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Whole Genome Sequencing of Gram-negative bacterial pathogens: identification and biochemical analysis of a new class A β-lactamase, the KPC-53 enzyme

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Summary

The β-lactamases are the most common mechanism of resistance to β-lactam antibiotics in Gram-negative bacteria. Two major families, extended-spectrum-β-lactamases (ESBLs) and metallo-β-lactamases, represent a serious risk for human health. β-lactam compounds are bactericidal agents that inhibit cell wall synthesis. Moreover, bacteria escape to their action by three different ways: 1) altered PBPs that exhibit low affinity for β-lactam antibiotics; 2) lack or diminished expression of outer membrane proteins; 3) production of β-lactamases which is the most common mechanism of bacterial resistance to β-lactams. β-lactamases are bacterial enzymes able to hydrolyse the β-lactam ring making the antibiotic inactive before it reaches the PBP target. These enzymes have been categorized into four classes based on their sequence homology (Ambler classes A, B, C and D). The enzymes belonging to classes A, C and D possess in their catalytic site an active residue of serine while class B enzymes (MBLs) require zinc ions for their activity [1]. The production of ESBLs is one of the most important responses of the bacteria to the excessive use of oxyimino-cephalosporins for the treatment of serious infection [2]. MBLs confer resistance to a wide range of β-lactams, including carbapenems [3]. The genes encoding serin- and metallo β-lactamases can be located on the bacterial chromosome, on plasmids, on transposons, or on integrons. The presence of resistance genes on Mobile Genetic Elements allows the spread of these genes among bacterial species by conjugation, transduction, or transformation. The genetic environment of the β-lactamase (*bla*) genes dictate whether the β-lactamases are produced in a constitutive or inducible manner. Transposable elements were identified as major contributors to bacterial genomic fluidity [4]. Plasmids harbor antibiotic resistance genes to most classes of antibiotics currently used in clinical practice including fluoroquinolones and aminoglycosides.

Various antibiotic treatments, as well as a total dependence on nurse care of Long Term Care Facilities (LTCFs) residents or hospitalized patients, expose them both to the selection and horizontal transmission of antibiotic resistant organisms. As a consequence, these facilities represent important reservoirs of antibiotic resistant strains, mainly ESBL- and carbapenemase-producing *Enterobacterales* [5]. In our study we decided to investigate the dissemination of antibiotic resistant genes and virulence factors in Gram-negative bacterial pathogens by Next Generation Sequencing (NGS) technology. In detail, this thesis draws attention on draft genome analysis of carbapenem resistant *Klebsiella pneumoniae* and *Escherichia coli* strains isolated from rectal swabs of residents in LTCFs located in Northern Italian Region. In addition, molecular analysis of draft genome analysis of *Acinetobacter baumannii* isolated from COVID-19 patients affected by sepsis admitted to the intensive care unit (ICU) of Spirito Santo Hospital in a Central Italian Region, was also performed.

The massive sequencing process of the whole genome performed on all these clinical strains has given the possibility to identify new β-lactamase variants: KPC-53. KPC-type enzymes are class A serine β-lactamases which have powerful carbapenemase activity. These enzymes are usually encoded by transferable plasmids. KPC β-lactamases are enzymes in continuous evolution: the new enzyme KPC-53 was identified in a *K. pneumoniae* resistant to ceftazidime-avibactam combination. The new enzyme showed a duplication of L167E168 residues in the Ω-loop. New mutations in the catalytic pocket of these enzymes, have changed the antibiotic profile hydrolysis of KPC natural variants.

Chapter 1: How does Antibiotic Resistance Spread?

1.1 Introduction

Infections caused by multi-resistant organisms significantly increase morbidity, mortality and health care costs. Antimicrobial resistant infections cause an important global health burden and were estimated to contribute to 4.95 million deaths worldwide in 2019 [6]. The U.S Center for Disease Control and Prevention (CDC) and the World Health Organization (WHO) categorize antimicrobial resistant pathogens as a looming threat to human health. In details, it is estimated that more than 2 million infections with a death tool of 29.000 occur in the USA per annum, at on attributable health care cost of more than 4.7 billion of dollars. In Europe, over 33.000 deaths and 874.000 disability-adjusted life years are attributed to hospital-acquired and community-acquired antimicrobial resistant infections each year, accounting of $1,5 billion in direct and indirect costs [7]. Moreover, the WHO predicts a global rate of 10 billion deaths per year due to drug-resistant bacterial diseases by 2050 if no actions are taken [8].

In the European Union/European Economic Area (EU/EEA) prior to December 2019, there were an estimated 2.9 million residents in 43,000 LTCFs (approximately 0.7% of the total population), with around 5.6% of the Italian population was potentially dependent, and 28.7% of them aged more than 65 in need of LTCFs [9].

On this matter, the European Centre for Disease Prevention and Control (ECDC) is monitoring healthcare-associated infections (HAIs) and antimicrobial use in European LTCFs, comparing data from different countries: the aim is to implement preventive and modulatory measures. *Klebsiella pneumoniae* and *Escherichia coli*, are the most frequent *Enterobacterales* resistant to numerous classes of antibiotics, causing infections of the urinary tract, lower respiratory tract and gastroenteric tract in the LTCFs [10].

Antibiotic-resistant bacteria (ARB) are a major cause of health care-associated infections around the world, mainly in long-term care facilities (LTCFs), that are considered important reservoirs of ARB because of their colonization of residents discharged from the hospital, or they become colonized inside the facility as a result of antibiotic selective pressure [11].

These are the main causes of serious infections among LTCF residents, and the recent emergence and spread of extended-spectrum β-lactamases (ESBLs) and/or carbapenemase-producing *Enterobacterales* firmly reduced the therapeutic options.

