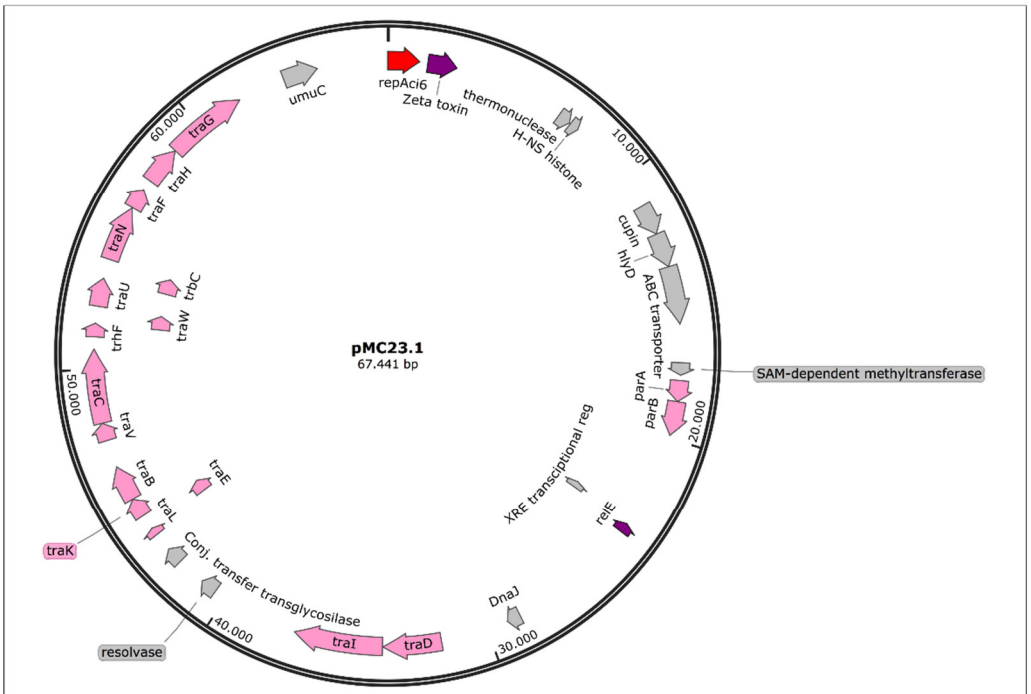

FIGURE 6 | Plasmid pMC23.1 in isolate MC23. Arrows represent predicted ORFs and the direction of the arrow represents the direction of transcription. Red arrow is used for the replicon. The toxins are indicated by violet arrows represent virulence genes. Transfer protein encoding genes, conjugal transfer protein encoding genes, and genes involved in plasmid partition and replication are shown in pink. Other genes are shown in gray. Hypothetical proteins are not shown.

different positions within the transposon (Vilacoba et al., 2013). In addition, the same inverted repeats (IR) generated by the insertion of the transposon were also found in pMC1.1 which together with the similar backbone with pA297-3 (**Figure 4**) suggest they share a common origin. The genes *aac(3)-IIa* and *aac(6′)-Ian* were associated with IS6 family IS and bracketed by two IS*CR1* in inverted orientation. IS*CR1* belongs to the IS91 family and has been described related to class 1 integrons and antimicrobial resistance genes in diverse Gram-negative species such as *Klebsiella pneumoniae*, *P. aeruginosa*, and *Citrobacter freundii* (Toleman et al., 2006). Different transfer genes (*tra*) were also found in this plasmid, as well as genes involved in plasmid partition and replication (*parB/repB* and *xerC*) that are related to segregational stability of plasmids. This plasmid also encoded a system called BREX type 1 (bacteriophage exclusion) which has been described to be involved in phage resistance (Goldfarb et al., 2015).

pMC1.2/pMC23.2

The 8.7 Kb plasmids found in MC1 and MC23 (pMC1.2 and pMC23.2) were identical (accession number MK531537), with a GC content of 34.3% (**Figure 5**). This small plasmid has often been found in IC1 *A. baumannii* isolates (Lean and Yeo, 2017). Annotation of this plasmid revealed ORFs encoding for a RepB replicon (Rep-3 superfamily, GR2) (Bertini et al., 2010; Lean and Yeo, 2017) a toxin-antitoxin system (BrnT-BrnA), that is involved in vertical stability; TonB-dependent receptor, related to the transmission of signals from the outside of the cell leading to transcriptional activation of target genes; a *septicolysin* gene encoding a cytolytic enzyme toward eukaryotic cells and is involved in pathogenesis; as well as *sel1* gene that encodes for a protein that has been described in diverse prokaryotic genera and has an important role in virulence.

pMC23.1

The largest plasmid in MC23 was the 67.5 Kb pMC23.1 (accession number MK531538) (**Figure 6**). It belonged to GR6 according to its replicase, *repAci6*. Its GC content was 33.7% and almost all of its putative protein encoding genes were related to conjugative plasmid transfer in a *tra* locus, some of them are part of a type IV (T4SS) secretion system. This T4SS is able to secrete or take up both proteins and DNA, and possibly is involved in natural competence, a feature of *A. baumannii* (Salto et al., 2018). Two toxin encoding genes were present in the plasmid,

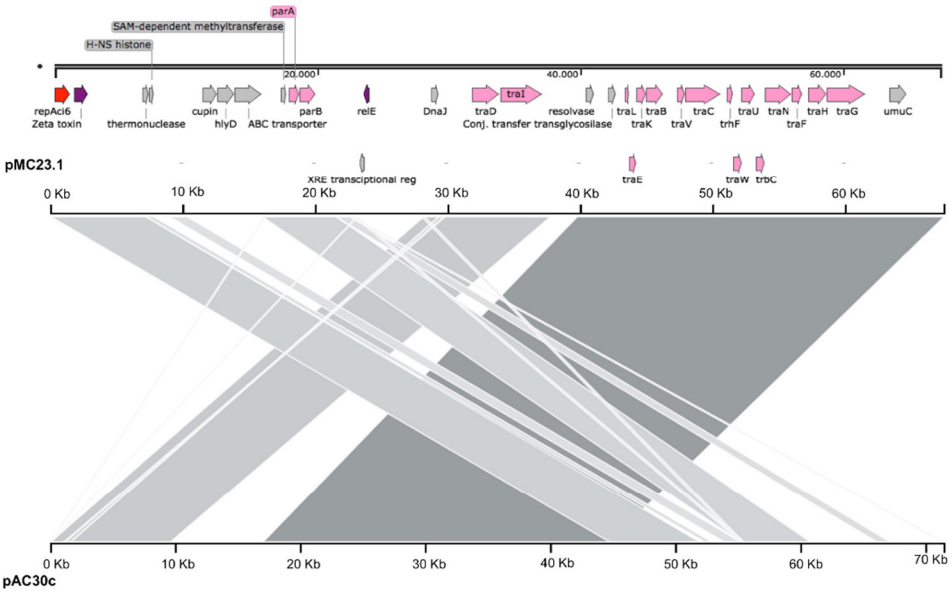

FIGURE 7 | Comparison of plasmids pMC23.1 and pAC30c. The top axis represents the pMC23.1, the bottom axis represents pAC30c. Gray shaded regions show the homologous regions between the two plasmids.

relE and zeta toxin, but no antitoxins were found, although they were present in a very similar plasmid (pAC30c) in an *A. baumannii* isolate belonging to ST195 (IC2) (**Figure 7**; Lean et al., 2016). In addition, the partition genes *parA/parB* were also encoded on pMC23.1. The backbone of pMC23.1 and pAC30c were very similar, with only a few differences. pMC23.1 lacked some hypothetical proteins present in pAC30c, and the region encoding for tellurite resistance (*telA* gene and IS*66*); while *traD*, a cupin-like protein (that is a superfamily of enzymes including dioxygenases, decarboxylases, hydrolases, or isomerases); HlyD protein, that exports proteins from the cytosol to the outside of the cell, and an ABC transporter were not present in pAC30.

pMC23.3

A 6 Kb small plasmid was present in the isolate MC23, pMC23.3 (accession number MK531539), 39.2% GC content, and was found to have 100% similarity with an already described plasmid, pRAY from an isolate in South Africa, encoding resistance to gentamicin, kanamycin and tobramycin (*aadB* gene) together with *mobA* and *mobC* genes, which are thought to encode mobilization proteins (Lean and Yeo, 2017). Many similar plasmids have been found in diverse *A. baumannii* isolates from different ICs and countries, suggesting a common origin and subsequent diversification in their evolution. Concurrent with other studies, no *rep* gene was found in the plasmid sequence, supporting the idea of the presence of a mechanism of replication relying in the host RNA polymerase (Lean and Yeo, 2017).

pMC75.1

Analysis of pMC75.1 (accession number MK531540) a large plasmid of 150 Kb revealed that it was very similar to pMC1.1 (sharing 80% of their sequences), it also carried a Tn*6172*, in which antimicrobial resistance genes such as *sul2, strB,* and *strA* are encoded, but lacking *tet(B)* and *arsR* that were present in pMC1.1 (**Figure 8**). The *mer* operon was also found in this plasmid, and many genes encoding conjugative transfer proteins. The BREX type 1 system was also present. A *stbA* gene was found, the protein encoded by this gene plays a role in plasmid stability as well as *parA/parB*. Several IS elements were also present, i.e., IS*Aba1*, IS*Aba125*, IS*Aba14*, IS*Aba42*, IS*1007*, and IS*Aha2*. However, this plasmid lacked the transposon carrying *aac(3)-IIa* and *aac(6′)-Ian*.

pMC75.2

The 13.9 Kb plasmid, pMC75.2 (accession number MK531541) (**Figure 9**) with a GC content of 40.3%, carried the broad-spectrum β-lactamase $bla_{\text{TEM-1B}}$ and the aminoglycoside resistance gene *aac(3)-IIa* flanked on both sides by IS15DIV; a toxin-antitoxin system, *brnT/brnA*; a TonB-dependant receptor, a septicolysin gene and *mobA/mobS*, which are involved in plasmid mobility. Conjugation experiments revealed that

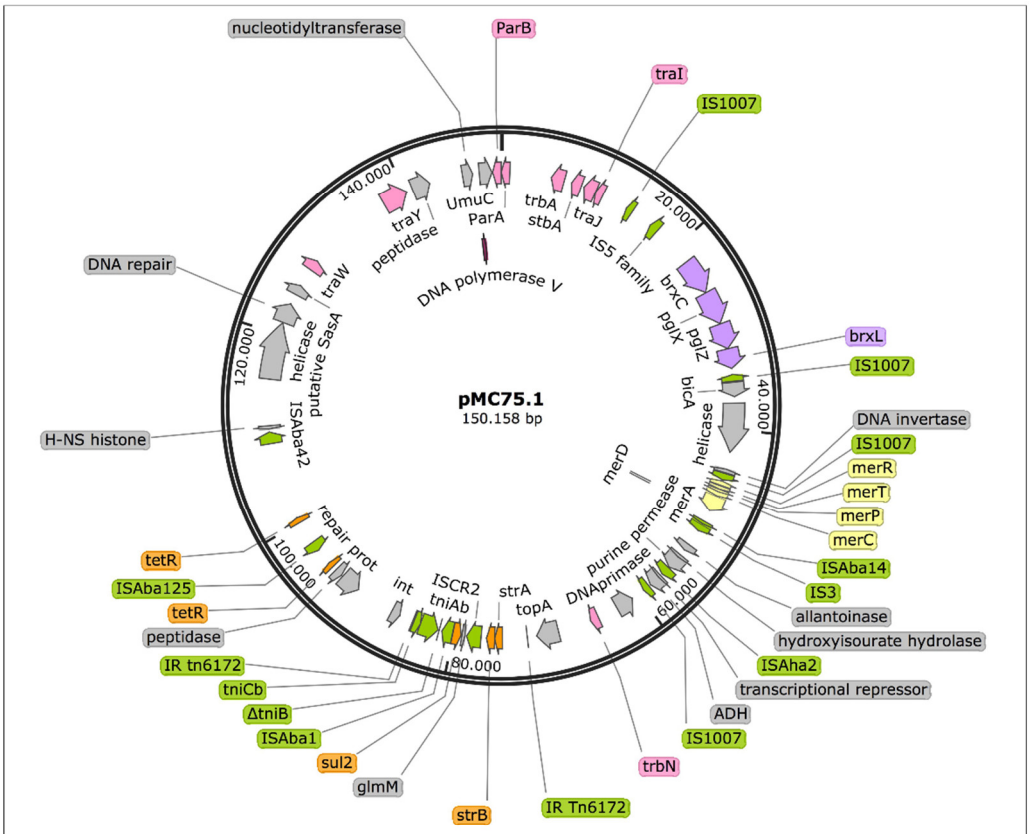

FIGURE 8 | Plasmid pMC75.1 in isolate MC75. Arrows represent predicted ORFs and the direction of the arrow represents the direction of transcription. Resistance genes are shown by orange arrows and transposon-related genes, recombinases and insertion sequences are indicated by green arrows. Transfer protein encoding genes, conjugal transfer protein encoding genes, and genes involved in plasmid partition and replication are shown in pink. The mercury resistance operon genes are indicated by yellow arrows and the BREX type 1 system is shown in purple. Other genes are indicated by gray arrows. Hypothetical proteins are not shown.

pMC75.2 was transferable into *A. baumannii* BM4547 but it was unstable and was lost after several passages. The replicon of this plasmid belonged to the RepB (Rep_3) superfamily with 100% homology. This plasmid shares a great homology with pMC1.2/pMC23.2, same RepB, toxin-antitoxin system, TonB-dependant receptor and septicolysin; it seems that one of them has lost or alternatively acquired the integron carrying the antimicrobial resistance genes and the mobility genes.

Recently, two similar plasmids to pMC75.1 and pMC75.2 were described in a Brazilian *A. baumannii* isolate representing the same ST (ST15). This illustrates that these plasmids can be very plastic by acquiring or losing genes, but can also be conserved within a ST (Matos et al., 2019).

The two carbapenem-resistant isolates carried the bla_{OXA-23} gene in Tn*2008*, which has been previously described in diverse ICs (Nigro and Hall, 2016; Ewers et al., 2017) including IC7 isolates recovered from a hospital in the same city, Cochabamba (Sennati et al., 2016). The Tn*2008* contributes to the overexpression of the carbapenemase encoding gene and to its mobilization. In addition, all three isolates harbored three aminoglycoside resistance genes such as *aac(3)-IIa*, *strA*, and *strB*; and *sul2* conferring resistance to sulphonamides; MC1 carried *tetB* conferring resistance to tetracycline as well. All the genes were found to be associated with IS elements, constituting transposons that lead to their mobilization and make genetic rearrangements more likely to happen. These genes were found both in the chromosome and in plasmids, demonstrating the plasticity of the *A. baumannii* genome and the mobility of these antimicrobial resistance

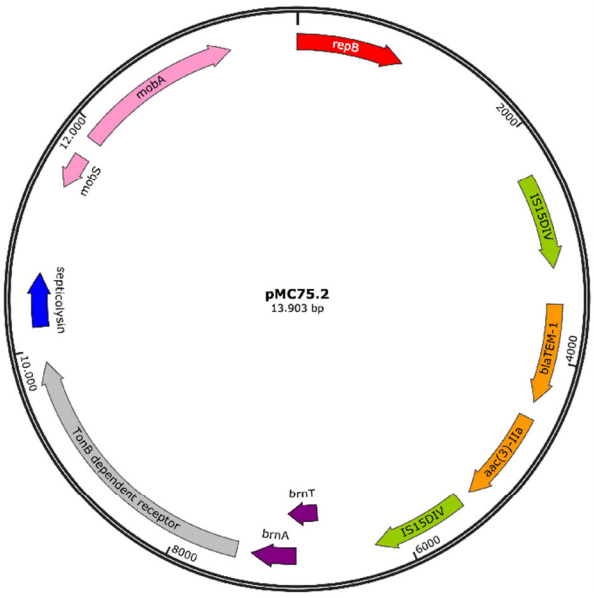

FIGURE 9 | Plasmid pMC75.2 in isolate MC75. Arrows represent predicted ORFs and the direction of the arrow represents the direction of transcription. Resistance genes are shown by orange arrows and insertion sequences are indicated by green arrows. Genes involved in plasmid mobility are shown in pink. The toxin-antitoxin system is shown in violet. Blue represents virulence genes. Other genes are indicated by gray arrows. Hypothetical proteins are not shown. Red arrow is used for the replicon.

determinants within MGEs such as transposons or plasmids.

CONCLUSION

In summary, these data further confirm that *A. baumannii* has a great ability to acquire antimicrobial resistance determinants and become a threat in hospitals. These are associated with different plasmids and many different IS elements, of which some are found in multiple genera. For these reasons it is important to study the dynamics and resistomes of the bacterial populations in order to understand the situation in each hospital or unit. The fact that some of these plasmids have been found in diverse *A. baumannii* clonal lineages mirrors the transfer and prevalence of these MGEs contributing to the spread of antimicrobial resistance worldwide.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank, MK531536, MK531538, MK531537, MK531539, MK531540, and MK531541.