1.2 Mobile genetic elements

Molecular analysis has revelated that widespread multi-resistance has commonly been achieved by the acquisition of determinants followed by amplification in response to selection. The capture, accumulation and dissemination of resistance genes are largely due to the actions of mobile genetic elements (MGEs), able to promote intracellular and intercellular DNA mobility (from chromosome to plasmid, between plasmids or between different bacteria, even belonging to different species).

Insertion sequences (ISs) and transposons (Tn) are DNA segments that are able to move themselves and also associated resistance genes almost randomly to new locations in the same or different DNA molecules within a single cell. Other elements, such as integrons (In), work by site-specific recombination, moving resistance genes in specific site; this kind of MGEs is often in multiple copies located in different part of genome, and this facilitates the homologous recombination [8].

There are several mechanisms of genetic exchange:

1. conjugation/mobilization, generally mediated by plasmids and integrative conjugative elements (ICEs);

2. transformation, that is an uptake of extracellular DNA;

3. transduction, mediated by bacteriophages.

Interactions between the various type of MGEs underpin the rapid evolution of diverse multi-resistant pathogens, which are insensitive to several antibiotic treatments (Figure 1).

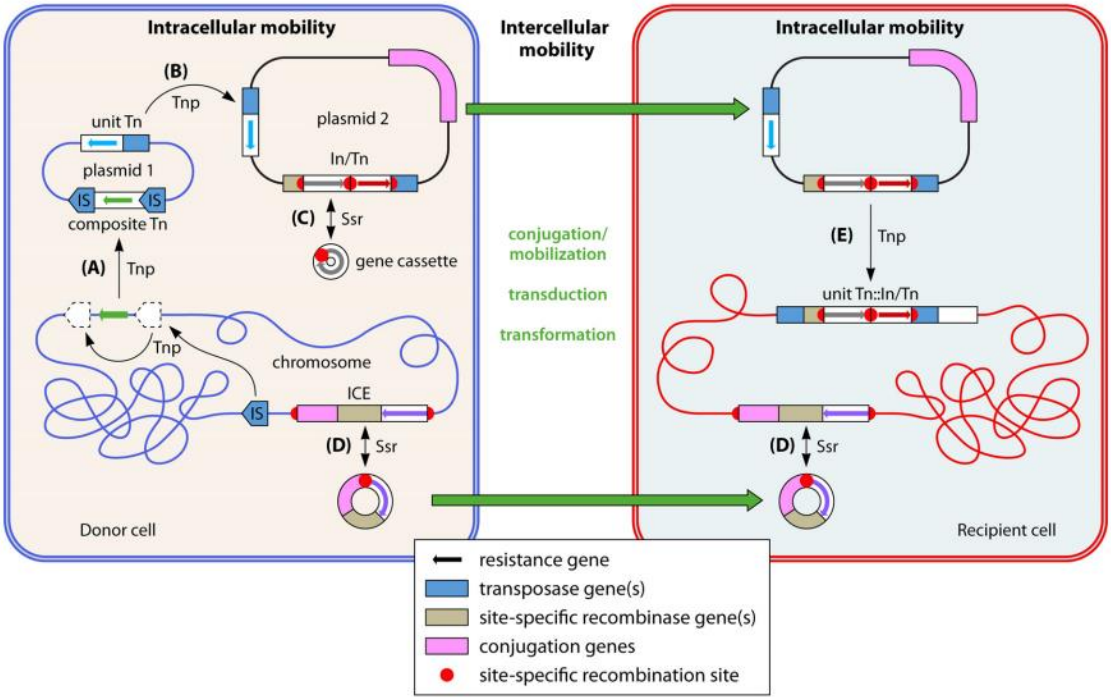

Figure 1 Examples of mobile genetic elements (MGE) and processes involved in intracellular mobility or intercellular transfer of antibiotic resistance genes [12].

These phenomena are particularly widespread in an important group of clinical pathogens, named ESKAPE that includes *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.*, and *Escherichia coli*. Through genetic mutations and the acquisition of MGEs, ESKAPE pathogens have developed resistance mechanisms against oxazolidinones, lipopeptides, macrolides, fluoroquinolones, tetracyclines, β-lactams, β-lactams/β-lactamases-inhibitors combinations, carbapenems, glycopeptides and clinically polymyxins. Overall, the constitutive and/or inducible expression of these drug resistance mechanisms has resulted in the increased representation of bacterial species with mechanisms-hospital-acquired infections [13].

1.2.1 Insertion Sequences (ISs)

ISs are generally small mobile elements that typically carry little more than one transposase (*tnp*) gene. These aelements are the simplest and smallest genetic elements at roughly 1 kb. They can be divided into groups based on

(1) active site motifs in_designed by key amino acids (most commonly Asp, Asp and Glu, but, sometimes also two His residues separated by a large hydrophobic amino acid); (2) and/or based on whether transposition that can be conservative, cut-and-paste mechanism (where the IS is simply excised from the donor and inserted into the recipient), or replicative. These mechanisms are often combined each other [14].

The ends of the most common type of ISs are generally defined by terminal inverted repeats (IR_L for left and IR_R for right) with respect to the direction of transcription of the *tnp* gene (figure 2). Transposition involves binding of the IR by the TNP protein, and as a result of repair of staggered cuts in different DNA strands during the transposition process [13]. Many ISs create short flanking direct repeats in insertion, also referred to as Target Site Duplications (TSD) (figure 3). ISfinder gives a complete database of IS based on BLAST approach [14]. Every IS is assigned names that include a code for the species in which the IS was first identified, and a number. IS elements are often named including the first three letters of the species in which they were discovered, thus *A. baumannii* IS elements are designated IS*Aba* [14]

IS elements can contribute to genomic variability as well as create significant phenotypic changes for the bacterial host, both favorable and unfavorable. Thus, they can promote antibiotic resistance by changing bacterial gene expression, such as by providing strong promoter sequences to downstream genes, disrupting promoters to decrease expression, or inactivating gene. IS elements are significant contributors to carbapenem-resistant *Acinetobacter*. This strain intrinsically encodes two classes of β-lactamases that normally do not confer clinical resistance at basal expression levels: (1) the chromosomally encoded *ampC* (also kwow as bla_{ADC} taht encodes a cephalosporinase) and (2) $bla_{OXA\text{-}51}$ (a carbapenemase) However, insertion of an IS element upstream of bla_{ampC} or $bla_{OXA\text{-}51}$ can provide a strong outward promoter and confer clinical resistance to cephalosporins or carbapenems, respectively. For example IS*Aba1* and IS*Aba125* have identified to promote *ampC* expression; IS*Aba1* is similarly associated with promoting the expression of the intrinsic $bla_{OXA\text{-}51}$. ISAba1 insertion was associated with significantly increased expression and carbapenem resistance. Moreover , carbapenem resistance conferred by the production of the NDM-1 is also thought to require IS-mediated activation of $bla_{NDM\text{-}1}$ that is not intrinsic to all *A. baumannii* strains [15].

Some of these can move resistance genes as part of a composite transposon, a region bounded by two copies of the same or related IS that can move as a single unit. Various examples of a single IS mobilizing an adjacent region that include one or more resistance genes are identified, particularly in Gram-negative bacteria. Many ISs include a strong promoter that drives expression of the captured gene (e.g. IS*Aba1* with $bla_{\text{OXA-51}}$ -like genes in *A. baumannii* that gives carbapenem resistance) [16].

The most common IS family in Gram-negative (founded also in Gram-positive) bacteria are the following:

IS*26* and related elements, which played a pivotal role in the dissemination of resistance determinants in Gram-negative (e.g resistance to phenicols, vancomycin, gentamicin, kanamycin, tobramycin, lincosamides, streptogramin A) [17].

IS*Ecp1* and related elements, first identified in *E. coli*, associated to resistance genes such as $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}25}$ group, bla_{ACC}, $bla_{CMY\text{-}2}$-like genes, $bla_{OXA\text{-}181}$-like genes, $bla_{OXA\text{-}204}$, *qnr* genes, *aac(3)-IIf-arr*, *aac(3')-IIb*, *rmtC*, bla_{BKC} and *aph(2")-Ie* [18].

IS*Apl1* and mrc-1. The IS30 family elements IS*Apl1* were first discovered in the pig pathogen *Actinobacillus pleuropneumoniae* and they are involved in capture and mobilization of the recently identified *mcr-1* gene (mobile colistin resistance gene) [19].

IS*91*-like and ISCR elements. Three related ISs , IS*91*,IS*801* and IS*1294*, lack conventional IR and move by rolling circle replication. IS*91* and IS*801* do not seem to have been involved in movement of known resistance genes, but *IS1294* and the variant IS*1294b* have transferred $bla_{CMY\text{-}2}$-like genes originally associated with IS*Ecp1*. ISCR family elements appear to have been responsible for capturing and moving different antibiotic resistance genes such as *dfrA10*, *catA2*, *armA*, $bla_{CMY/MOX\text{-}}$*like genes*, *qnrB genes*, *sul2*, *tet(31)*, $bla_{AIM\text{-}1}$, $bla_{SPM\text{-}1}$,*floR*. One of these elements, IS*CR27*, may have been responsible for mobilization of a precursor of bla_{NDM} from an identified source organism to *A.baumannii*, while IS*CR1* may have contributed to subsequent movement [20].
(Figure 2, Figure 3).

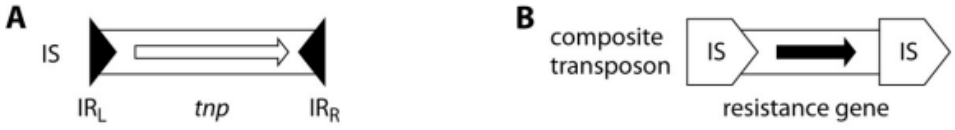

Figure 2 Insertion sequences and composite transposons. (A) Components of a typical IS. (B) Composite transposon. IS are shown as block arrows, with the pointed end corresponding to IRR, and a captured resistance gene is shown as a black arrow [12].

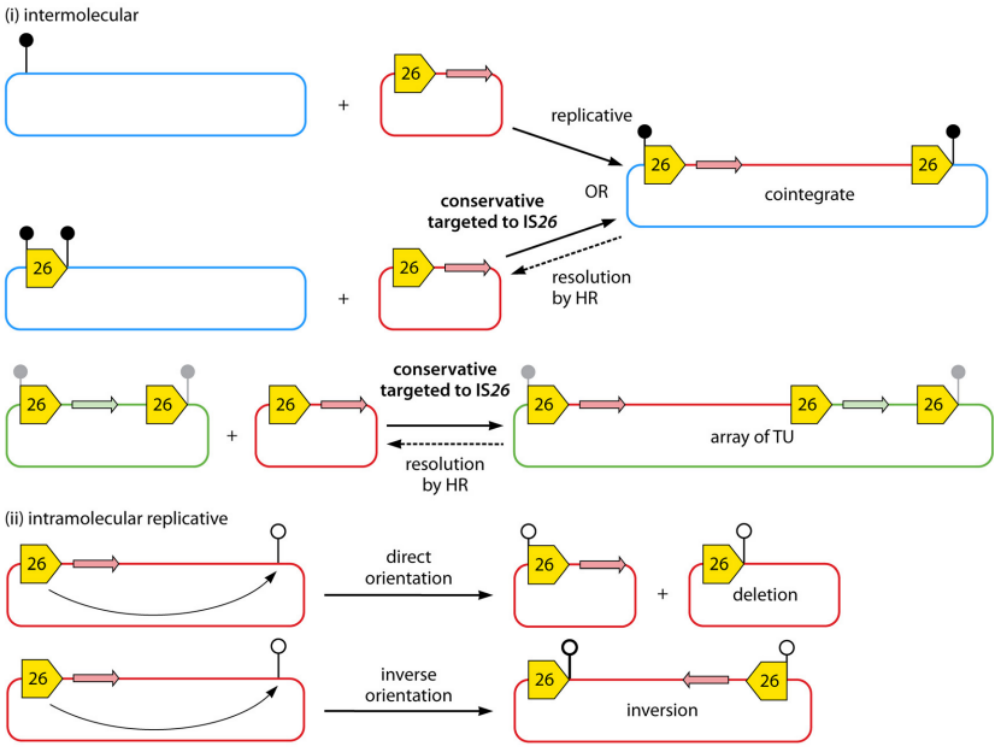

Figure 3 Outcomes of transposition by IS26. (i) Intermolecular replicative transposition. (ii) Intramolecular replicative transposition [12].