AUTHOR CONTRIBUTIONS

MC, KX, JW, and PH contributed to the design of the experiments. MC, KX, and JW performed the experiments. MC, KX, JW, OK, HS, LG, and PH analyzed and interpreted the data. MC, KX, and PH wrote the manuscript. All authors contributed to critical manuscript revision, read, and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.6 Antibiotic resistance and MGEs in XDR *K. pneumoniae* ST147 recovered from Germany

Steadily increasing rates of resistance to different classes of antibiotics are being reported in *K. pneumoniae* with not only MDR but also XDR and PDR phenotypes over the years. In *K. pneumoniae* antibiotic resistance can be mostly ascribed to MGEs, such as conjugative plasmids or transposons.[2, 94]

In the present manuscript, we analysed three XDR *K. pneumoniae* isolates colonizing three patients from a German university hospital. The isolates were assigned to the high-risk clone ST147 and were clonal by cgMLST (up to one allele difference). Furthermore, all three isolates harboured 8 plasmids in total. The carbapenemase $bla_{\text{OXA-181}}$, an OXA-48 variant, was encoded on a ColKP3 plasmid. Moreover, 12 antibiotic resistance determinants, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1B}}$, *aac(6')Ib-cr*, *aac(3)-IIa*, *strA*, *strB, qnrS1*, *sul1*, *dfrA1*, *tet*(A), *catB3*-like, were encoded on an MDR IncR plasmid which harboured in addition a large number of IS elements. The latter plasmid showed a high degree homology among the three isolates but presented also diversity including inversion or deletion of DNA segments in the vicinity of MGEs. For instance, the IncR plasmids varied in a 4 kb region and in the opposite orientation of two composite transposons (3,8 kb and 13 kb), events that can be ascribed to MGEs. These results demonstrated that the dynamics of MGEs can facilitate genome plasticity and contribute to the genetic variation within closely related isolates.

My contribution to the present study involved plasmid analysis, WGS, phenotypic and genotypic characterization, *in silico* analysis, data interpretation, and writing the following manuscript.

antibiotics

MDPI

Article

Antibiotic Resistance and Mobile Genetic Elements in Extensively Drug-Resistant *Klebsiella pneumoniae* Sequence Type 147 Recovered from Germany

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Abstract: Mobile genetic elements (MGEs), especially multidrug-resistance plasmids, are major vehicles for the dissemination of antimicrobial resistance determinants. Herein, we analyse the MGEs in three extensively drug-resistant (XDR) *Klebsiella pneumoniae* isolates from Germany. Whole genome sequencing (WGS) is performed using Illumina and MinION platforms followed by core-genome multi-locus sequence typing (MLST). The plasmid content is analysed by conjugation, S1-pulsed-field gel electrophoresis (S1-PFGE) and Southern blot experiments. The *K. pneumoniae* isolates belong to the international high-risk clone ST147 and form a cluster of closely related isolates. They harbour the $bla_{\text{OXA-181}}$ carbapenemase on a ColKP3 plasmid, and 12 antibiotic resistance determinants on an multidrug-resistant (MDR) IncR plasmid with a recombinogenic nature and encoding a large number of insertion elements. The IncR plasmids within the three isolates share a high degree of homology, but present also genetic variations, such as inversion or deletion of genetic regions in close proximity to MGEs. In addition, six plasmids not harbouring any antibiotic resistance determinants are present in each isolate. Our study indicates that genetic variations can be observed within a cluster of closely related isolates, due to the dynamic nature of MGEs. The mobilome of the *K. pneumoniae* isolates combined with the emergence of the XDR ST147 high-risk clone have the potential to become a major challenge for global healthcare.

Keywords: carbapenem resistance; carbapenemase; whole genome sequencing; long reads, plasmid; *Klebsiella pneumoniae*; extensively drug-resistant; molecular typing

1. Introduction

The evolution and spread of antibiotic-resistant pathogens has emerged as one of the most important public health problems worldwide over the last decades (https://www.who.int/en/news-room/fact-sheets/detail/antibiotic-resistance). In bacterial genomes, capture, accumulation and dissemination of antibiotic resistance determinants are often associated with mobile genetic elements (MGEs) like plasmids, transposons and insertion sequences (ISs) [1]. Plasmids are often assemblies of different MGE modules and are the most efficient intra- and interspecies DNA transfer mechanism among prokaryotes [2]. This is well exemplified by the global spread of the KPC carbapenemase involving the incompatibility group FIIk (IncFIIk) plasmids in *Klebsiella pneumoniae* [3]. Moreover, bla_{NDM-1} in *K. pneumoniae* has been mainly associated with broad host range IncA/C2, IncHI1, IncX3 and IncN2 plasmids [4]. In *Acinetobacter baumannii*, the transposon Tn*125*, harbouring the insertion element IS*Aba125*, is considered as the main vehicle for the dissemination of NDM-1 enzymes [5,6].

K. pneumoniae, belonging to the Enterobacterales family, is a natural inhabitant of the gastrointestinal tract of humans and animals. Nevertheless, it is also encountered as a nosocomial pathogen causing various infections such as pneumonia, urinary tract infection and bloodstream infection [4]. Of concern is the rapid expansion of carbapenem-resistant *K. pneumoniae*, mainly associated with those carbapenemases which are endemic in certain countries, such as KPC-positive *K. pneumoniae* in Greece and Italy [7,8]. OXA-48-like is the most common carbapenemase in Enterobacterales in some regions of the world including Germany. Other frequently encountered carbapenemases in Germany include VIM-1 and NDM-1 [9,10]. The successful propagation of OXA-48-positive Enterobacterales is reinforced by the global distribution of certain high-risk clones (e.g., *K. pneumoniae* sequence type (ST) 307 or *Escherichia coli* ST38) as also its association with MGEs, e.g., OXA-48 linked with different Tn*1999* variants on highly transferable IncL plasmids [10,11]. The expansion of high-risk *K. pneumoniae* clones with a multidrug-resistant (MDR) or extensive drug-resistant (XDR) phenotype has been observed in recent years [4]. *K. pneumoniae* ST147 has been reported as an emerging high-risk clone associated with plasmid-encoded extended-spectrum β-lactamases (ESBLs) like $bla_{CTX-M-15}$, or carbapenemases such as bla_{OXA-48} and bla_{NDM-1} [4,12–17].

In the present study, we characterise the content and genetic structure of MGEs and the clonal relatedness of three OXA-181-producing *K. pneumoniae* ST147 clinical isolates recovered in Germany.

2. Results and Discussion

Dissemination of antibiotic resistance is driven by clonal expansion or horizontal gene transfer, including mainly MGEs [1,2]. In the present study, all three isolates colonising haematology/oncology patients were identified as *K. pneumoniae* ST147 and were the only representatives of this ST among 40 in total collected *K. pneumoniae* isolates. The three isolates were also characterised by their capsular type KL64 (*wzi* allele 64). MDR *K. pneumoniae* ST147 isolates represent a successful clone with a global spread and these isolates are often armed with carbapenemases and ESBLs [15,18]. The German National Reference Centre for Multidrug-Resistant Gram-negative Bacteria and the Robert Koch Institute reported, between 2008 and 2014, 13 carbapenemase-producing ST147 *K. pneumoniae* isolates in Germany. In particular, 9/42 OXA-48-, 3/34 KPC-2- and 1/5 NDM-1-producing isolates were assigned to ST147 [19].

In the present study, the isolates HKP0018, HKP0064 and HKP0067 were analysed by whole genome sequencing (WGS) and harboured on the chromosome a gene encoding the intrinsic SHV-11, as well as *oqxAB* and *fosA* genes, belonging to the core genome of the KpI–III phylogroups [20]. The plasmid-encoded resistome of the investigated isolates, summarised in Table 1, was identical

and included beta-lactam, aminoglycoside, fluoroquinolone, tetracycline and other antimicrobial resistance determinants. Antimicrobial susceptibility testing showed that all three *K. pneumoniae* isolates exhibited an XDR phenotype; resistant to ampicillin, aztreonam, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, meropenem, minocycline, tetracycline, ticarcillin, tigecycline, and trimethoprim and susceptible only to amikacin and colistin (Table 2). MDR and XDR *K. pneumoniae* isolates involved in nosocomial outbreaks have been widely reported [4,21,22]. Between June and October 2109, an outbreak of XDR *K. pneumoniae* producing NDM-1 and OXA-48 was reported in four medical facilities in Mecklenburg-Western Pomerania, Germany [23]. Molecular characterisation using core genome multi-locus sequence typing (cgMLST) analysis revealed that the three investigated isolates were closely related and formed a cluster with 0–1 allelic differences (data not shown). One could speculate that the closely related isolates were likely transmitted within the hospital. All three patients had been hospitalised in the same department (Table 3) and two of the patients had an overlapping hospitalisation at the same ward (C5A). However, a direct connection to HKP0018 could not be established within the study.

Table 1. Plasmid encoded antimicrobial resistance determinants, plasmid content and plasmid size of the isolates.

Plasmid	Replicon	Size (bp)	Antimicrobial Resistance Determinants	Isolate No.		
				HKP0018	HKP0064	HKP0067
pHKP0018.1	ColKP3	6103	blaOXA$_{-181}$	+	+	+
pHKP0018.2	IncR	66,330	*bla*$_{\text{CTX-M-15}}$[b], *bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM-1B}}$, *aac(6')Ib-cr*, *aac*(3)-*IIa*, *strA*, *strB*, *qnrS1*, *sul1*, *dfrA1*, *tet*(A), *catB3*-like	+	-	-
pHKP0064.2	IncR	70,762	*bla*$_{\text{CTX-M-15}}$[b], *bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM-1B}}$, *aac(6')Ib-cr*, *aac*(3)-*IIa*, *strA*, *strB*, *qnrS1*, *sul1*, *dfrA1*, *tet*(*A*), *catB3*-like	-	+	+
pHKP0018.3	IncFIB	113,014	-	+	+	+
pHKP0018.4	NT [a]	57,450	-	+	+	+
pHKP0018.5	Col-like	8428	-	+	+	+
pHKP0018.6	Col-like	5499	-	+	+	+
pHKP0018.7	NT [a]	2044	-	+	+	+
pHKP0018.8	Col-like	1459	-	+	+	+

[a] NT, not typeable; [b] gene present in two copies.

Table 2. Antimicrobial susceptibility of the three *K. pneumoniae* isolates.

Antimicrobial Agent	MIC (mg/L)			Susceptibility [a]
	HKP0018	HKP0064	HKP0067	
Amikacin	8	8	8	S
Ampicillin	>128	>128	>128	R
Aztreonam	>128	>128	>128	R
Ceftazidime	128	128	128	R
Chloramphenicol	32	32	32	R
Ciprofloxacin	128	128	128	R
Colistin [b]	1	2	1	S
Gentamicin	128	128	128	R
Imipenem	8	8	8	R
Levofloxacin	64	64	64	R
Meropenem	32	32	32	R
Minocycline [c]	64	64	64	R
Rifampicin [d]	64	64	64	-
Tetracycline [c]	128	128	128	R
Ticarcillin	>128	>128	>128	R
Tigecycline [b]	2	2	2	R
Trimethoprim	128	128	128	R

[a] R, resistant; S, susceptible; [b] tested by broth microdilution method; [c] only CLSI breakpoint available; [d] no breakpoint available.

Table 3. *K. pneumoniae* clinical isolates information.

Isolate	Date of Isolation	Source	Department	Ward	ST
HKP0018	16.02.2015	Rectal swab	Haematology/Oncology	C5A	147
HKP0064	08.05.2015	Throat swab	Haematology/Oncology	1G	147
HKP0067	19.05.2015	Rectal swab	Haematology/Oncology	C5A	147

Phylogenetic analysis of 30 ST147 *K. pneumoniae* isolates from different countries showed several branches (Figure 1). The isolates HKP0018, HKP0064 and HKP0067 were on the same branch with ST147 isolates from different countries, such as Switzerland, USA, United Kingdom and Singapore, illustrating the worldwide spread of this clone. In addition, the three investigated isolates clustered together with 7 ST147 *K. pneumoniae* isolates recovered between 2013 and 2014 in Göttingen, Germany. The latter MDR isolates harboured the carbapenemase OXA-48 on a 63.6 kb IncL plasmid [15]. The close genetic relatedness observed between the German isolates suggests that an OXA-48-like producing ST147 clone is circulating in the country.

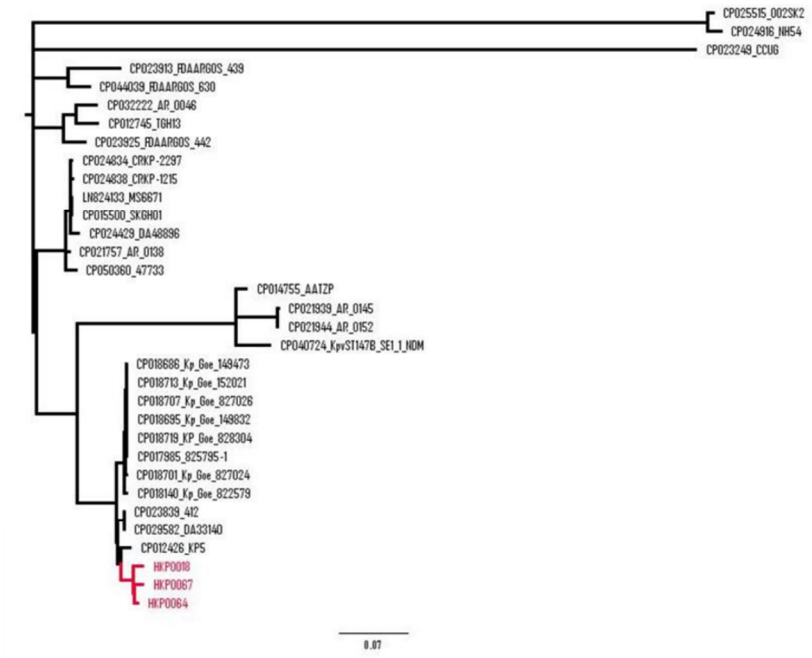

Figure 1. Phylogenetic analysis of HKP0018, HKP0064, HKP0067 and 30 ST147 *K. pneumoniae* isolates. Phylogenetic maximum-likelihood tree was generated using the FigTree v1.4.3 software of the SNP analysis performed using the kSNP3 tool (Galaxy version 3.1) software at the ARIES Galaxy server (https://aries.iss.it/).

In the present study, plasmid analysis revealed eight closed plasmids for each individual *K. pneumoniae* isolate.

OXA-48-like is the most prevalent carbapenem-hydrolysing β-lactamase in Enterobacterales isolates from Germany [9,24]. MDR *K. pneumoniae* ST147 encoding OXA-48 on a conjugative IncL plasmid have been recently reported in Germany [15]. In the present study, all three investigated isolates harboured OXA-181 on an identical 6103 bp ColKP3 plasmid, pHKP0018.1. This plasmid also encoded the mobilisation genes *mobA*, *mobB*, *mobC* and *mobD*, and, upstream of $bla_{\text{OXA-181}}$ gene, 170 bp of a disrupted IS*Ecp1* was present. A blastn analysis to compare pHKP0018.1 to sequences available in the GenBank database revealed high similarities mainly with three groups of plasmids, of which Carbapenemase OXA-232_ColKP3 (Acc. No CP050165), pKP3-A (Acc. No JN205800) and p50595_OXA_181 (Acc. No CP050375) were chosen as exemplars for a more detailed comparison. The first one, with a size of 6141 bp, showed an identity of 99.98% to our plasmid, has a longer fragment of the interrupted IS*Ecp1* (208 bp) and carries the $bla_{\text{OXA-232}}$ gene, a $bla_{\text{OXA-181}}$ variant from which it differs by a single nucleotide, leading to the Arg214-Ser amino acid substitution, and from which it probably originated (Figure 2) [25]. Plasmid pKP3-A, obtained from a clinical *K. pneumoniae* isolate in 2010, is a ColKP3 plasmid carrying $bla_{\text{OXA-181}}$, proved to be mobilisable but not self-transmissible. It showed 99.95% similarity when compared to pHKP0018.1, from which it differs by the presence of the complete IS*Ecp1* element. In this plasmid, the carbapenemase gene was described as part of the Tn*2013* transposon, made up by the 3139 bp module IS*Ecp1*-$bla_{\text{OXA-181}}$-Δ*lysR*-Δ*ereA* [26]. In plasmid pHKP0018.1 this transposon was disrupted, with only the two right inverted repeats (IRR1 and IRR2) and the 3′ target site duplication (ATATA) still identifiable (Figure 2) [26]. Lastly, p50595_OXA_181 plasmid depicts the group of X3-ColKP3 plasmids of approximately 51 kb in size, which held 50% of the pHKP0018.1 plasmid, with an identity of 100%. This portion contained the interrupted Tn*2013* (ΔIS*Ecp1*-$bla_{\text{OXA-181}}$-Δ*lysR*-Δ*ereA*) and an almost complete *repA* gene of ColKP3, inserted between the two insertion sequences IS*3000* and IS*Kpn19* (Figure 2). The sequence comparative analysis also showed that $bla_{\text{OXA-181}}$ seems to be almost uniquely located on X3-ColKP3 plasmids, frequently harboured

by *E. coli* isolates, while its variant $bla_{OXA-232}$ is primarily located on ColKP3 plasmids harboured predominantly by *K. pneumoniae*. Nevertheless, both variants are distributed on a global scale, including not only clinical isolates but also animal and environmental ones. Indeed, OXA-181 and OXA-232 represent, respectively, the second and third most common and widespread OXA-48-like enzyme and both are described as part of the Tn*2013* transposon, which, together with its localisation on plasmids like ColE-type, IncX3, IncN1 and IncT, is responsible for their dissemination [10].