1.2.2 Unit Trasposons

Unit transposons were traditionally thought of as elements larger than IS, bounded by IR rather than by a pair of IS, and including a transposase gene and an internal "passenger" gene which may encode antibiotic resistance.

Tn3 family transposons: the archetype of Tn3 family and the close relatives Tn1 and Tn2 were some of the earliest unit transposons to be identified in Gram-negative bacteria. However, antibiotic resistance genes are often associated with Tn3 family [21]. Tn2, instead, is the most common in clinical isolates. *bla*$_{TEM}$ gene, have always been found within Tn1, Tn2, Tn3 or variants, hybrids, or fragments of these transposons. Exists, also, a derivate of Tn3, Tn1331, that carries additional resistance genes in a region derived from a class1integron. Hybrid Tn1331 are quite common associated with *bla*$_{KPC}$ gene [22]. Neverthless, Tn3 family includes Tn21 subfamily transposons, which often carry a mercury resistance gene (*mer*) operon, but they are also important in movement of antibiotic resistance determinants such as *tet(A)* involved in tetracycline resistance. Tn4401, included in this subfamily, carries *bla*$_{KPC}$ variant. In addition, Tn4401 has two promoters driving *bla*$_{KPC}$ expression (Figure 4) [23].

Tn7 family transposons: another transposon superfamily, named Tn7-like transposon, includes members associated with antibiotic resistance, such as Tn7 and Tn402-like elements in Gram-negative bacteria, and Tn552 in *Staphylococcus* resistant to penicillin (Tn552-like elements are believed to be the origin of all β-lactamase genes in *Staphylococcus*) (Figure 4) [24].

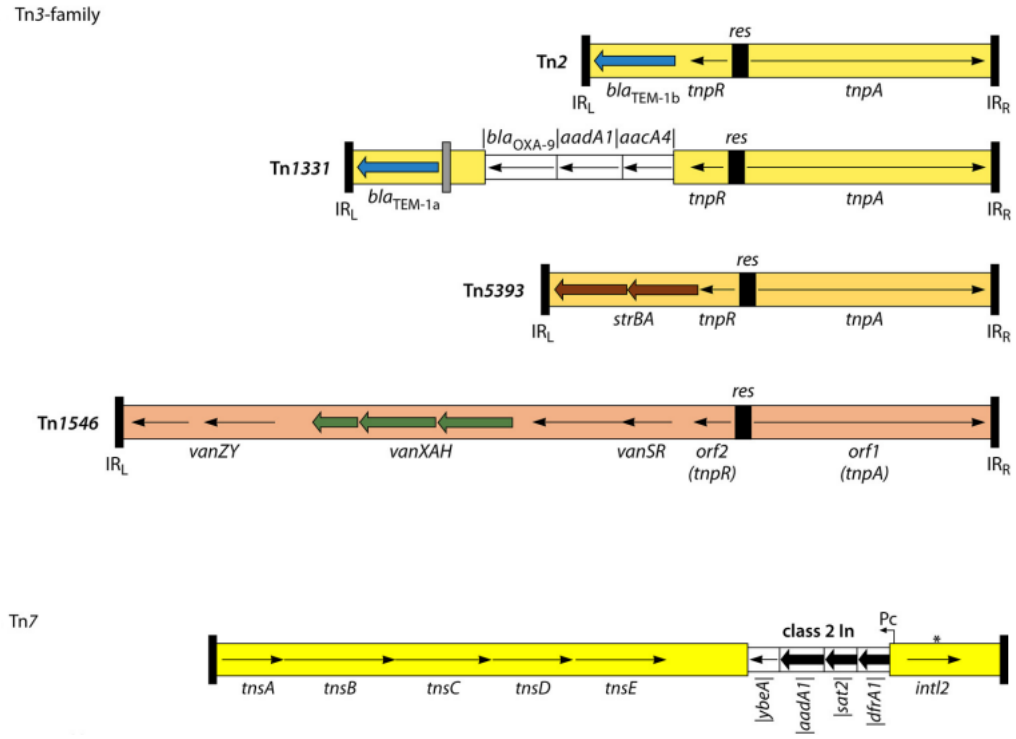

Figure 4 Tn3 family transposons and Tn7 family transposons structures [12].

1.2.3 Gene cassettes and integrons

Integrons are genetic elements that have the ability to capture genes of various origin and make them express. Integrons are not inserted randomly but they are highly selective on the insertion site. Integrons are characterized by an *IntL* gene, encoding an atypical site-specific tyrosine recombinase; they contain also a specific sequence of DNA, *aatl*, which is a recombination site, and a promoter (Pc). Integrons can be part of transposases, plasmids and, even, the bacterial chromosome [25].

A gene cassette is a small mobile element (0.5 to 1kb) consisting of a single gene (occasionally two), typically lacking a promoter, and an *attC* recombination site [25]. Gene cassettes can exist in a free circular element, but they are non-replicative and are usually found inserted into an integron by which they can be transcripted (Figure 5). Multiple cassettes may be inserted into the same integron to create a cassette array that may confer multi-resistance.