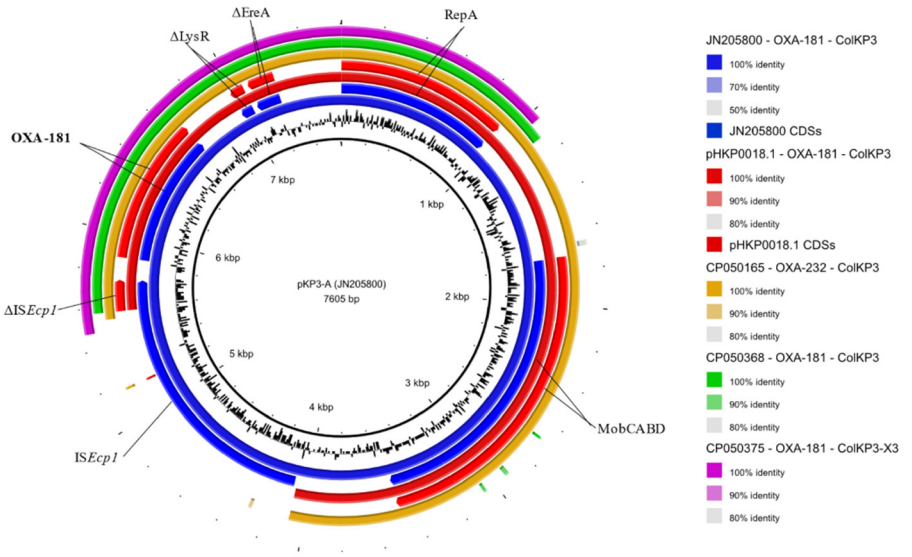

Figure 2. Graphical representation of $bla_{OXA-181}$/$bla_{OXA-232}$-carrying plasmids sequence comparison. Starting from the inner ring: GC content of pKP3-A plasmid sequenced (here used as reference), $bla_{OXA-181}$-positive pKP3-A plasmid sequence (JN205800), pKP3-A CDSs, $bla_{OXA-181}$-positive pHKP0018.1 plasmid sequence (CP061063.1), pHKP0018.1 CDSs, $bla_{OXA-232}$-positive Carbapenemase (OXA-232)_ColKP3 plasmid sequence (CP050165), $bla_{OXA-181}$-positive p47733_OXA_181 plasmid sequence (CP050368), $bla_{OXA-181}$-positive p50595_OXA_181 plasmid sequence (CP050375). CDS's arrows indicate their transcription direction. Hypothetical proteins are not displayed. The figure was generated with BRIG v0.95.

S1-PFGE, Southern blot and WGS analysis revealed that all three isolates harboured an IncR plasmid, pHKP0018.2, pHKP0064.2 and pHKP0067.2, presenting only the *repB* gene and lacking the *repE* and *repA* genes and encoding the same antibiotic resistance determinants (Figure 3). The MDR region included a mosaic structure of 12 antibiotic resistance genes, including β-lactamases $bla_{CTX-M-15}$ (present in two copies on each IncR plasmid), bla_{OXA-1}, bla_{TEM-1B}, aminoglycoside modifying enzymes *aac(6')Ib-cr*, *aac(3)-IIa*, *strA*, *strB*, as well as the resistance determinants *qnrS1*, *sul1*, *dfrA1*, *tet*(A) and *catB3*-like (Table 1). The MDR region was highly recombinogenic and encoded several copies of different ISs (n = 9). Furthermore, pHKP0018.2, pHKP0064.2 and pHKP0067.2 encoded a *higB/higA* toxin-antitoxin (TA) module and *parA/parB* partitioning genes, contributing to plasmid stabilisation and inheritance. As many others previously described, containing only the *repB* gene alone, the IncR plasmid of this study did not harbour known conjugative loci, and consequently attempts to transfer by conjugation IncR and to mobilise the ColKP3-OXA-181 into *E. coli* J53 were not successful. The IncR plasmids showed high sequence homology to IncR plasmids pKp_Goe_304-4 (Acc. No CP018724.1), pKp_Goe_021-4 (Acc. No CP018718.1), pKp_Goe_024-4 (Acc. No CP018705.1), and CP017989.1 of a ST147 *K. pneumoniae* isolate collected in Germany in 2014, and to the IncR plasmid pSg1-NDM (Acc. No CP011839.1) identified in a ST147 *K. pneumoniae* isolate from Singapore [18].

Sequence analysis revealed that pHKP0064.2 and pHKP0067.2 were identical and 70,762 bp in size. Nevertheless, comparative analysis revealed a rearrangement of a composite transposon flanked

by two inverted copies of IS*26* and containing *catB3*-like, *aac(6')Ib-cr* and bla_{OXA-1} genes. This 3826 bp region was inserted in the same position in the two IncR plasmids but in opposite orientation. Similarly, another reshuffling of a 13,957 bp region was observed for pHKP0064.2 and pHKP0067.2. This genomic region was flanked by two copies of IS*Ecp1* in inverse orientation and harboured a truncated transposase, Tn3 resolvase, bla_{TEM-1B}, *qnrS1*, recombinase, IS*Kpn19*, *umuC*, HAMP-domain and IS*26* (Figure 3). In the isolate HKP0018 an IncR plasmid, pHKP0018.2, with a size of 66,330 bp was identified. The plasmids pHKP0064.2 and pHKP0067.2 shared a high degree of sequence homology with pHKP0018.2, apart from a 4432 bp region which was missing from the latter plasmid. The missing region was part of the 13,957 bp genomic region involved in the rearrangement in pHKP0064.2 and pHKP0067.2. This subregion was comprised of genes encoding for the error-prone DNA polymerase V subunit (*umuC*) and a sensor histidine kinase (HAMP-domain) followed by the MGE IS*Kpn19* (Figure 3). These results indicate that within a group of clonal isolates, diversity can still be observed. Genetic variation within clonal bacterial groups caused by homologous recombination has been described in *E. coli* [27]. While genetic rearrangement, such as inversion or duplication, caused by MGEs have been confirmed by diverse studies [28–30].

An identical 113,014 bp IncFIB-like plasmid, pHKP0018.3, was identified in the *K. pneumoniae* isolates and did not encode any known antibiotic resistance determinants (Figure S1). However, this plasmid harboured a tellurite/colicin resistance determinant, phage-related genes, and was lacking known conjugative transfer genes. Furthermore, the plasmid harboured two members of the IS3 family, IS*Kpn1* and IS2. The IncFIB-like plasmid showed high homology (coverage 97%, identity 100%) to pSG1.1 (Acc. No CP012427.1) from an NDM-1 positive ST147 *K. pneumoniae* isolate from Singapore and also with other ST147 IncFIB plasmids (Acc. No CP021940.1, CP021945.1 and CP014756.1), indicating that this plasmid might be intrinsic to this ST [18].

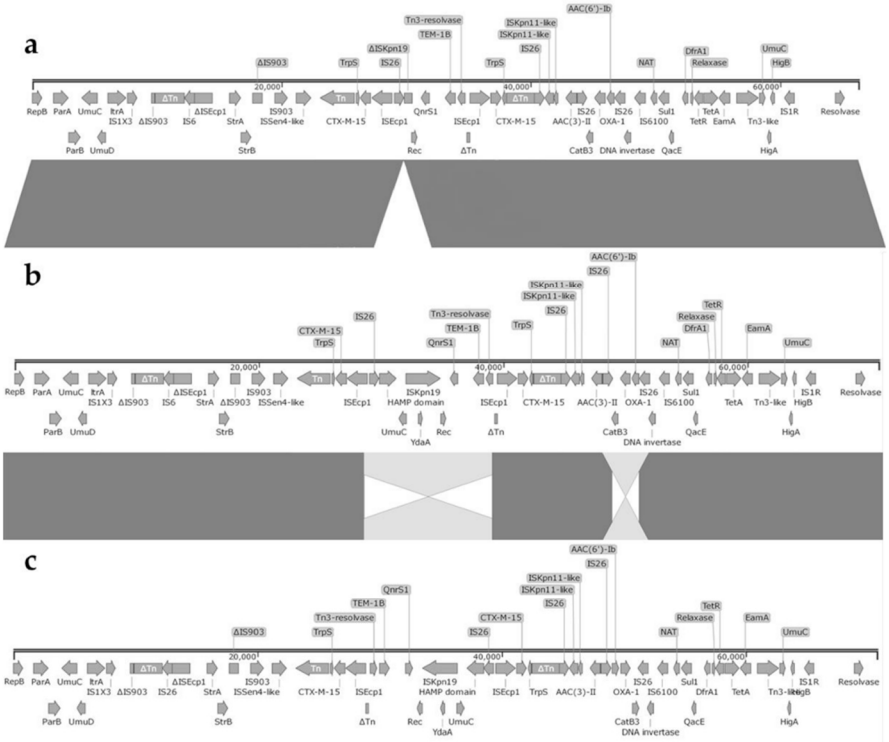

Figure 3. Major structural features of the IncR plasmids, pHKP0018.2 (**a**), pHKP0064.2 (**b**) and pHKP0067.2 (**c**), identified in *K. pneumoniae* isolates HKP0018, HKP0064 and HKP0067, respectively. Arrows indicate the deduced open reading frames (ORFs) and their orientations. Hypothetical proteins are not shown. The figure was generated with EasyFig 2.1 [31].

Southern blot and WGS revealed that the ST147 isolates carried also an identical 54,750 bp plasmid, pHKP0018.4 (Figure S2). The plasmid showed similarity (coverage 78%, identity 99%) to *K. pneumoniae* ST147 plasmids recovered from Singapore, pSg1-3 (Acc. No CP012429) [18]. pHKP0018.4 exhibited also similarity (coverage 62%, identity 83%) to phiKO2 of a *Klebsiella oxytoca* isolate which was described as a prophage able to replicate as linear plasmids with covalently closed ends [32].

Small plasmids, often present in high copy numbers, can serve as an important reservoir for antibiotic resistance determinants, such as small ColE plasmid derivatives encoding *qnrS1* in *Salmonella enterica* [33–35]. In the present study, apart from the ColKP3 $bla_{\text{OXA-181}}$-encoding plasmid, the ST147 *K. pneumoniae* isolates harboured in addition four identical small plasmids, which varied in size from 1.4 kb to 8.4 kb and did not encode known antimicrobial resistance determinants. An identical 8428 bp plasmid, pHKP0018.5, was identified in the three isolates. The plasmid carried two Col-like replication initiation proteins and showed similarity to pKpvST147B_4 (Acc. No CP040727.1, coverage 56%, identity 100%) from a ST147 *K. pneumoniae* isolate recovered at a hospital in south-east England (Figure S3). Another plasmid, 5499 bp in size and identical for the investigated ST147 isolates (pHKP0018.6), was detected and typed as a Col-like plasmid (Figure S4).

Moreover, a 2044 bp plasmid identical for the three *K. pneumoniae* plasmids, pHKP0018.7, was detected and encoded two hypothetical proteins, with no conserved domains. This plasmid could not be assigned to a replicon type and was identical (coverage 100%, identity 100%) to plasmids p4_1_2.4 (Acc. No CP023843.1) and pDA33140-2 (Acc. No CP029584.1) both from ST147 *K. pneumoniae* isolates recovered in Sweden (plasmid map not shown). Finally, an identical 1459 bp plasmid, pHKP0018.8, replicon typed as Col-like and bearing a hypothetical protein was identified. The Col-like plasmid was identical (coverage 100%, identity 100%) to plasmids found in *E. coli*, such as pEC881_8 (Acc. No CP019021.1) and pEC648_7 (Acc. No CP008721.1), which can be a result of interspecies plasmid transfer (plasmid map not shown).

3. Materials and Methods

3.1. Bacterial Isolates and Transformants

The isolates HKP0018, HKP0064 and HKP0067 were recovered in 2015 from throat and rectal swabs of three patients on admission to a university hospital in northern Germany (Table 3). The isolates were collected as part of the CONTAIN multicentre cohort study of the German Centre for Infection Research (DZIF) on the efficiency of infection control measures to prevent the transmission of ESBL producing Enterobacterales in haematology/oncology units [36]. The selection of the three isolates for further investigation was based on their clonal relatedness, ST, and acquired resistome. The surveillance swabs were plated on selective media (chromID® ESBL; bioMérieux, Nürtingen, Germany) and incubated for 18–24 h. The species identification was performed with MALDI-TOF mass spectrometry. Additionally, plasmid DNA was extracted from the isolate HKP0018 with the PureYield Plasmid Midiprep System (Promega, Madison, WI, USA) and then used to transform One Shot MAX Efficiency DH5α-T1R Competent Cells (Thermo Fisher Scientific, Waltham MA, USA). Selection of transformants was performed using ampicillin (40 mg/L) and tetracycline (30 mg/L) and was confirmed by PCR (Supplementary data).

3.2. Antimicrobial Susceptibility Testing

MICs for ampicillin, tetracycline, trimethoprim, gentamicin (Sigma–Aldrich, Steinheim, Germany), amikacin, aztreonam, imipenem, meropenem, minocycline, rifampicin, (Molekula, Newcastle-upon-Tyne, UK), levofloxacin (Sanofi Aventis, Frankfurt, Germany), ciprofloxacin (Bayer Pharma AG, Berlin, Germany) and ticarcillin (Carl Roth GmbH, Karlsruhe, Germany) were determined using the agar dilution method [37]. MICs for colistin and tigecycline were determined by broth microdilution method (Merlin Diagnostika GmbH, Bornheim, Germany). *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923 were used as

quality control strains. MICs were interpreted using the resistance breakpoints for Enterobacterales from EUCAST (Version 10.0, January 2020, http://www.eucast.org/clinical_breakpoints/) and CLSI (https://clsi.org/standards/products/microbiology/documents/m100/).

3.3. S1-Pulsed-Field Gel Electrophoresis (S1-PFGE) and Southern Blot Hybridisation

Plasmid linearisation by S1 nuclease followed by PFGE was used to determine the size and total number of plasmids. Bacterial DNA embedded in agarose plugs was digested using 50 Units S1 nuclease (Thermo Fisher Scientific, Waltham, MA, USA) per plug slice and incubated according to the manufacturer's instructions. Samples were run on a CHEF-DR II system (Bio-Rad, Munich, Germany) for 17 h at 6 V/cm and 14 °C while initial and final pulses were conducted at 4 and 16 s, respectively. The Lambda PFG Ladder and λ DNA-Mono Cut Mix (New England Biolabs, Frankfurt, Germany) were used as markers. The approximate plasmid size was calculated using Image Lab™ software (Bio-Rad, Munich, Germany).

Southern blot hybridisation was performed to determine the plasmid/chromosomal gene location by hybridisation with digoxigenin (DIG)-labelled probes (Roche, Mannheim, Germany). For the IncR replicon and *strA* of pHKP0018.2 and for the terminase pHKP0018.4 specific probes were used respectively (Table S1). Signal detection was performed according to the manufacturer's instructions using CDP-Star®ready-to-use (Roche, Mannheim, Germany) chemiluminescent substrate by autoradiography on a X-ray film (GE Healthcare, Buckinghamshire, United Kingdom). Chromosomal location was shown by colocalisation with a *rpoB* probe.

3.4. Whole Genome Sequencing (WGS) and Bioinformatics

Total DNA from the bacterial isolates and transformants was extracted using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) and plasmid DNA was extracted using PureYield Plasmid Midiprep System according to manufacturer's instructions and used for short-read sequencing. Sequencing libraries were prepared using a Nextera XT library prep kit (Illumina GmbH, Munich, Germany) for a 250 bp paired-end sequencing run on an Illumina MiSeq platform. The obtained reads were de novo assembled with the Velvet assembler integrated in the Ridom SeqSphere+ v. 7.2.1 software, and SPAdes 3.11 [38]. Finally, where necessary, overlapping assembly contigs and predicted gaps were filled and confirmed by PCR-based gap closure as described previously [39].

DNA extraction for long-read sequencing was performed using the Genomic-Tips 100/G kit and Genomic DNA Buffers kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Libraries were prepared using the 1D Ligation Sequencing Kit (SQK-LSK108) in combination with Native Barcoding Kit (EXP-NBD103) and Rapid Barcoding Kit (SQK-RBK004) in accordance with the manufacturer's instructions (Oxford Nanopore Technologies, Oxford, United Kingdom) and were loaded onto a R9.4 flow cell (Oxford Nanopore Technologies, Oxford, United Kingdom). The run was performed on a MinION MK1b device (Oxford Nanopore Technologies, Oxford, United Kingdom). Collection of raw electronic signal data and live base-calling was performed using the MinKNOW software and Albacore (Oxford Nanopore Technologies, Oxford, United Kingdom). *De novo* assembly of the MinION long-reads was performed using Canu [40]. The Illumina short-reads were assembled with the MinION long-reads using hybridSPAdes and Unicycler [41,42]. Additionally, plasmidSPAdes was implemented to identify plasmid sequences [43].

The assembled genomes generated in this project have been deposited in the NCBI under the BioProject ID PRJNA660340 (BioSample accessions: HKP0018, SAMN15946735; HKP00164, SAMN15946736; HKP0067, SAMN15946737).

3.5. Molecular Epidemiology, Resistome, Mobilome and Genome Annotation

The Pasteur multi-locus sequence typing (MLST) scheme was used to assign the ST (https://bigsdb.pasteur.fr/index.html). The molecular epidemiology was investigated with a validated cgMLST scheme, including 2358 target alleles, using the Ridom SeqSphere+ v. 7.2.1 software [44]. Capsular type

(KL-type) were assigned using Kaptive Web [45]. The resistome and plasmidome were analysed using ResFinder v.3.2.0 (https://cge.cbs.dtu.dk/services/ResFinder/) and PlasmidFinder v.2.0.1 [46,47]. Genome sequences were annotated using the RAST server (http://rast.nmpdr.org/) and partially manually edited. Plasmids were graphically depicted using SnapGene (http://www.snapgene.com/).