There are different classes of integrons (Int):

Integrons of class 1, that often carry *sul1* gene, encoding resistance to the sulphonamide antibiotics;

Integrons of class 2, associated with Tn7 and variants;

Integrons of class 3, mostly of them carry cassettes that encode β-lactamases;

Integrons ofclass 4, founded in *Vibrio cholerae* chromosome

Integrons of class 5 that is very rare [26,27].

A wide variety of gene cassettes containing resistance genes, have been identified. The most clinically relevant are those carrying genes encoding β-lactamases or aminoglycoside-modifying enzymes. The former includes metallo-β-lactamases, especially VIM and IMP types. Cassette-borne genes also encode class A GES enzymes and class D OXA-10-like and OXA-1 like enzymes. Variants of the common *aacA4/aac(6')-Ib* cassettes may confer resistance to tombramycin plus gentamicin and amikacin or low level resistance to fluoroquinolones. Certain cassette arrays e.g. |*dfrA17*|*aadA5*| and |*dfrA12*|*gcuF*|*aadA2*|, give resistance to trimethoprim, streptomycin and spectinomycin, and they are very common in class1 integrons [28].

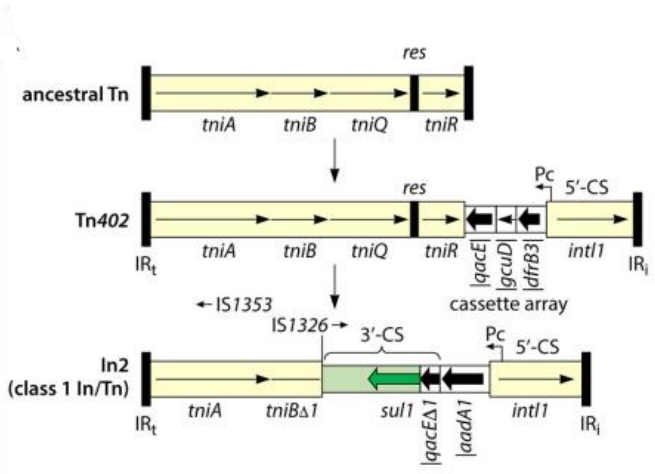

Figure 5 Evolution of class 1 In/Tn. The diagrams show capture of intI1/attI1/Pc and gene cassettes, with qacE in the last position, by a Tn5053-like transposon. Subsequent deletion of parts of the final cassette and tni region and insertion of sul1 create the 3=-CS, giving a typical "clinical" class 1In/Tn which is not self-transposable [12].

1.2.4 Incompatibility Plasmids

Plasmids are important vehicles for the carriage of other mobile genetic elements and antimicrobial resistance genes in both Gram-negative and Gram-positive bacteria. Their size ranging from less than a Kilobase to several megabases. Their extrachromosomal existence stems from their ability to replicate and be inherited in a growing population of host cells. In addition, conjugation or mobilization functions may also be present, allowing plasmids to spread horizontally. All genes encoding these functions create a "backbone" that represents the core of plasmids features. In resistance plasmids these regions are typically made up of one or more resistance genes and associated mobile elements, such as IS, Tn and In. Plasmid replication initiates at a specific region called origin (*ori*) triggered by an RNA transcript or by the binding of an initiation protein (*rep*) encoded by *rep* gene, located on the plasmid. These elements, usually, exploit the host's chromosomally encoded replication mechanism for DNA synthesis [29]. Replication initiation proteins often possess some conserved domains which define the type of replication system [30]. There are three modes of plasmid replication:

1. Rolling circle replication, that is mostly used by small plasmids in Gram-positive;

2. Theta-mode replication, used by small to very large plasmids;

3. Strand-displacement replication, where both DNA strands are replicated continuously in opposite directions from the origin and it is generally used by small plasmids.

Some plasmids, both in Gram-negative and Gram-positive, commonly have multiple replication regions, suggesting that fusions and cointegrations between one or more plasmids, occur frequently [31].

Once replicated, plasmids must be distributed between daughter cells. For small plasmids with an high copy number can be achieved by random segregation; instead, larger plasmids, that usually exist at low copy number, possess functional modules that contribute in segregational stability [31]. These include three systems:

1. Resolution system that converts plasmid multimers into monomers that can be segregated independently;

2. Partitioning system, that actively distributes plasmid copies to daughter cells;

3. Post-segregational killing system, that kills progeny cells that fail to inherit a copy of the plasmid.

Plasmid propagation can take place not only via vertical transmission, but also via horizontal transmission to other bacterial cells. Thus, conjugative plasmids include genetically complex system for horizontal plasmid transfer which encode proteins for Mating Pair Formation (MPF) that function as a specialized secretion system pore, as well as DNA transfer replication proteins that process plasmid DNA [32]. In Gram negative bacteria, this system assembles a conjugative pilus, a filamentous surface appendage that mediated interactions with recipient cells. In the donor cell there is the nucleoprotein complex involved to accept the plasmid. Conjugative plasmids carry also genes encoding "entry exclusion proteins" that prevent the host from acting as a recipient cell for the same or related plasmid [33]. On the other hand, nonconjugative plasmids exploit the MPF system provided by a conjugative plasmid, to be transferred horizontally.