3.6. Conjugation Experiments

Broth mate conjugation experiments were performed using the sodium azide-resistant *E. coli* J53 as recipient. Selection of transconjugants was performed using sodium azide (200 mg/L) and ampicillin (40 mg/L), or tetracycline (30 mg/L). Transconjugants were tested by PCR for the presence of the $bla_{\text{OXA-181}}$ and *tet*(A) genes, while their susceptibility to meropenem (10 µg) and tetracycline (30 µg) was tested using the disk diffusion method, according to EUCAST recommendations (Version 10.0, January 2020, http://www.eucast.org/clinical_breakpoints/).

4. Conlusions

In conclusion, the present study describes a complex variety of plasmids within three clonal ST147 *K. pneumoniae* isolates recovered from haematology/oncology patients hospitalised in the same German hospital. The ST147 *K. pneumoniae* isolates harboured the $bla_{\text{OXA-181}}$ carbapenemase gene on a small ColKP3 plasmid, but also a complex array of 12 antibiotic resistance determinants on an MDR IncR plasmid, severely limiting treatment options. The recombinogenic nature of the MDR IncR plasmid encoding a large number of ISs can serve as genome plasticity mediators. The IncR plasmids of the studied isolates differed overall in a 4 kb region which could be attributed to an IS transposition event, as also in the opposite orientation of two composite transposons (3.8 kb and 13 kb). These results indicate that within a cluster of closely related isolates, variation can be observed due to the dynamic nature of MGEs. The abundant mobilome and resistome of the *K. pneumoniae* isolates combined with the emergence of ST147 as an international high-risk clone has the potential to become a major challenge for the healthcare setting and requires special attention and vigilance.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6382/9/10/675/s1. Table S1: List of oligonucleotides used in the present study, Figure S1: Major structural features of the IncFIB plasmid pHKP0018.3 identified in K. pneumoniae isolates HKP0018, HKP0064 and HKP0067. Arrows represent predicted open reading frames (ORFs) and their direction represents the direction of transcription. Hypothetical proteins are not shown, Figure S2: Major structural features of the pHKP0018.4 plasmid identified in K. pneumoniae isolates HKP0018, HKP0064 and HKP0067. Arrows represent predicted ORFs and their direction represents the direction of transcription. Hypothetical proteins are not shown, Figure S3: Major structural features of pHKP0018.5 identified in K. pneumoniae isolates HKP0018, HKP0064 and HKP0067. Arrows represent predicted ORFs and their direction represents the direction of transcription. Hypothetical proteins are assigned as hp, Figure S4: Major structural features of pHKP0018.6 identified in K. pneumoniae isolates HKP0018, HKP0064 and HKP0067, respectively. Arrows represent predicted ORFs and the direction of the arrow represents the direction of transcription. Hypothetical proteins are assigned as hp.

Author Contributions: Conceptualization, K.X., A.C, H.S. and P.G.H.; methodology, K.X, A.C. and P.G.H.; software, F.F., O.K. and C.F; formal analysis, K.X., A.C., C.F., L.V., P.G.H.; investigation, K.X., J.W., P.G.H.; data curation, K.X.; writing—original draft preparation, K.X. and P.G.H.; writing—review and editing, K.X., A.C., J.W., L.M.B., H.R., F.F., O.K., L.V., C.F., H.S., P.G.H.; supervision, A.C. and P.G.H.; funding acquisition, H.S. 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.

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Supplementary Materials

Table S1. List of oligonucleotides used in the present study.

Target	Primer	Sequence (5′ to 3′)	Experiment	Reference
IncR	L7	TCGCTTCATTCCTGCTTCAGC	Southern blot	This study
	L8	GTGTGCTGTGGTTATGCCTCA		
StrA	L3	CTGTCAGAGGCGGAGAATCT	Southern blot	This study
	L4	ATCCGCTCCAGACAGATCAG		
RpoB	KX65	TGGTAAACGTCCACAAGTTCTG	Southern blot	This study
	KX66	CTGTAGCTCTGAATCGGGAAT		
Terminase	KX103	TTCAAGCTCTTTCATCGCCG	Southern blot	This study
	KX104	TTATGAAATGGGCGTGCTGG		
pHKP0018.1	KX37	CGGTAATGTTAGGGGCTGGA	PCR-based gap closure	This study
	KX38	CACTGACAATGGTGCCACTC		
pHKP0018.3	KX45	GATCTGGTGGCGGACTATCA	PCR-based gap closure	This study
	KX46	TCAGGAACGTTTAAAGGCGC		
pHKP0018.3	KX47	CCGGACAAGTATTTCTGCGG	PCR-based gap closure	This study
	KX48	CTGCTTTGTCGTCCGGTAAG		
pHKP0018.4	KX61	CCACGTTCAACAGCTCCATC	PCR-based gap closure	This study
	KX62	CTGACCTCCTGAAACGCTCT		

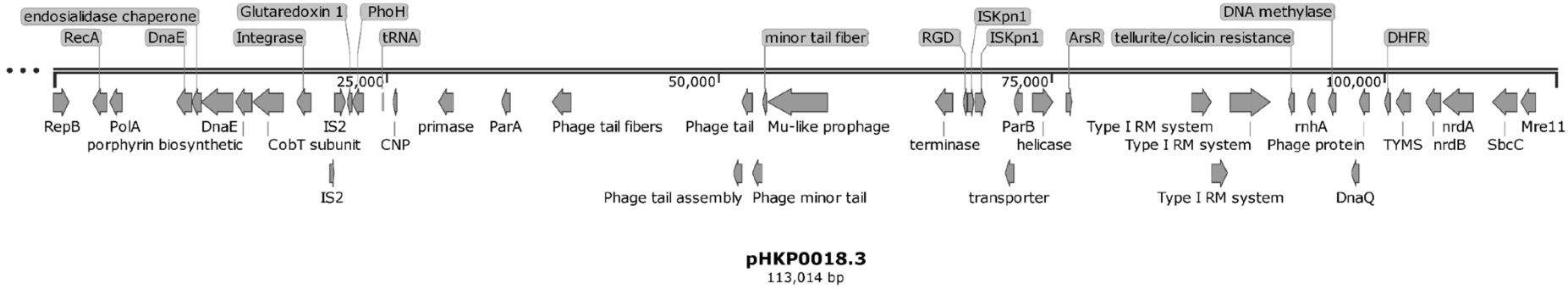

Figure S1. Major structural features of the IncFIB plasmid pHKP0018.3 identified in *K. pneumoniae* isolates HKP0018, HKP0064 and HKP0067. Arrows represent predicted ORFs and their direction represents the direction of transcription. Hypothetical proteins are not shown.

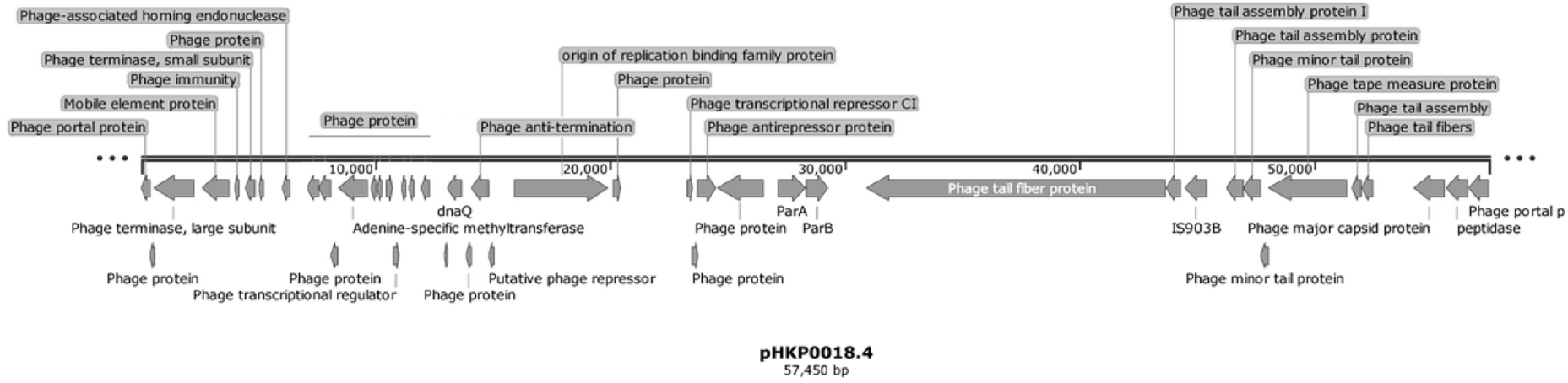

Figure S2. Major structural features of the pHKP0018.4 plasmid identified in *K. pneumoniae* isolates HKP0018, HKP0064 and HKP0067. Arrows represent predicted ORFs and their direction represents the direction of transcription. Hypothetical proteins are not shown.

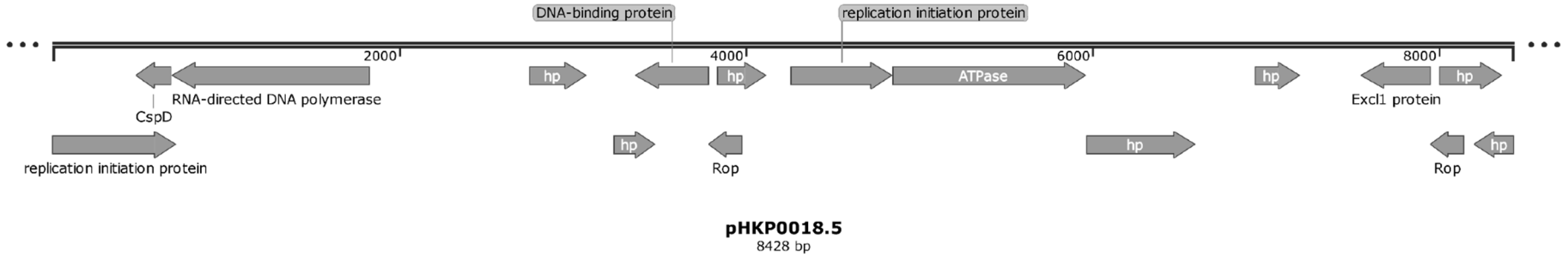

Figure S3. Major structural features of pHKP0018.5 identified in *K. pneumoniae* isolates HKP0018, HKP0064 and HKP0067. Arrows represent predicted ORFs and their direction represents the direction of transcription. Hypothetical proteins are assigned as hp.

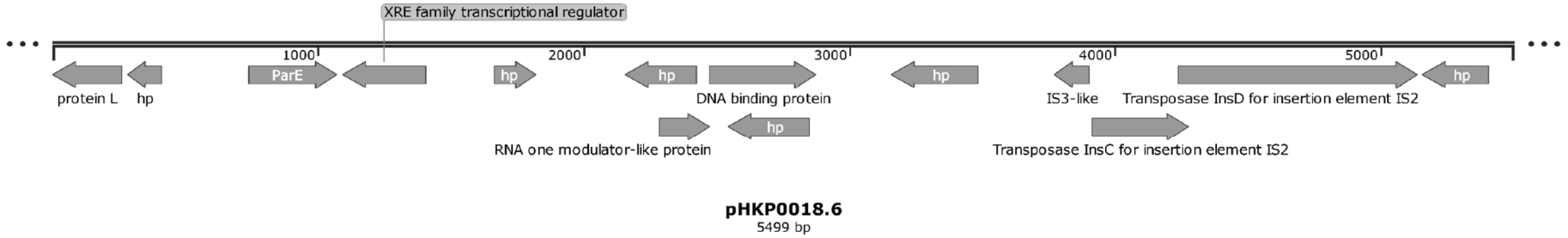

Figure S4. Major structural features of pHKP0018.6 identified in *K. pneumoniae* isolates HKP0018, HKP0064 and HKP0067, respectively. Arrows represent predicted ORFs and the direction of the arrow represents the direction of transcription. Hypothetical proteins are assigned as hp.

2.7 Molecular characterization of *bla*$_{\text{NDM-1}}$ from an *A. baumannii* outbreak in a German university hospital

NDM producing *A. baumannii* have been associated with outbreaks in hospital settings around the world.[95, 96] However, in Germany these outbreaks are rare. A CRAb outbreak in a university hospital in Germany was investigated. The index patient was a 85-year-old woman who was transferred from Egypt where she had been hospitalized before. In total, 10 invasive and colonising *A. baumannii* isolates were recovered from nine patients between May and September 2019. The patients were hospitalised in different wards in an internal medicine department. The antimicrobial susceptibility of the isolates was assessed by agar dilution and microbroth dilution methods. MICs were interpreted using the resistance breakpoints for *Acinetobacter* spp. from EUCAST (Version 10.0, January 2020, http://www.eucast.org/clinical_breakpoints/) and CLSI (https://clsi.org/standards/products/microbiology/documents/m100/). For tigecycline, the EUCAST PK-PD (non-species related) breakpoint of 0.5 mg/L was used. The isolates were subjected to WGS using short- (Miseq) and long-read (MinION) technologies followed by hybrid- (Unicycler) and non-hybrid (Velvet and SPAdes) methods for *de novo* genome assembly.[97-99] cgMLST (Ridom® SeqSphere+) was used to investigate the molecular epidemiology and the assembled genomes were used for seven-loci MLST (Pasteur scheme: https://pubmlst.org/organisms/acinetobacter-baumannii) and resistome analysis.[66] The genetic location of the MBL was confirmed by S1-PFGE followed by Southern blot hybridization for *bla*$_{\text{NDM-1}}$ and the intrinsic *bla*$_{\text{OXA-51-like}}$, as a chromosomal marker.

The *A. baumannii* isolates were assigned as IC2, encoded the intrinsic *bla*$_{\text{OXA-66}}$ (a *bla*$_{\text{OXA-51}}$ variant) and were identified as ST570$^{\text{Pas}}$. All investigated isolates presented the same resistance phenotype and were resistant to amikacin, chloramphenicol, ciprofloxacin, gentamicin, levofloxacin, meropenem, tetracycline, and tigecycline. The *A. baumannii* isolates were susceptible only to minocycline (Table 3). Molecular epidemiology analysis revealed a cluster of closely related isolates which differed in less than one allele (Figure 5). Because of the clonal relatedness of the investigated isolates an outbreak within the hospital could be traced.

Table 3. Antimicrobial susceptibility profile of the NDM-1-positive CRAb isolates. Abbreviations: AMK, amikacin; CHL, chloramphenicol; LVX, levofloxacin; MEM, meropenem; MIN, minocycline; TET, tetracycline; TGC, tigecycline.

Isolate	MIC (mg/L)								
	AMK	CHL[a]	CIP	GEN	LVX	MEM	MIN[a]	TET[a]	TGC*
AML_0669	>128	>128	>128	>128	128	>128	4	64	2
AML_0670	>128	>128	>128	>128	128	>128	4	64	2
AML_0675	>128	>128	>128	>128	128	>128	4	64	2
AML_0676	>128	>128	>128	>128	128	>128	4	64	1
AML_0686	>128	>128	>128	>128	128	>128	4	64	2
AML_0688	>128	>128	>128	>128	128	>128	4	64	2
AML_0689	>128	>128	>128	>128	128	>128	4	64	2
AML_0691	>128	>128	>128	>128	128	>128	4	64	2
AML_0712	>128	64	>128	>128	32	>128	0.5	16	1
AML_0718	>128	>128	>128	>128	128	>128	4	64	2

[a]only CLSI breakpoint available; *tested by broth microdilution method

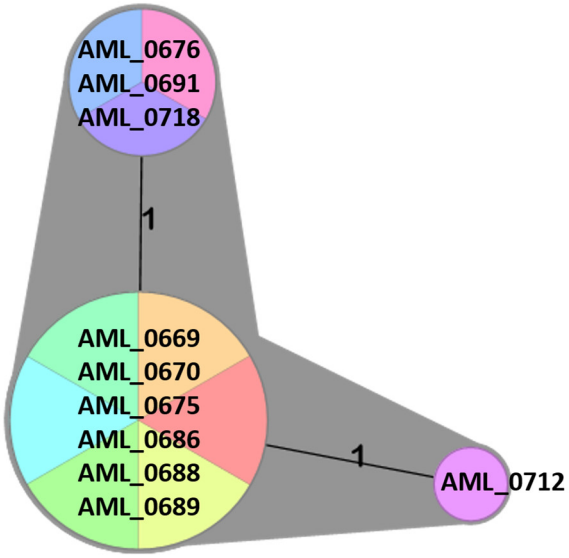

Figure 5. Minimum spanning tree generated using Ridom SeqSphere+ for the 10 CRAb isolates coloured by ST. Each coloured circle or segment within a circle represents one individual isolate based on sequence analysis of 2390 cgMLST target genes and ignoring missing values. Closely related genotypes (up to nine alleles different) are shaded grey.

Resistome analysis revealed that nine out of the 10 CRAb isolates encoded both *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-23}}$ carbapenemases. The *bla*$_{\text{NDM-1}}$ was encoded on the chromosome based on S1-PFGE and Southern blot hybridization experiments. Using a hybrid assembly approach, the genetic environment of *bla*$_{\text{NDM-1}}$ could be elucidated. The carbapenemase NDM-1 was encoded on a 19 kb Tn*125*-like transposon and was located upstream of IS*Aba14-aphA6* (Figure 6). However, one patient was found colonized in addition with a CRAb isolate (AML_0676) that was *bla*$_{\text{OXA-23}}$-positive but lacked *bla*$_{\text{NDM-1}}$ and *aphA6* (Table 4).

Table 4. Resistome of the CRAb outbreak isolates.

Isolate No.	Resistome		
	Aminoglycoside-modifying enzymes	β-lactamases	Other
AML_0669 AML_0670 AML_0675 AML_0686 AML_0688 AML_0689 AML_0691 AML_0712 AML_0718	*aadA1* *aac(6')Ib-cr*-like *aph(3')-Ic*-like *aphA6* *armA*	*bla*$_{\text{ADC-25-like}}$ *bla*$_{\text{NDM-1}}$ *bla*$_{\text{OXA-23}}$ *bla*$_{\text{OXA-66}}$ *bla*$_{\text{TEM-1D}}$	*catB8* *mph(E)* *msr(E)* *sul1*
AML_0676	*aadA1* *aac(6')Ib-cr*-like *aph(3')-Ic*-like *armA*	*bla*$_{\text{ADC-25-like}}$ *bla*$_{\text{OXA-23}}$ *bla*$_{\text{OXA-66}}$ *bla*$_{\text{TEM-1D}}$	*catB8* *mph(E)* *msr(E)* *sul1*

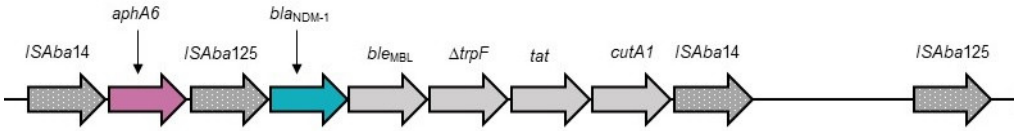

Figure 6. Schematic diagram of the genetic environment of *bla*$_{\text{NDM-1}}$ in *A. baumannii*. Arrows indicate the deduced open reading frames (ORFs) and their orientations. Hypothetical proteins are not shown.

In the present study, we demonstrated the transmission of an NDM-1-positive CRAb clone between patients within a hospital setting in Germany. In the investigated outbreak two closely related CRAb isolates could be recovered from one patient (AML_0676 and AML_0691). One of these isolates lost the carbapenemase *bla*$_{\text{NDM-1}}$ but remained carbapenem-resistant because of the presence of *bla*$_{\text{OXA-23}}$ and was also missing the aminoglycoside-modifying enzyme *aphA6*. The antibiotic resistance determinants NDM-1 and AphA6 were in close proximity to ISs and therefore the observed loss could be attributed to a transposition event. For the conditions tested here, the loss of a resistance gene had no effect on the phenotype. These results demonstrate that MGEs can cause genetic diversity during the course of an outbreak.

Preliminary data of this work was accepted as a poster presentation at the 30th European Congress of Clinical Microbiology and Infectious Diseases in Paris, France in April 2020. The conference has been cancelled due to the SARS-CoV-2 pandemic.

2.8 Characterization of a vancomycin-resistant and a vancomycin-susceptible *E. faecium* isolate from the same blood culture

The successful spread of VREfm is the result of the clonal expansion of certain lineages but also of MGEs.[100] In enterococci, the glycopeptide resistance determinants are organized in inducible operons while clinically relevant are the *vanA* and *vanB* operons. The main vehicle of the *vanA* resistance determinant is the Tn*1546* transposon while the conjugative transposon Tn*1549*/Tn*5382* is the most common vector of the *vanB* operon. Glycopeptide resistance determinants can be chromosomally- or plasmid-encoded.[23, 76]

In the present manuscript we investigated two isolates obtained from the same patient with a different phenotype. Both isolates were assigned ST203 while, one was VREfm and the second was vancomycin-susceptible *E. faecium* (VSEfm). By cgMLST analysis, the VREfm and VSEfm isolates were identical. The VREfm isolate harboured the *vanA* gene cluster as part of a Tn*1546*-type transposon, which was encoded on a 49 kb multi-replicon (*rep1*, *rep2* and *rep7a*) plasmid (pAML0157.1). In the VSEfm isolate, a 12 kb *rep2* plasmid (pAML0158.1) was found and was present in full length as part of the pAML0157.1 from the VREfm isolate. The *vanA*-encoding pAML0157.1 was a chimera of the pAML0158.1 and a DNA fragment encoding *vanA*, *ant(6)-Ia*, *erm*(B) and *cat*-like and the replicons *rep1* and *rep7a*. Our results demonstrate the impact of MGEs in the dissemination of vancomycin resistance and the genotypic and phenotypic diversity within clonal isolates.

My contribution to the present study included the molecular characterization, WGS, typing, plasmid profiling and genetic location determination of the two *E. faecium* isolates. Furthermore, I completed data interpretation, analysis design and compiled the manuscript.

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Characterization of a vancomycin-resistant *Enterococcus faecium* isolate and a vancomycin-susceptible *E. faecium* isolate from the same blood culture

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Objectives: To characterize two *Enterococcus faecium* isolates with different resistance phenotypes obtained from the same blood culture.

Methods: The isolates were identified by MALDI-TOF MS and antimicrobial susceptibility testing (AST) was performed using a VITEK® 2 AST P592 card and Etest. WGS was performed on the MiSeq and MinION sequencer platforms. Core-genome MLST (cgMLST) and seven-loci MLST were performed. Plasmid analysis was performed using S1-PFGE followed by Southern-blot hybridization.

Results: Both *E. faecium* isolates were ST203. AST revealed that one was a vancomycin-resistant *E. faecium* (VREfm) isolate and the other was a vancomycin-susceptible *E. faecium* (VSEfm) isolate. The VREfm isolate harboured the *vanA* gene cluster as part of a Tn*1546*-type transposon encoded on a 49 kb multireplicon (*rep1, rep2* and *rep7a*) plasmid (pAML0157.1). On the same plasmid, *ant(6)-Ia, cat*-like and *erm*(B) were encoded. The VSEfm isolate harboured a *rep2* plasmid (pAML0158.1), 12 kb in size, which was present in full length as part of pAML0157.1 from the VREfm isolate. The *vanA*-encoding pAML0157.1 was a chimera of the *rep2* pAML0158.1 and a second DNA segment harbouring *vanA, ant(6)-Ia, erm*(B) and *cat*-like, as well as the replicons *rep1* and *rep7a*. By cgMLST analysis, the VREfm and VSEfm isolates were identical.

Conclusions: Our results demonstrate that the VREfm and VSEfm blood culture isolates represented ST203 and were identical. The investigated heterogeneous resistance phenotypes resulted from the acquisition or loss of plasmid segments in the enterococcal isolates. These data illustrate that mobile genetic elements may contribute to the spread of vancomycin resistance among enterococci and to the genotypic and phenotypic variation within clonal isolates.

Introduction

Enterococci are natural inhabitants of the gastrointestinal tract and a major cause of healthcare-associated infections, with an increasing incidence globally.[1] *Enterococcus faecalis* and *Enterococcus faecium* are the clinically most important species among enterococci. Of major concern is the continuous increase in vancomycin-resistant *E. faecium* (VREfm) causing healthcare-associated bloodstream infections associated with increased mortality.[2,3] In Germany, an increase in the percentage of VREfm causing invasive infections from 11.9% in 2016 to 23.8% in 2018 was reported.[4]

Clinically relevant glycopeptide resistance is mainly mediated by the *vanA* and *vanB* operons, which can be either chromosomally or plasmid encoded. *vanA*-positive *Enterococcus* spp. are highly resistant to vancomycin and cross-resistant to teicoplanin due to the presence of a *vanZ* cassette in the resistance operon.[5] The mobile genetic element (MGE) Tn*1546* is the main reservoir carrying the *vanA* gene cluster and contributing to its dissemination by transposition.[6] The *vanA* genotype used to be the predominant determinant of glycopeptide resistance in Germany and other countries in Europe, but, in recent years, a decreasing prevalence of *vanA* VREfm and a shift to the *vanB* VREfm genotype has been observed.[7,8]

The aim of the present study was to characterize a VREfm isolate and a vancomycin-susceptible *E. faecium* (VSEfm) isolate obtained from the same blood culture.

Materials and methods

Bacterial isolates, species identification and antimicrobial susceptibility testing (AST)

A patient with a transjugular intrahepatic portosystemic shunt ('TIPSS') implanted to manage portal hypertension resulting from liver cirrhosis was hospitalized in an ICU in University Hospital Cologne. The patient had had multiple relapses of device-related VREfm bloodstream infections over the past 5 months. During the current septic episode in November 2016, two morphologically identical enterococcal isolates (AML0157 and AML0158) were recovered from a blood culture, with one isolate showing growth within the vancomycin inhibition zone on the Mueller–Hinton agar plate inoculated for preliminary disc diffusion testing. Species identification was performed using MALDI-TOF MS (Bruker Daltonic GmbH, Bremen, Germany). AST was carried out using a VITEK® 2 AST P592 card and Etest (bioMérieux, Nürtingen, Germany). MICs were interpreted using the EUCAST (v.10.0; http://www.eucast.org/clinical_breakpoints/) and CLSI (https://clsi.org/standards/products/microbiology/documents/m100/) breakpoints for *Enterococcus* spp.

WGS

DNA was extracted using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) and prepared using the Nextera XT Library Prep Kit (Illumina GmbH, Munich, Germany) for a 250 bp paired-end run on an MiSeq followed by *de novo* assembly using Velvet and SPAdes.[9,10] For long-read sequencing, DNA was extracted using the Genomic-Tip 100/G Kit and the Genomic DNA Buffer Kit (Qiagen). Libraries were prepared using the Ligation Sequencing Kit (SQK-LSK109) combined with the Native Barcoding Kit (EXP-NBD103) (Oxford Nanopore Technologies, Oxford, UK) and were loaded onto a R9.4 flow cell (Oxford Nanopore Technologies). The short reads and long reads were assembled using Unicycler.[11] Core-genome MLST (cgMLST) analysis was performed using Ridom SeqSphere⁻ v.6.0.7 software (Ridom GmbH, Münster, Germany).[12] The MLST scheme for *E. faecium* was used to assign the ST (https://pubmlst.org/). Genomes were analysed using ResFinder v.3.2, PlasmidFinder v.2.0.1, VirulenceFinder v.2.0 (https://cge.cbs.dtu.dk/services/) and VFanalyzer (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer). Predicted gaps were filled and confirmed by PCR-based gap closure (Table S1, available as Supplementary data at *JAC* Online).

S1-PFGE and Southern-blot hybridization

Bacterial DNA embedded in agarose plugs was digested using 50 U of S1-nuclease (Thermo Fisher Scientific, Waltham, MA, USA), followed by PFGE on a CHEF-DR II system (Bio-Rad, Munich, Germany) for 17 h at 6 V/cm and 14°C (4 and 16 s initial and final pulses, respectively). The Lambda PFG and λ DNA-Mono Cut Mix (New England Biolabs, Frankfurt, Germany) were used as markers. Plasmid size was calculated using Image Lab™ software (Bio-Rad). Southern-blot hybridization was performed to determine the plasmid/chromosomal location using digoxigenin-labelled probes (Roche, Mannheim, Germany). A *vanA*-specific probe was generated, while chromosomal location was shown by co-localization with an *rpoB* probe (Table S1). In addition, a *rep2* probe (specific for pAML0157.1 and pAML0158.1) and a *fliS* probe (*fliS* being a single-copy gene specific for pAML0157.4) were prepared for hybridization experiments (Table S1). Signal detection was performed using CDP-Star® ready-to-use (Roche) chemiluminescent substrate by autoradiography on X-ray film (GE Healthcare, Buckinghamshire, UK).

Determination of growth rates

Isolates were grown overnight in brain heart infusion (BHI) broth. The cultures were diluted 1:100 into 200 μL of BHI broth in a microplate and incubated at 37°C with shaking for 10 h. Absorbance was measured at 600 nm every 15 min using an Infinite M1000 microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

Data availability

The raw sequencing reads generated in this project were submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/ena/) under nucleotide accession numbers ERR2789865 (AML0157) and ERR2789866 (AML0158). The assembled genomes have been deposited in the NCBI under the BioProject ID PRJNA648658 (BioSample accessions: AML0157, SAMN15643623; and AML0158, SAMN15643624).

Results and discussion

The two bloodstream isolates were identified as *E. faecium*. AST revealed that the isolate AML0157 was resistant to vancomycin (MIC >256 mg/L) and to teicoplanin (MIC >256 mg/L) and was therefore classified as VREfm, while the isolate AML0158 was susceptible to vancomycin (MIC 0.5 mg/L) and teicoplanin (MIC 1 mg/L) and was therefore classified as VSEfm (Table 1). Both isolates were resistant to ampicillin and imipenem, and susceptible to linezolid and daptomycin. The isolate AML0157 was resistant to chloramphenicol, erythromycin and streptomycin. Based on AST results, the isolates AML0157 and AML0158 recovered from the same blood culture would have been classified as two unrelated *E. faecium* isolates. Nevertheless, by seven-loci MLST both isolates were ST203, while cgMLST analysis revealed that the isolates were identical (with no allele differences) and were assigned as complex type (CT) 20. In 2016, ST203 was the second most prevalent ST in Germany.[13] A recent study reported a decreasing prevalence of ST203 from 30.4% in 2014 to 2.6% in 2018 in Germany.[8]

The VREfm isolate (AML0157) harboured the *vanA* gene cluster as part of a Tn*1546*-type transposon (Tn3 transposon family). The Tn3 transposon family has been linked to the spread of the *vanA* operon and high-level glycopeptide resistance.[14] PFGE and Southern-blot hybridization revealed that the *vanA* operon was encoded on a 49 122 bp plasmid (pAML0157.1). The plasmid pAML0157.1 showed high homology (100% coverage, 100% identity) to a 49 kb *vanA*-encoding plasmid (CP020486.1) from another ST203 VREfm recovered in Denmark in 2014. In addition, *ant(6)-Ia*, *erm*(B) and *cat*-like were encoded on pAML0157.1, contributing to increased MICs of streptomycin, erythromycin and chloramphenicol, respectively (Table 1). *ant(6)-Ia* (encoding an aminoglycoside-modifying enzyme) was flanked by IS*1216E*, while the erythromycin resistance determinant *erm*(B) was bracketed by two inverted copies of IS*Enfa3* and IS*1542*. pAML0157.1 encoded a ζ/ε toxin–antitoxin ('TA') module contributing to plasmid stabilization and inheritance. Finally, three replicons (Inc18 family, *rep1* and *rep2*; and Rep_trans family, *rep7a*) were identified in pAML0157.1, suggesting a plasmid fusion.

A *rep2* replicon was also identified and confirmed by Southern-blot hybridization in the VSEfm isolate AML0158, located on a 12 131 bp plasmid (pAML0158.1). Comparative analysis of pAML0157.1 and pAML0158.1 revealed a fusion event. pAML0158.1 from the VSEfm isolate was present in full length as part of the pAML0157.1 from the VREfm isolate (Figure 1). These data indicate that the *vanA*-encoding pAML0157.1 is a chimera containing pAML0158.1 and a second DNA segment of plasmid origin, encoding *vanA*, *ant(6)-Ia*, *erm*(B) and *cat*-like, and the

Table 1. Antimicrobial susceptibility phenotype, antimicrobial resistance determinants and plasmid content of the two *E. faecium* isolates

		MIC (mg/L)		Acquired resistome	
Antimicrobial class	Antimicrobial agent	AML0157	AML0158	AML0157	AML0158
Aminoglycoside	streptomycin	192	32	*ant(6)-Ia*	ND
	amikacin	12	12	*aac(6') Ii* like	*aac(6') Ii* like
β-Lactam	ampicillin	>32	>32		
	imipenem	>16	>16		
Glycopeptide	teicoplanin	>256	1	*vanHAX*	ND
	vancomycin	>256	0.5		
Macrolide	erythromycin	>8	<0.25	*erm*(B)	ND
				msr(C)-like	*msr*(C)-like
Oxazolidinone	linezolid	3	4	*crf(B)*-like	*crf(B)*-like
Tetracycline	tetracycline	8	8	*tet*(M)-like	*tet*(M)-like
Other	chloramphenicol	>256	12	*cat*-like	ND
	daptomycin	4	4		
		Plasmid content			
	pAML0157.1	pAML0157.2	pAML0157.3	pAML0157.4	pAML0158.1
Size (bp)	49 122	261 677	6173	94 256	12 131
Replicon	*rep1*, *rep2*, *rep7a*	*repUS15*-like	*rep11a*	ND	*rep2*
Resistance genes	*ant(6)-Ia*, *cat*-like, *erm*(B), *vanHAX*	ND	ND	ND	ND
Isolate AML0157	+	+	+	+	−
Isolate AML0158	−	+	+	−	+

ND, not detected.