Plasmid classification is commonly relied on the phenomenon of incompatibility, based on the observation that closely related plasmids cannot coexist stably in the same cell. This is due to cross talk between the replication system of the two plasmids that "confuse" copy number control and the

two different plasmids are perceived as the same. Incompatibily plasmids are classified in the same Inc group (PlasmidFinder is one of the most important classification systems for plasmids from *Enterobacterales* and is a usefull strarting point for identifying plasmid types in WGS) [34]. It is also essential the analysis of Plasmid Multilocus Sequence Typing (pMLST) for plasmids belonging to the same Inc groups, that assigns allele numers and sequence types (pST). Often, this kind of characterization has been useful in identification of relationships between plasmids for epidemiological purposes, including information about the geographic distribution of plasmids, the source of the outbreak and the associations with resistance genes. Although plasmids are now often assigned to a group on the bases of sequence homology rather than information about true incompatibility, (the laborious nature of incompatibility testing is based on hybridization, then PCR-based replication typing abd sequencing-based approaches), known resistance plasmids given the same Inc designation do mainly share backbones with similar organizations and functions [35]. The following are the major Inc categories identified in *Enterobacterales*.

A/C plasmids: incompatibilty group IncA/C plasmids, discovered in multidrug-resistant *Aeromonas hydrophila* and *Vibrio* strains, are large, low-copy plasmids with their unique modular structure. The literature suggests an emergence of this plasmid type in food, animal and human enteric bacteria, followed by the rapid acquisition of resistance gene modules, associated with antibiotics used in animal, agriculture and human therapy. Circulation of IncA/C plasmids in Gram negative pathogenesis now common, and these plasmids bring with them the ability to encode resistance to broad array of antimicrobial agents. *bla$_{CMY-2}$* gene is often localized to IncA/C plasmids (Figure 6) [36].

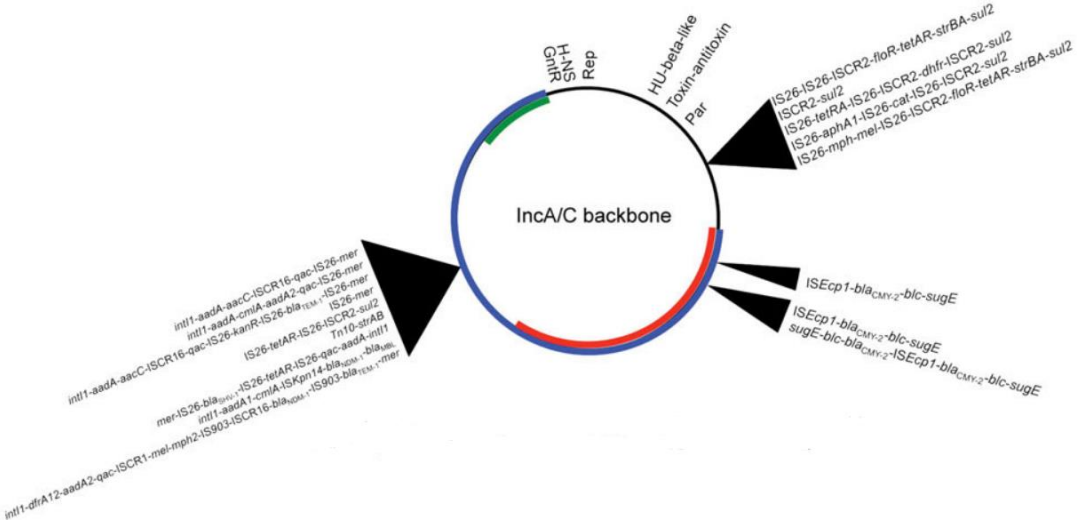

Figure 6 Backbone structure of IncA/C plasmids. Integration hotspots are depicted with black arrows, and inserted modules of sequenced IncA/C plasmids are summarized next to the arrows. The replicon (Rep), transcriptional regulators (HU-b-like, GntR, and H-NS) [36].

F plasmids: the F ("fertility factors") plasmid was the first example of a conjugative plasmid found in bacteria. These plasmids carry genes encoding conjugative factors (such as F factor necessary for bacterial conjugation), virulence factors and resistance genes such as *bla$_{CTX-M-15}$*, *bla$_{CTX-M-27}$*, *bla$_{KPC}$*, *bla$_{NDM}$* and *mcr-1* [37].

HI plasmids: HI plasmids encode serologically related pili similar to the F-pilus. They are larger than other conjugative plasmids and they may encode heavy metal, phage and/or colicin resistance factor

in addition to antibiotic resistance genes (*bla*$_{IMP}$, *bla*$_{CTX-M}$, *bla*$_{NDM}$, *mcr-1* and *mcr-3*). HI plasmids are mostly found in *Salmonella* and *E. coli* but also in *S. enterica* and *S. marcescens* [38].

I-complex plasmids: plasmids classified as Inc Types Iα, Iγ, B/O, K and Z were grouped into I complex due to the similar serologies and morphologies of their pili. I-complex plasmids generate both a thick pilus for DNA transfer and thin pilus that appears to stabilize the mating apparatus in liquid media but not on solid surface. Most examples of I plasmids are from *E. coli* or *Salmonella* and many of them carry resistance genes, commonly *bla*$_{CMY-2}$, some variants of *bla*$_{CTX-M}$ and *mcr-1* [39].

I2 plasmids: this group has many features in common with the I-complex, such as encoding pili and carrying resistance genes [40].

L/M plasmids: these plasmids have a broad host range, even though mostly are from *Enterobacterales*. Most sequenced L palsmids carry *bla*$_{OXA-48}$-like genes, resulting in a high conjugation frequency [55]. Futhermore, M plasmids often carry *ISEcp1*, *bla*$_{CTX-M-3}$ and/or clinically important genes, including *armA, bla*$_{NDM}$, *bla*$_{IMP-4,}$ *bla*$_{KPC}$, *bla*$_{SHV}$ (ESBL) *or bla*$_{FOX}$ *(ampC)* [41].

N plasmids; N plasmids are relatively small conjugative plasmids. They confer a variety of antibiotic resistances, and especially carbapenem and third generation cephalosporin resistance, to *K. pneumoniae* and *E. coli* worldwide. IncN plasmids were also described to encode the NDM β-lactamase and other genes including blaKPC, blaIMP and blaCTX-M. IncN is one of the most frequent plasmid types in *Enterobacterales*, becoming particularly widespread in the clinical setting [42].

P/P1 plasmids: although these plasmids do not generally confer resistance to the most clinically important antibiotics, it is recently discovered that some plasmid from this clade, often carry genes coding for some antibiotics, heavy metals and quaternary ammonium, compounds used as disinfectants. In particular, IncP plasmids are widely distributed in Gram negative bacteria, mainly *E. coli, Pseudomonas spp., K. pneumoniae* and *Sphingomonas* [43].

R plasmids: IncR plasmids carry various resistance genes such as *bla*$_{KPC-2}$, *bla*$_{NDM-1}$, *bla*$_{VIM-1}$, *qnrS1 or armA* in clinical *Enterobacterales*, especially in *K. pneumoniae* strains over the world. The pool of resistance genes carried by IncR replicon plasmids may spread through transposition or plasmid recombination events, contributing to the high plasticity of multiple-replicon plasmids. These often coexist with other replicons such as IncC, IncN, IncHI and IncFII, and, for this reason they are frequently involved in the emergence of multidrug resistant strains [44].

T plasmids**:** few T plasmids have been reported so far; they are associated with *bla*$_{CTX-M-2}$ and *bla*$_{NDM-1}$. The gene encoding carbapenemase OXA-181 (an OXA-48 variant) was identified in *Citrobacter freundii* isolate co-producing NDM-1, located in a T-type plasmid [45].

U and G/P-6 plasmids: the *E. coli* IncG and IncU plasmids, and *P. aeruginosa* IncP-6 plasmids, have recently sequenced and has been revealed that their replication proteins are related. Particularly, when cloned at high copy-number, introns from IncG replicon cause a strong incompatibility with IncU plasmids. This suggests that a single incompatibility group exists. *bla*$_{KPC}$ has been reported to be present in P-6 plasmids of *P. aeruginosa* [46].

W plasmids: W plasmids are the smallest conjugative plasmids found in *Enterobacterales*. There are reports of detection of W plasmids carrying *bla*$_{IMP-1}$, *bla*$_{VIM1/4}$ and *bla*$_{KPC}$ [47].

X plasmids: IncX plasmids carry various antibiotic resistance genes, e.g. *PMQR* genes (*qnrS1*, *oqxAB*), ESBL genes (bla_{TEM-52}, $bla_{CTX-M-15}$, bla_{SHV-12}), carbapenemase genes (bla_{NDM-1}, bla_{KPC2-5}) and others. All these findings highlight the great diversity of the IncX family and the potent role of these plasmids in dissemination of antibiotic resistance genes among *Enterobacterales* [48].

Y plasmids: the Y group of plasmids correspond to the prophage form of P1 phage, which infects *E. coli* and other *Enterobacterales*. These kinds are normally identified from *Enterobacterales* such as *E. coli, salmonella* and *K. pneumoniae* and ordinarily exists stably as a low-copy number plasmid that replicate independently rather than integrating into the host chromosome; they can also transfer between bacterial cells as virus particles. The common antibiotic resistance gene, carried by IncY plasmids, is $bla_{CTX-M-15}$ ESBL. Also mcr-1 gene was found within this class [49].

Q plasmids: plasmids of IncQ-family are distinguished by having a unique strand-displacement mechanism of replication that is capable of functioning in a wide variety of bacterial hosts. In addition, these plasmids are small and highly mobilizable and therefore very promiscuous. Q-plasmids carry mainly genes conferring resistance to "older" antibiotics, but someone encodes also bla_{GES-5}, bla_{GES-1} (ESBLs) and *qnrS2* (conferring a low-level quinolone resistance [50].

ColE1 and related plasmids: ColE1 is a small plasmid that encodes colicin E1 and colicin immunity factor. Also, *qnrB12* and bla_{TEM} have been detected in this clade. Plasmid related to ColE1 that carries a colicin gene, appear to be common in *K. pneumoniae* isolates, including ST258, which has been endemic in Italy for many years [51].

Project's aim

The increase of bacteria harboring antimicrobial resistance genes, and in particular multidrug-resistant bacteria, is a global problem in veterinary and public health. Thus, identification and characterization of microorganisms that cause infections are crucial for successful treatment and safety of patients. Moreover, not every species can be successfully cultured and analyzed by traditional microbiology methods but, in the same time, unrecognized pathogens can easily cause hospital outbreaks and aggravate the medical conditions of the patient. Molecular methods are frequently used as an effective strategy to aid in epidemiological investigation following an outbreak; molecular characterization of antimicrobial resistance determinants is also used for local, national or even global surveillance of antimicrobial resistance. Currently, systems like European Antimicrobial Resistance Surveillance System (EARSS), European Food Safety Authority (EFSA) and the European Center for Disease Control (ECDC) are involved in monitoring of AMR in bacteria from food, animals and humans; most of the data are based on phenotypic characterization of isolates, although genotypic detection of AMR genes is increasingly performed by a molecular point of view [52].

Over the last decade, Whole Genome Sequencing (WGS) has been identified as one of the most promising and revolutionary technique in clinical microbiology, both in research and diagnostics. NGS allows sequencing of the whole genome of numerous pathogens in one sequence run, either from bacterial isolates of patients, or from multiple species present in material from an individual patient. A great advantage of NGS is that a single protocol can be used for all pathogens for both identification, characterization and typing; this enables to reduce time and costs. At the moment, a number of NGS platforms are available. However, the different NGS platforms use different sequencing technologies; today, the most common platforms for high-throughput sequencing of bacterial genomes are Illumina (used in our project) and Ion Torrent [53].

WGS analysis provides all relevant informations relating input genome: genotype;serotype; Multi-Locus Sequence Typing profile (MLST); genome size; presence, classification and subtyping of Mobile Genetic Elements; virulence and antibiotic resistance profile; Single Nucleotide Polymorphisms (SNP); phylogenetic background. These informations are useful to obtain the overall molecular features with a high discrimination among closely related strains.