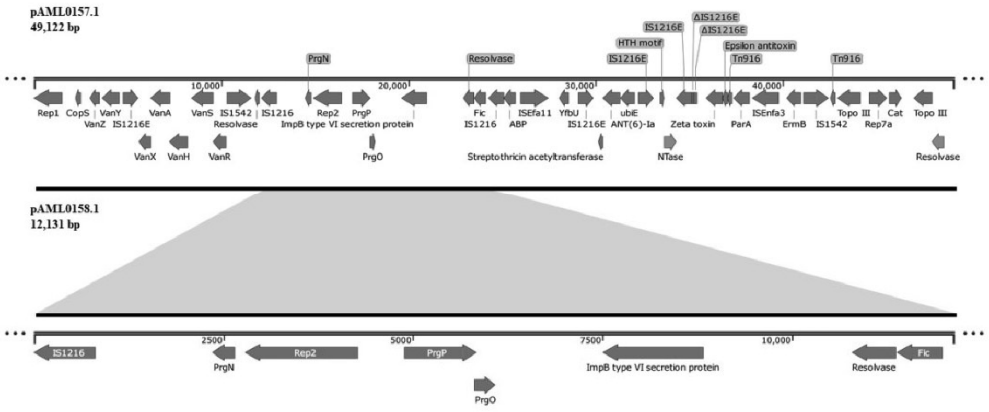

Figure 1. Major structural features of the plasmids pAML0157.1 and pAML0158.1 identified in the isolates AML0157 and AML0158, respectively. Arrows indicate the deduced ORFs and their orientations. Grey shading represents 100% nucleotide identity. Hypothetical proteins are not shown.

replicons *rep1* and *rep7a*. Furthermore, both plasmids were identified as derivatives of the Inc18 conjugative plasmid pRE25 (Figure S1). In enterococci, mosaic plasmids have been previously reported, such as fusions between pRUM, Inc18 and pheromone plasmids.[15] Furthermore, a transposon-mediated fusion of a *vanA*-encoding plasmid with a sex pheromone-response plasmid has also been described in *E. faecium*.[16]

Investigation of the virulence genes, e.g. *acm*, *bopD*, *ebpA*, *ebpB*, *ebpC*, *ecbA*, *efaAfm*, *esp*, *cpsA*, *cpsB*, hyl_{Efm}, *ptsD*, *scm*, *sgrA* and *srtC*, revealed no difference between the VREfm and VSEfm

isolates. Moreover, the growth rates of both isolates were identical, demonstrating no fitness cost associated with pAML0157.1 (Figure S2). In cgMLST analysis, the VREfm and VSEfm formed a cluster of closely related isolates with CT20 representatives from different regions in Germany (Figure S3). All plasmids harboured by the VREfm and VSEfm isolates are summarized in Table 1.

Our study has certain limitations. Perhaps most significantly, we could not determine the origin of the *vanA* operon. Furthermore, we were unable to ascertain whether pAML0157.1 acquired or pAML0158.1 lost a plasmid segment. Considering the fact that the patient had multiple episodes of VREfm bacteraemia before VSEfm was identified for the first time it can be assumed that pAML0158.1 lost the plasmid segment that contained the *vanA* operon.

The VREfm and VSEfm paradigm demonstrates the challenges in diagnostics and treatment of enterococcal infections when strain variants cannot be morphologically distinguished, e.g. an inadequate treatment if only the VSEfm is identified by the routine diagnostics. Similarly, coinfection with a linezolid-resistant *E. faecium* and variants of it with different resistance and virulence phenotypes in the same blood culture has been reported.[17] Diversity within a clonal population has also been reported in other species.[18,19]

In conclusion, the present study revealed that the VREfm and VSEfm blood culture isolates were identical by cgMLST. The heterogeneous resistance phenotypes of the clonal isolates resulted from the presence of a mosaic *vanA*-encoding plasmid. WGS-based bacterial typing helped to delineate the dynamics of plasmids and transposons within a VREfm population. Our data demonstrate that MGEs may contribute to the spread of vancomycin resistance among enterococci and can be the source not only of genetic but also of phenotypic variation within enterococcal isolates.

Acknowledgements

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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Supplementary data

Table S1. List of oligonucleotides used in the present study

Target	Primer	Sequence (5´ to 3´)	Experiment	Reference
vanA	M50	GGATAGCTACTCCCGCCTTT	Southern blot	This study
	M51	CCGAAACAGCCTGCTCAATT		
rpoB	N45	GCAAGCGACTCAAGAACAGA	Southern blot	This study
	N46	TCACGTTCAGGGTCTCTTGG		
rep2	O60	GACGTGGCTGGTGGAAATTT	Southern blot	This study
	O61	CGCCATTTCTTCCTCGTGTT		
fliS	O62	TGGAGGGTTGCTTGGTGATA	Southern blot	This study
	O63	GATCTCTGCTCCTGATCGAG		
pAML0158.1	O67	CTTCTCGAAATGGGTGAGCG	PCR-based gap closure	This study
	O68	TGGCATCGGAAAATTGTGGT		
pAML0157.4	O78	TACTGGCGATATAGGGTAGG	PCR-based gap closure	This study
	O79	ACAGTGAGTGAAGTAGCTTG		

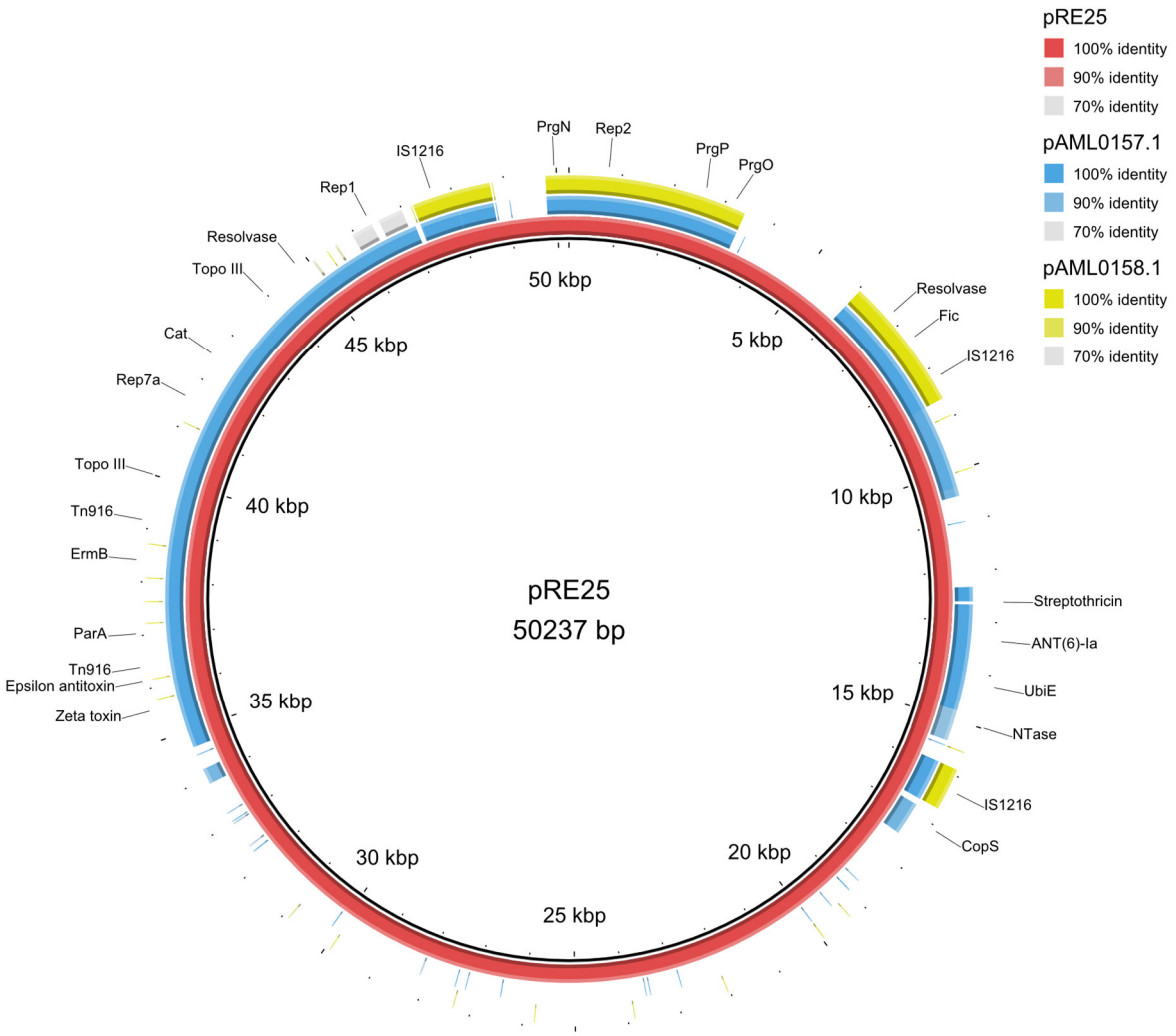

Figure S1. Comparative analysis of the plasmids pAML0157.1 and pAML0158.1 with the pRE25 (Acc. No. NC_008445.1) used here as a reference plasmid. The figure was generated using BRIG v0.95.

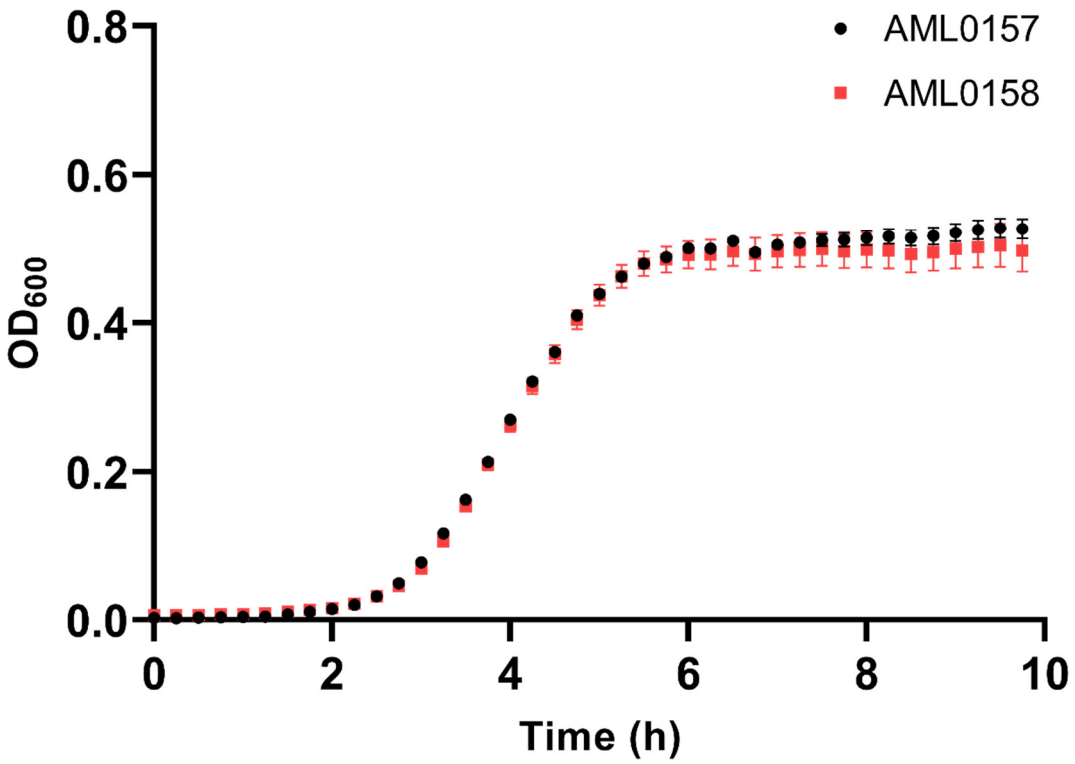

Figure S2. Growth curve of the VREfm and VSEfm, AML0157 and AML0158 respectively, in BHI broth. The data are representative examples from two separate experiments and are shown as means ± standard errors of the means

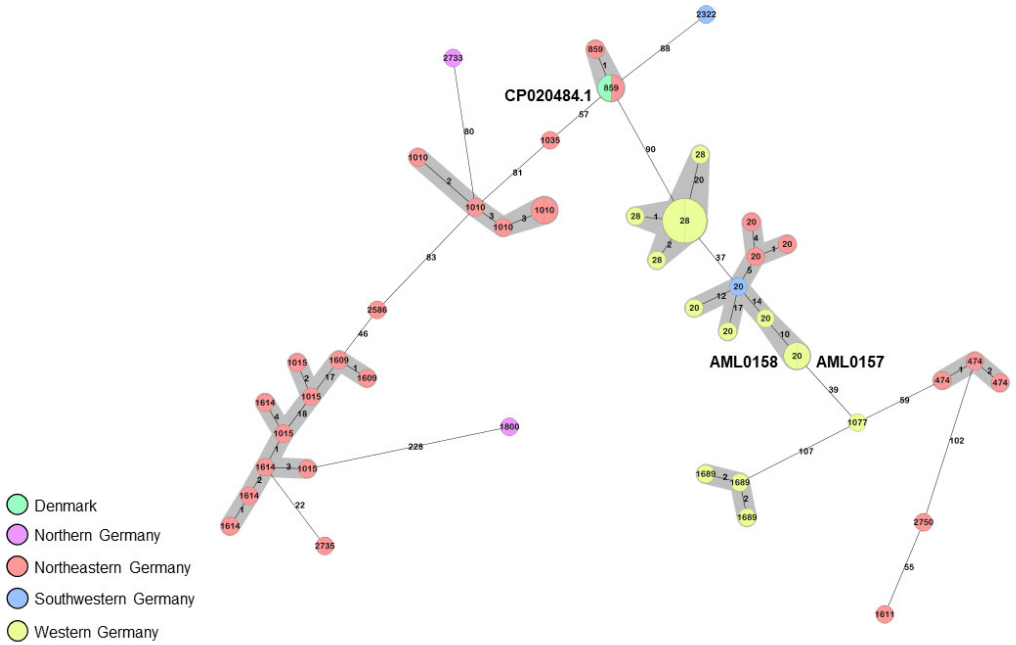

Figure S3. Minimum spanning tree generated using Ridom SeqSphere+ for ST203 VREfm isolates (n=49) from Germany, published[8] and unpublished data, ignoring missing values. The isolate CT859 from Denmark (Acc. No. CP020484.1) harboring a highly similar plasmid to pAML0157.1 has been also included. The ST203 isolates are grouped and coloured by region and labelled by CT. Each circle represents one isolate from an individual patient based on sequence analysis of 1423 cgMLST target genes. The size of the circle represents the number of isolates with no allelic difference. Numbers between the nodes indicate the number of allelic differences while closely related genotypes (≤20 alleles different) are shaded grey.

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3. Concluding discussion

The present cumulative work provides an insight into the population genomics and clonal expansion of three members of the ESKAPE pathogens, VREfm colonising isolates from Germany (see 2.1), 3GCR and carbapenem-resistant *K. pneumoniae* (see 2.2) and CRAb isolates from Bolivia (2.3).[87, 101, 102] Furthermore, the contribution of MGEs to the spread of AMR and the genetic diversity among closely related isolates in *A. baumannii* (see 2.4-2.5, 2.7), *K. pneumoniae* (see 2.6) and *E. faecium* (see 2.8), was explored.[103-106] Taken together, these studies provide significant new knowledge of the molecular epidemiology and of the mobile fraction of the genome of three nosocomial bacterial pathogens.

3.1 Paradigms of clonality in bacterial populations in the hospital

Enterococci compose less than 1% of the human gut microbiome and are intrinsically resistant to several antimicrobials including cephalosporins and aminoglycosides but can also accumulate mutations and acquire antibiotic-resistance determinants. In 1988, vancomycin-resistant enterococci were reported for the first time and since then, they have spread and pose a challenge to the healthcare system around the world.[23, 107-110] The VREfm epidemiology in Europe varies significantly between countries. In 2018 the national percentage of VREfm causing invasive infections varied from 0.0% (Luxembourg) to 59.1% (Cyprus) while, only 12 of 30 reporting countries had prevalence rates lower than 5%. In Germany, the rates of VREfm involved in invasive infections increased from 10.5% in 2015 to 23.8% in 2018.[21] In the present study, we have assessed the prevalence of VREfm colonising patients on hospital admission, i.e. the burden of bacterial import into the hospital (see 2.1). Our results indicated a steady increase (2014, 0.8%; 2015, 1.2%; 2016, 1.3%; 2017, 1.5%; 2018, 2.6%) in the prevalence of VREfm in the six tertiary care university hospitals in Germany.[101] However, the majority of the neighbouring countries of Germany reported in 2018 prevalence rates lower than 2.1%, apart from the Czech Republic and Poland, with 20.7% and 35.8%, respectively.[21]

E. faecium single nucleotide variants (SNVs) analysis revealed a distinct clustering of hospital-associated, farm animal and commensal isolates, clades A1, A2 and B respectively.[111] Furthermore, clade A1 isolates are almost always resistant to ampicillin, in contrast to the other clades which are mostly susceptible to ampicillin.[112] In our surveillance study, investigating the prevalence of MDR pathogens recovered from screening samples obtained from patients at admission to six university hospitals located in different regions in Germany, 99.6% of the

VREfm isolates were also resistant to ampicillin.[101] Within the hospital-associated clade the vast majority of VREfm infections have been linked to specific STs. For instance, in Germany between 2011 and 2014, ST117, ST203 and ST192 were the predominant clones causing bloodstream infections. However, the epidemiology of VREfm in Germany has changed over time. The Robert Koch Institute (RKI) reported in 2018 that ST117 (58%) was the predominant clone among bloodstream isolates followed by ST80 (19%).[113] In concordance with these findings we have demonstrated the dominance of ST117 among colonising VREfm isolates and also that the previously prominent ST203 and ST192 became increasingly rare in our study sites.[101] Before 2003 VanB-type enterococci were rare in Germany but, already in 2009 41.5% of the VREfm isolates analysed by the RKI were *vanB*-positive.[114] We also identified *vanB* as the predominant in our cohort of VREfm. Moreover, an increase of the VanB-type isolates (2014, 78.3%; 2015, 66%; 2016, 69.7%; 2017, 79.4%; 2018, 89.5%) during the study period was consistent with the increase of ST117 isolates (2014, 21.7%; 2015, 29.8%; 2016, 36.4%; 2017, 72.1%; 2018, 79%).[101] Previous studies at selected sites also reported an increasing prevalence of ST117/CT71/*vanB* positive isolates in Germany.[79, 115] In our study the subpopulation CT71 of ST117/*vanB* VREfm formed one large multi-centre cluster of closely related isolates spread throughout Germany. This remarkable clonal dissemination of the ST117/CT71/*vanB* isolates overcoming geographical boundaries indicates the presence of an epidemic clone circulating in Germany.[101] Patients already colonised with VREfm upon hospital admission could increase the risk for import and transmission of new clones within the healthcare setting, and consideration must be given to prevention interventions especially in high-risk settings.[80]

Among the *K. pneumoniae* complex, *K. pneumoniae* is the clinically most relevant species. Nevertheless, other members of the complex have already gained attention e.g. implication of *K. variicola* in nosocomial outbreaks of colonization in neonatal ICUs. [116] In the present study, the vast majority of isolates were identified as *K. pneumoniae* (94.7%) while a few representatives of the species *K. variicola* subsp. *variicola* (3.9%) and *K. quasipneumoniae* subsp. *quasipneumoniae* (2.6%) were detected among the colonising isolates (see 2.2). *K. pneumoniae* is next to enterococci another common colonizer of the gastrointestinal tract of healthy humans, nevertheless, the global spread of 3GCR isolates is of particular concern.[2] In 2018 the European Antimicrobial Resistance Surveillance Network (EARS-Net) reported that in Germany 12.9% of invasive *K. pneumoniae* isolates were 3GCR.[21] Although our work

indicated an increase in the prevalence of 3GCR *K. pneumoniae* over the study period (2016, 0.8%; 2017, 0.9%; 2018, 1.1%), overall rates of 3GCR among colonising isolates on hospital admission were rather low. Comparable to our results, in an admission prevalence study in 2014 among 4376 patients screened on hospital admission 37 3GCR *K. pneumoniae* isolates have been recovered (0.8%). In the latter study, 90.2% of the isolates encoded an ESBL and CTX-M-1 group (67.3%) enzymes were the most frequent β-lactamases.[117] In the present study, the CTX-M-1 group, including CTX-M-1, CTX-M-3, CTX-M-15, were the predominant acquired β-lactam resistance gene followed by TEM-1B.

Increasing trends in carbapenem-resistant *K. pneumoniae* have been reported in Europe, from 6.8% in 2015 to 7.5% in 2018. Although in 22 of the 30 participating countries the resistance rates were lower than 5%, a gradient from south and east Europe to northern Europe can be observed. In some Mediterranean countries such as Greece and Italy high resistance rates for carbapenems of 63.9% and 23.8% have been reported, respectively.[21] In our study, carbapenem resistance among *K. pneumoniae* carriage isolates was rare. Only one *K. variicola* isolate showed a carbapenem non-susceptible phenotype and harboured the acquired carbapenemase $bla_{\text{OXA-181}}$. The prevalence rates of carbapenem-resistant *K. pneumoniae* in Germany reported by the EARS-Net were lower than 0.5% in 2018.[21] Furthermore, the German Antimicrobial Resistance Surveillance System reported also low rates (0.63%) of carbapenem non-susceptibility in *K. pneumoniae* in Germany.[118] Based on these results, it becomes evident that no epidemic carbapenem-resistant *K. pneumoniae* clone is circulating in Germany in contrast to some other countries.[119]

In our collection of isolates, no dominant *K. pneumoniae* clone could be identified. Certain STs, such as ST307, and ST45 (9.2% for both STs), followed by ST17 (5.3%), ST219 (5.3%), ST14 (3.9%) were found more commonly but formed only a limited number of small clusters of closely related isolates. Healthcare-associated infections caused by *K. pneumoniae* are mainly due to diverse clones with a wide origin. However, certain clones succeeded to spread widely and are considered as problematic or high-risk clones. A high-risk *K. pneumoniae* clone was defined by Navon-Venezia *et al.* a clone that caused at least four outbreaks and that was reported in more than 10 countries. For instance, ST258 and ST147 are emerging high-risk clones that contribute disproportionately to *K. pneumoniae* infections.[2, 120] In general, a rather diverse composition of STs and clones was observed among the colonising isolates in the present study. Nevertheless, representatives of emerging problematic clones were identified,

such as ST307, ST17 and ST14, forming partially small clusters indicating that high-risk clones are already circulating in Germany.[2, 121, 122]

Carbapenem resistance in *A. baumannii* has evolved in the last decades as a major public-health threat.[19] In the US in 2017, CRAb infections and deaths in hospitalized patients were estimated as 8500 and 700, respectively (https://www.cdc.gov/drugresistance/biggest-threats.html). In CRAb isolates, OXA-23 is the most pervasive carbapenem resistance determinant throughout all clonal lineages and dominates over the other carbapenemases also in Latin America.[91, 123, 124] Molecular epidemiology studies made clear that the majority of CRAb cluster within a few clonal lineages, referred as ICs, distributed across the continents. Currently, 9 ICs have been identified in total while IC2 represents the most prevalent and widespread clonal lineage.[88, 123, 125] Nevertheless, for certain clonal lineages a regional endemicity has been observed, such as IC5 found mainly in Latin America.[88, 123] Studies about the molecular epidemiology of CRAb in Bolivia and Uruguay reported IC7 as the most prevalent lineage.[126] Recently, a high prevalence of XDR *A. baumannii* IC7 isolates has been reported at a children hospital in Bolivia.[127] In our study (see 2.3), all IC7 CRAb isolates obtained from two hospitals in Bolivia carried $bla_{\text{OXA-23}}$ as part of the transposon Tn*2008* (consisting of IS*Aba1*-$bla_{\text{OXA–23}}$-Δ*ATPase*).[104] In the latter transposon an IS*Aba1* copy is located upstream of the OXA-23 providing a strong promoter leading to overexpression of $bla_{\text{OXA-23}}$ and carbapenem resistance.[128] The Tn*2008* was identified as the main vehicle of the carbapenemase OXA-23 in *A. baumannii* in China.[129] In concordance with previous findings from Latin America, we identified IC7 CRAb isolates as the predominant clonal lineage in two hospitals in Cochabamba, Bolivia.[126] By cgMLST the CRAb isolates clustered in five STs, separated by ≥ 40 alleles, within the CC25 associated with IC7. Taken together, our study emphasized the presence of several endemic IC7 OXA-23-positive CRAb clones in two hospitals in Bolivia.[104]

Bacterial isolates that are genetically indistinguishable can be described as a clone that has arisen from a recent common ancestor.[130] Certain bacterial clones, such as ST131 *Escherichia coli*, are primed for success and dominance over other clones.[131] Here we described three paradigms of bacterial spread in the hospital: the success story of ST117/*vanB* VREfm clone, the diverse population of 3GCR *K. pneumoniae* in Germany and the endemic IC7 CRAb clones in two hospitals in Bolivia.[87, 101, 102] These results demonstrate two sides of bacteria involved in nosocomial infections, i.e. clonality and diversity. All three pathogens described here can survive or persist up to several months on surfaces constituting a continuous source for further

transmission in the hospital setting: a key factor for the spread of bacteria. The longer bacteria can survive on dry surfaces, the longer they can serve as a source for transmission putting into risk healthcare workers and susceptible patients.[132] Nevertheless, other factors such as virulence traits or a wide repertoire of ARGs harboured by some bacterial clones can contribute to their clonal expansion. *E. faecium* is often encountered in plants, soil and in the gastrointestinal tract of humans, mammals, and insects. Nevertheless, a distinct *E. faecium* clone associated with the hospital has emerged due to acquired genes involved in antimicrobial resistance, biofilm formation or metabolism and contributed to its successful spread.[133] On the other hand, carbapenem-resistant *K. pneumoniae* isolates exhibit a notable clonal expansion in some countries, such as Greece, but they are rare in Germany.[134, 135] Therefore, clonal spread or diversity of bacterial pathogens in the hospital environment is complex, can be broken down to several contributing factors and is individual for each organism.

3.2 MGEs and clonal diversity in antibiotic-resistant bacterial pathogens

MGEs are the main drivers of the evolution and diversification in bacteria and can spread accessory genes, such as antibiotic resistance or virulence genes, offering a survival advantage in specific niches. In parallel with the emergence of cutting-edge WGS technologies the knowledge about the mobile gene pool of prokaryotes has rapidly increased.[35, 136]

NDM-6, an NDM-1 variant, has been first described on a plasmid in *E. coli* in a patient who also harboured an NDM-1-positive *Proteus mirabilis* in New Zealand.[137] In our study we identified the *bla*$_{\text{NDM-6}}$ for the first time in *A. baumannii* embedded in a Tn*125*-like transposon and present in two adjacent copies which constitutes a novel genetic environment (see 2.4).[105] Multicopy carbapenemases have been previously reported, such as *bla*$_{\text{OXA-235}}$ embedded in a composite transposon found simultaneously on the chromosome and on a plasmid in *A. baumannii* isolates.[15] The multicopy NDM-6 was associated with the recently described IC9, a clonal lineage with a wide distribution that has already been associated with MBLs.[96, 138] MGEs were present to a great extent also in representatives of IC4, IC5 and IC7 CRAb from Bolivia analysed in the present work (see 2.5). Mobile structures were linked to resistance genes, such as a 6 kb plasmid, pMC23.3, harbouring the aminoglycoside modifying enzyme *aadB* in the IC5 isolate, or *bla*$_{\text{OXA-23}}$ embedded in Tn*2008* on the chromosome in IC4 and IC7 isolates.[104] Tn*2008* (IS*Aba1*-*bla*$_{\text{OXA-23}}$) and its variants have been reported from different

countries in different continents.[139] Furthermore, the same resistance genes were found on various MGEs in different isolates, such as *strA* and *strB* along with *sul2* and *floR* were embedded in a RI, (RI1.MC23) in the IC5 isolate, while in the IC7 CRAb the *strA*, *strB* and *sul2* were encoded on a Tn*6172* on a 150 kb plasmid (pMC75.1).[104] Similar plasmids to pMC75.1 and pMC75.2, encoding $bla_{\text{TEM-1}}$ and *aac(3)-IIa*, have been found in *A. baumannii* isolates from Brazil with the same ST to MC75, ST15.[140] On the other hand, identical plasmids can be found in unrelated isolates, such as an 8.7 kb plasmid (pMC1.2/pMC23.2) found in IC7 and IC5 isolates from Bolivia.[104] MGEs in *A. baumannii* are not well studied but the field of HGT is gaining more interest. The present work provides further knowledge on MGEs and transferable resistance in four important but not well studied clonal lineages of *A. baumannii*, IC4, IC5, IC7 and IC9.

MGEs are a source and shuttle of antibiotic resistance in *K. pneumoniae*, an organism often harbouring plasmids, and armed with a wide repertoire of antibiotic resistance determinants. We have analysed three XDR *K. pneumoniae* isolates from northern Germany assigned to the high-risk clone ST147 (see 2.6). Of note, the ST147 was not detected among the *K. pneumoniae* colonising isolates investigated in the present surveillance study (see 2.2). Nevertheless, the ST147 isolates clustered by phylogenetic analysis with ST147 isolates recovered in Göttingen, Germany, indicating that this clone might be circulating in northern Germany. [138] Furthermore, the three isolates were armed with the carbapenemase OXA-181 on a ColKP3 plasmid and an IncR plasmid encoding 12 resistance determinants resulting in a highly resistant phenotype and potentially contributing to the selective success of this pathogen. The isolates were closely related (one allele difference) but showed diversity including inversion or deletion of DNA features in the vicinity of MGEs. For instance, the IncR plasmids varied in a 4 kb region and in the opposite orientation of two composite transposons (3,8 kb and 13 kb), events that can be ascribed to MGEs.[103] Genomic variation within clonal bacterial isolates as a result of homologous recombination and structural rearrangements because of MGEs have been previously described.[141, 142] Our results demonstrated that the dynamics of MGEs can facilitate genome plasticity and contribute to the genetic variation within closely related isolates.

Numerous studies on the clonal expansion of CRAb isolates are available that underline the presence of clonal lineages and endemicity of certain clones.[88, 123] However, the extent of diversity within clonal isolates is not well studied. We have analysed a CRAb outbreak in a German hospital (see 2.7). The CRAb outbreak isolates harboured both the acquired β-

lactamases OXA-23 and NDM-1. Although the carbapenemase NDM-1 gene is prevalent in different countries, it has been rarely encountered in Germany.[43, 143] The investigated isolates were closely related and encoded the NDM-1 gene on the chromosome as part of a Tn*125*-like transposon. The latter transposon is considered the main vehicle of NDM-1 carbapenemase in *A. baumannii*.[42] In close proximity to the NDM-1 the aminoglycoside modifying enzyme AphA6 was found. However, one of the patients was co-infected with an NDM-1-positive CRAb isolate and an isogenic variant of it not encoding the NDM-1 and AphA6. The observed diversity can be traced back to MGEs as both enzymes, NDM-1 and AphA6 were embedded in a transposon. Furthermore, the NDM-1-negative isolate remained carbapenem-resistant because of the presence of OXA-23 and was phenotypically indistinguishable from the remaining outbreak isolates. In a study of a cystic fibrosis patient chronically colonized with *P. aeruginosa* a large degree of diversity was observed within clonal isolates including phenotypes such as colony morphology, motility or antibiotic resistance.[144] An organism can undergo alterations through the course of infection that could affect not only the genotype but also the phenotype. The individuality within closely related isolates and their implication in routine diagnostics needs to be further investigated.

Molecular epidemiology of MGEs in VREfm identified a linkage between the *vanA* gene cluster carried by the Tn*1546* and Inc18-, pHTbeta- and pRUM-related plasmids.[145] Particularly worrisome is the transfer of the VanA-type plasmid borne Tn*1546* from enterococci to *S. aureus* resulting in vancomycin-resistant *S. aureus* indicating the potential of MGEs in the dissemination of ARGs across species.[146] Moreover, VREfm bloodstream infections have been found associated with increased in-hospital mortality and worse outcome compared to VSEfm.[147] The VREfm and VSEfm blood culture isolates analysed here (see 2.8) were identical in a genetic context but showed a heterogeneous resistance phenotype due to the presence of a mosaic plasmid, encoding the resistance determinants *vanA* operon*, ant(6)-Ia*, *erm*(B) and *cat*-like in the VREfm isolate.[106] Mosaic plasmids have been previously reported in enterococcal species, such as fusions between pRUM, Inc18 and pheromone plasmids.[148] But also, transposon-mediated fusion events between sex pheromone plasmids and *vanA*-positive plasmids have been described.[149] A co-infection with a linezolid-resistant *E. faecium* and variants of it with different resistance and virulence phenotypes in the same blood culture have been reported.[150] The VREfm and VSEfm paradigm illustrates not only the contribution of MGEs in genotypic and phenotypic diversity among closely related isolates, but also

challenges in bloodstream infection diagnostics. The manifestation of a co-infection with clonal isolates with variable phenotypes could complicate patient treatment, e.g. in case only the VSEfm variant would have been detected by routine diagnostic. The detection of such type of heterogeneity becomes more complex when the strain variants cannot be morphologically distinguished and when only one colony is used for phenotypic and genotypic investigation.

MGEs are key agents of the bacterial evolution and contribute to the adaptation of bacteria to new environments.[34] In this thesis, numerous examples of ARGs associated with MGEs including plasmids, transposons and ISs in three clinically relevant bacteria, *A. baumannni*, *E. faecium* and *K. pneumoniae* are described.[103, 106] At present, the vast majority of studies and bacteria sequenced have clinical origin and represent a snapshot of a rather narrow environment ignoring other niches outside of the hospital setting to elucidate potential linking and transmission routes. Furthermore, the extent of diversity within a clonal population of bacteria has not been widely studied. A considerable diversity within-host was observed in an MRSA outbreak in a veterinary hospital, where multiple colonies of *S. aureus* recovered from animal patients and hospital workers were investigated.[151] Similar findings were described in the present study, including closely related isolates which differ in genotype or phenotype. These examples represent a real-world scenario uncovered by deep genome analysis. Usually in genomic studies, only individual isolates are analysed representing a snapshot and resulting in underrepresented heterogeneity in bacterial populations. A study based on theoretical modelling demonstrated that using a single isolate can be misleading for analysing transmission routes when diversity occurs within the host.[152] In conclusion, this thesis highlights the contribution of MGEs to the dissemination of antimicrobial resistance, their complexity and the potential pitfalls of clonality and genetic diversity in bacterial pathogens.

4. Summary

Over the past decades antimicrobial resistance rates are rapidly growing and have provoked the emergence of multidrug-resistant bacterial pathogens. Of particular concern are nosocomial outbreaks and the clonal spread of antibiotic-resistant bacteria representing a major threat for the healthcare system and especially to susceptible patient populations. One of the aims of this PhD project was to investigate the spread of three bacterial species commonly involved in nosocomial infections: vancomycin-resistant *Enterococcus faecium* (VREfm) and third-generation cephalosporin-resistant (3GCR) *Klebsiella pneumoniae* recovered from screening samples of patients admitted to six university hospitals in Germany, and carbapenem-resistant *Acinetobacter baumannii* (CRAb) from two hospitals in Bolivia.

Between 2014 and 2018 a steady increase in the prevalence of VREfm (2014, 0.8%; 2015, 1.2%; 2016, 1.3%; 2017, 1.5%; 2018, 2.6%) was observed in six university hospitals in Germany. In the present cohort the sequence type (ST) 117 was identified as the predominant clone followed by ST80, ST203, ST78 and ST17. Furthermore, *vanB* was the most common glycopeptide resistance determinant among the colonising isolates while the vast majority of the ST117 isolates were *vanB*-positive. A remarkable clonal dissemination of the *vanB*-positive cluster type (CT) 71 subpopulation of the ST117 clone was identified among VREfm. In contrast, other STs, such as ST80, formed mostly local clusters restricted to single study centres. The ST117/CT71/*vanB* VREfm clone was widespread over six geographically separated centres and was endemic and indicated that the epidemiological profile of VREfm in Germany has changed in the past five years.

Conversely, patients on hospital admission in Germany were colonised with a diverse population of 3GCR *K. pneumoniae* complex isolates. Over the study period an increase in the prevalence of 3GCR *K. pneumoniae* complex (2016, 0.8%; 2017, 0.9%; 2018, 1.1%) was observed. The clinically relevant *K. pneumoniae* was the predominant species in the present study, but a few representatives of *Klebsiella quasipneumoniae* and *Klebsiella variicola* were also identified. Among 3GCR *K. pneumoniae* isolates CTX-M-15 was the most common acquired β-lactamase followed by TEM-1B. Carbapenem-resistance was rare among the colonising isolates with only one *K. variicola* isolate harbouring a carbapenemase, OXA-181. Lastly, no clonal expansion and only small clusters of closely related isolates were identified, suggesting that a diverse *K. pneumoniae* population is circulating in the participating study centres.

The clonal spread of CRAb isolates obtained from two hospitals in Bolivia was demonstrated in the present thesis. Among *A. baumannii* 51 were identified as carbapenem-resistant. The vast majority of the CRAb isolates were identified as ST25 or a single locus variant of it, ST991. The latter ST was only identified in one of the hospitals while ST25 was predominant in the second hospital. Moreover, all CRAb isolates harboured OXA-23 on a Tn*2008* transposon while no other carbapenemase was detected. All but one of the CRAb isolates belonged to the international clone (IC) 7 and formed five transmission clusters. Furthermore, one CRAb isolate was assigned as IC4. These results suggested that several IC7 OXA-23-positive CRAb clones are endemic and are circulating in two hospitals in Bolivia.

Horizontal gene transfer and mobile genetic elements (MGEs) are the main drivers of the evolution, diversification and spread of antibiotic resistance genes (ARGs) in bacteria. This thesis analysed and characterised MGEs including plasmids, transposons and insertion sequences (ISs) catalysing the mobilization of ARGs in *A. baumannni* clinical isolates. For the first time the carbapenemase *bla*$_{\text{NDM-6}}$, a *bla*$_{\text{NDM-1}}$ variant, was identified in a CRAb isolate recovered in northern Spain in 2019. The ST85 CRAb clustered together with the recently described and widespread clonal lineage IC9. Furthermore, in the latter isolate NDM-6 was detected in two copies and in a novel genetic environment linked to MGEs. Novel insights into MGEs harbouring ARGs in *A. baumannii* IC4, IC5 and IC7 representatives from Bolivia were observed. A diverse array of transposons, plasmids and resistance islands, such as *strA* and *strB* harboured by a large plasmid in the IC4 CRAb and by a chromosome encoded resistance island in the IC5 isolate, were identified in the present study. Furthermore, MGEs, e.g. a small plasmid or the Tn*2008* carrying the *bla*$_{\text{OXA-23}}$, were found across different ICs. These data reflect the prevalence of MGEs in *A. baumannii* and the strong link between the spread of ARGs and the mobilome.

Bacteria can be clonal but yet different. The extent of clonal heterogeneity associated with MGEs in bacterial populations was also discussed for three extensive drug-resistant *K. pneumoniae* colonising isolates from Germany. The isolates were assigned to the high-risk clone ST147 and were armed in addition with a wide repertoire of ARGs, including the plasmid encoded carbapenemase OXA-181. Of particular interest was an IncR plasmid harbouring in total 12 resistance determinants and a wide variety of ISs. The *K. pneumoniae* isolates were closely related but diversity including inversions or deletions in the proximity to MGEs within the IncR plasmid was identified. Nevertheless, this genetic variation did not affect the

resistance phenotype of the three *K. pneumoniae* isolates. These data demonstrated the influence of MGEs on genome plasticity and their contribution to heterogeneity within closely related isolates.

Another example of clonal heterogeneity was detected in a CRAb outbreak in a German university hospital. The CRAb isolates harboured the acquired β-lactamase NDM-1 embedded in a transposon adjacent to the aminoglycoside modifying enzyme AphA6. Moreover, a second carbapenemase, OXA-23, was carried by the CRAb isolates. By cgMLST analysis the isolates were closely related and formed a transmission cluster. However, one patient was co-infected with two near identical CRAb isolates, i.e. one NDM-1-positive isolate and one isolate lacking NDM-1 and AphA6. Nevertheless, the NDM-1-negative isolate was still carbapenem-resistant because of the presence of $bla_{\text{OXA-23}}$. The loss of the antibiotic resistance determinants NDM-1 and AphA6 could be attributed to a transposition event. MGEs associated with ARGs can lead to genetic variation during the course of an outbreak but not necessarily alter the resistance phenotype.

Also, a co-infection of two closely related but phenotypically different *E. faecium* isolates was discussed in the present study. Both isolates were obtained from the same blood culture and were identified as ST203. Furthermore, one isolate was VREfm while the second was vancomycin-susceptible *E. faecium* (VSEfm). Molecular epidemiology of the two isolates revealed that the VREfm and VSEfm were closely related. In the VSEfm isolate, a 12 kb plasmid was detected which was also present in the VREfm isolate. However, in the VREfm the latter plasmid carried also the *vanA* gene cluster embedded in a Tn*1546*-type transposon as also the resistance genes *ant(6)-Ia*, *erm*(B) and *cat*-like and two more replicons indicating the presence of a chimeric plasmid. Here, MGEs and clonal diversity led to a heterogenous resistance phenotype within closely related isolates.

Taken all together, this thesis documents the clonal spread and diversity of three hospital-acquired bacterial species but also highlights the dynamics of MGEs contributing to genome plasticity and the genetic diversity within closely related isolates.

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Abbreviations

AMR	antimicrobial resistance
ARG	antibiotic resistance gene
bp	base pairs
CC	clonal complex
cgMLST	core-genome multilocus sequence typing
CRAb	carbapenem-resistant *Acinetobacter baumannii*
DNA	deoxyribonucleic acid
DR	direct repeat
dsDNA	double stranded deoxyribonucleic acid
EARS-Net	European Antimicrobial Resistance Surveillance Network
ESBL	extended-spectrum-β-lactamase
HGT	horizontal gene transfer
3GCR	third-generation cephalosporin resistant
GI	genomic island
IC	international clone
ICE	integrative and conjugative element
ICU	intensive care unit
IR	inverted repeat
IRL	left inverted repeat
IRR	right inverted repeat
IS	insertion sequence
ITR	inverted terminal repeat
kb	kilobase
Mb	megabase
MBL	metallo-β-lactamase
MDR	multidrug-resistant
MGE	mobile genetic element
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
MRSA	methicillin-resistant *Staphylococcus aureus*
NDM	New Delhi metallo-β-lactamase
NGS	next-generation sequencing

No.	number
ORF	open reading frame
ori	origin of replication
OXA	oxacillinase
PBP	penicillin-binding protein
PDR	pan-drug resistant
PFGE	pulsed-field gel electrophoresis
RI	resistance island
SLV	single locus variant
SNV	single nucleotide variant
ST	sequence type
TA	toxin-antitoxin
TE	transposable element
VREfm	vancomycin-resistant *Enterococcus faecium*
VSEfm	vancomycin-susceptible *Enterococcus faecium*
WGS	whole genome sequencing
WHO	world health organization
XDR	extensive-drug resistant

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Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht. Die in der vorliegenden Dissertation generierten Primärdaten und Materialien sind am Institut für Medizinische Mikrobiologie, Immunologie und Hygiene der Uniklinik Köln, Arbeitsgruppe Seifert, gesichert und zugänglich.

Eingebundene Publikationen

Xanthopoulou K., Wille J., Zweigner J., Lucaßen K., Wille T., Seifert H., Higgins P.G. Characterization of a vancomycin-resistant and a vancomycin-susceptible *Enterococcus faecium*. Journal of Antimicrobial Chemotherapy, 2020, DOI: 10.1093/jac/dkaa532

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Teilpublikationen – Konferenzbeiträge

Xanthopoulou K., Wille J., Zweigner J., Lucassen K., Seifert H., Higgins P.G. Molecular characterization of *bla*$_{\text{NDM-1}}$ from an *Acinetobacter baumannii* outbreak in a German university hospital. Accepted as a poster presentation at the 30th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in Paris, France in April 2020. The conference has been cancelled due to the SARS-CoV-2 pandemic.

Xanthopoulou K., Wille J., Walker S., Stelzer Y., Persy V., Imirzalioglu C., Lucassen K., Seifert H., Higgins P.G. Prevalence and molecular epidemiology of high-risk clones among third-generation cephalosporin-resistant and carbapenem-resistant *Klebsiella pneumoniae* in Germany. Accepted as a poster presentation at the 30th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in Paris, France in April 2020. The conference has been cancelled due to the SARS-CoV-2 pandemic.

Xanthopoulou K., Wille J., Walker S., Stelzer Y., Imirzalioglu C., Seifert H., Higgins P.G. Prevalence and molecular epidemiology of high-risk clones among third-generation

cephalosporin-resistant and carbapenem-resistant *Klebsiella pneumoniae* in Germany. Poster presentation at the DGI and DZIF Joint Annual Meeting, Bad Nauheim, Germany, November 2019

Köln, 11. August 2021 Kyriaki Xanthopoulou

Appendix

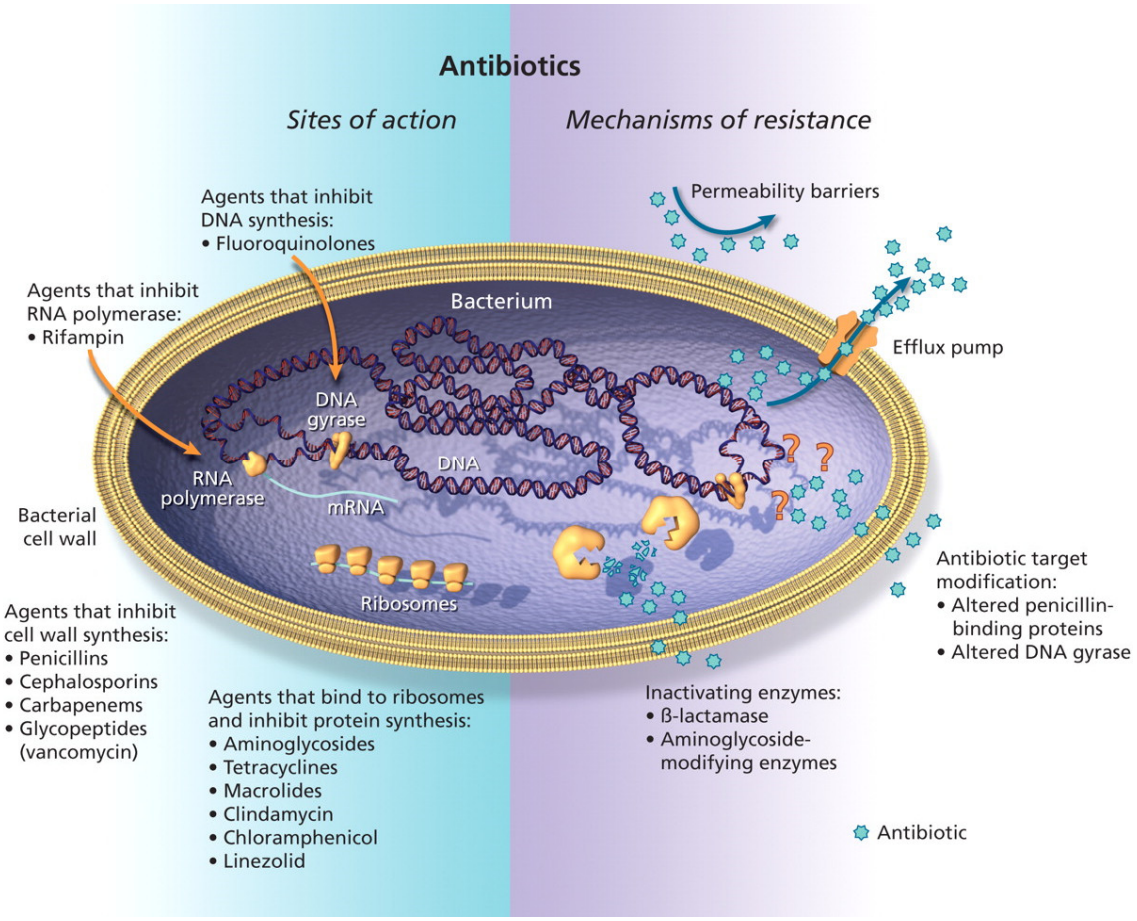

Figure S1. Overview of the site of action and mechanism of resistance to antibiotics. The figure was reprinted from the Canadian Medical Association Journal.[153]

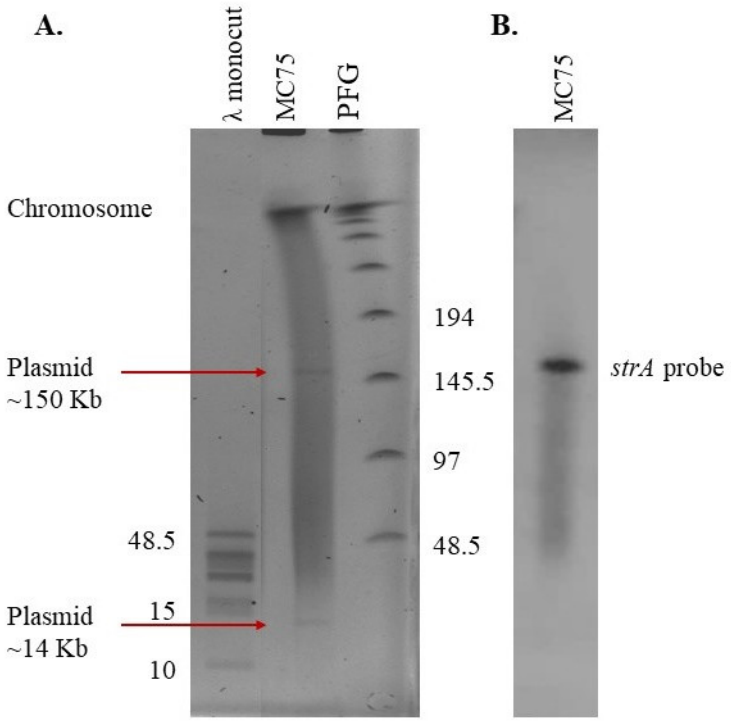

Figure S2. Localization of the *strA* gene determined by S1-PFGE and Southern blot using a DIG-labelled probe. S1 treatment of DNA embedded in agarose plugs of the MC75 *A. baumannii* isolate followed by PFGE (A) and Southern blot (B) with a *strA* probe. λ monocut indicates the lambda monocut ladder and PFG the lambda pulsed-field gel electrophoresis marker (molecular sizes in kilobases are shown on the left). The PFGE figure is a composite of different lanes from the same gel.

Table S1: Tools for *in silico* sequence analysis.

	***In silico* tool**	**Usage**	**URL**
Database	Genbank	genetic sequence database	https://www.ncbi.nlm.nih.gov/genbank/
	ISfinder	bacterial insertion sequences database	https://isfinder.biotoul.fr/
	National Database of Antibiotic Resistant Organisms	antimicrobial resistance database	https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/
Genome analysis	Blast	basic local alignment search tool	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	Center for Genomic Epidemiology	online individual tools for typing, phenotyping, phylogeny	https://cge.cbs.dtu.dk/services/
	JSpecies	prokaryotic species circumscription based on pairwise genome comparison	http://jspecies.ribohost.com/jspeciesws/
	Minimap2	sequence alignment	
	Multalin	multiple sequence alignment	http://multalin.toulouse.inra.fr/multalin/
	PubMLST	molecular typing	https://pubmlst.org/
	Galaxy	sequence analysis web-based platform	https://usegalaxy.org/
Genome assembly	Canu	long-read assembler	https://github.com/marbl/canu
	SPAdes, hybridSPAdes, plasmidSPAdes	short-read, hybrid and plasmid assembler	https://github.com/ablab/spades
	Velvet	short-read assembler	https://www.ebi.ac.uk/~zerbino/velvet/

	Unicycler	short-read, long-read, hybrid assembler	https://github.com/rrwick/Unicycler
Genome annotation	Prokka	prokaryotic genome annotation	https://github.com/tseemann/prokka
	RAST	prokaryotic genome annotation	https://rast.nmpdr.org/
Visualization	Artemis	genome browser	https://www.sanger.ac.uk/tool/artemis/
	SnapGene	genome and plasmid browser	https://www.snapgene.com/