The advantages of using whole genome sequencing - based typing, is promoting the implementation of NGS for epidemiological studies and public health investigations. It is especially important and helpful in outbreak detection and monitoring the evolution and dynamics of multi-drug resistant pathogens . Thus, for a rapid and improved molecular epidemiological surveillance of pathogens at regional and national scale, the role of WGS is undeniable. NGS is also helpful in detection of novel resistance genes in bacteria, both in current as well as in historical strain collections. Novel variants of antibiotic resistance genes (ARG) can be identified using NGS, and further experiments can be performed to determine if these genes are indeed responsible for the observed antibiotic resistance pattern [54].

The main objective of this thesis was to identify and characterize antibiotic resistance genes and virulence factors in clinical isolates resistant to different classes of antibiotics. Among all determinants of the resistome, a new variant, the β-lactamase KPC-53, was isolated and characterized from a biochemical point of view.

In details, the aim of the present project focuses on two key points:

1. Identification and molecular characterization of resistome and viruloma in Gram-negative pathogenic bacteria using Next-Generation Sequencing technologies. 68 strains of *Klebsiella pneumoiniae* and 43 strains of *Escherichia coli* were isolated from subjects residing in long term care facilities (LCTFs) located in the Veneto region. 8 strains of *Acinetobacter baumannii* were, instead, isolated from patients affected by COVID-19 hospitalized at the Spirito Santo hospital in Pescara. Molecular characterization of clinical isolates was performed by genome analysis using the MiSeq platform (Illumina), a massive sequencing system [55,56,57].

2. Characterization of carbapenemase KPC-53, a natural variant of KPC which has a L167E168 duplication in the **Ω**-loop structure, was carried out. The kinetic profile and the molecular modeling of KPC-53 was performed to study the structure/function relationships of this natural KPC variant.

Chapter 2: Identification and molecular characterization of resistome and virulome of Carbapenem-Resistant *Klebsiella pneumoniae*

2.1 Introduction

One of the most important crises is the worldwide spread of the carbapenem-resistant *K. pneumoniae* (*CR-KPN*) during the last decade. Indeed, several studies have reported that carbapenem-resistant hypervirulent *K. pneumoniae* isolates have emerged in the health care setting causing severe hospital-related infections, such as bloodstream infections, urinary tract infections, surgical-site infections and pneumoniae.

The increasing prevalence of antimicrobial drug resistance is an overstated problem, especially long term care facilities by increasing vast amounts of resistance mechanisms, leading to high mortality and morbidity rates [58]. The data from the European antimicrobial resistance Sourveillance Network displayed that carbapenems-producing Enterobacterales are responsible for the majority of human infections [59].

K. pneumoniae is an opportunistic, Gram-negative pathogen that is ubiquitously found on the surface of mucosa in animals or in the environment. In the era of pre-antibiotics, *K. pneumoniae* was a vital pathogen of community-acquired pneumonia, especially in diabetics and alcoholics. In the era of antibiotics that followed, it became a major cause of medical-related infections in hospitals, and a risk factor of severe community-acquired infections. Resistance can develop in *K. pneumoniae* isolates notably producing β-lactamases, including extended spectrum β-lactamases (ESBLs).

Carbapenem-resistant *K. pneumoniae* (CRKP) deactivates the carbapenems through two main mechanisms: (1) acquisition of carbapenemase genes that encode for enzymes capable of hydrolyzing carbapenemsm (the three most important carbapenemase types are KPC-type enzymes, metallo-β-lactamases such as VIM, IMP, NDM, and OXA-48 type enzymes); (2) reduction in the accumulation of antibiotics by a quantitative and/or qualitative deficiency of porin expression in combination with overexpression of β-lactamases that possess weak affinity for carbapenems [60]. Furthermore, the genome of *K. pneumoniae* continues to receive antibiotic-resistance genes by acquiring plasmids and transferable genetic elements, resulting in the emergence of multidrug-resistant (MDR) and extremely-drug-resistant (XDR) strains [61].This is the mainly reason for the rapid worldwide dissemination of *K. pneumoniae*.

In this part of the thesis will be shown the dissemination of antibiotic resistance genes in *K. pneumoniae* isolated from rectal swabs of residents in the Long Term Care Facilities of the Veneto region.

2.2 Materials and methods

2.2.1 Setting and strains selection of Carbapenem -resistant *K.pneumoniae*

Between July 2018 and June 2019, we conducted a point-prevalence survey among the residents of 27 LTCFs in the Veneto Region, Northern Italy, on basis a voluntary participation. The study-specific data were collected on a single day for each LTCF in volved even if in the LTCFs with a high number of beds, data collection was spread over two or more consecutive days. The 27 facilities were not involved simultaneously but at different times based on the local Ethic Committee's approval, the

willingness of local personnel to collaborate with researchers in the collection of the study-specific biological samples and the possibility of the reference microbiology laboratory to accept and process them. However, all the beds in one ward were surveyed on the same day. Only subjects housed in the facility for at least 48 h were asked for consent to participate. A total of 118 variables, such as hospitalization and surgery during the previous year, antibiotics within the last three months, and the presence of medical devices (i.e., urinary catheter; peripheral vascular catheter; central venous catheter; nasogastric tube; and percutaneous endoscopic gastrostomy) were collected from each enrolled participant. Additionally, the enteric carriage of ESBL and carbapenemase-producing Gram-negative bacteria was assessed collecting a rectal swab from every enrolled resident. For privacy, each facility has been named by his acronym.

2.2.2 Antimicrobial Susceptibility of *K. pneumoniae*

The rectal swabs were collected and inoculated onto ChromID ESBL agar (bi- oMerieux, Marcy l'Etoile, France) with an Ertapenem disk (10 µg) and on Mac Conkey agar with a Meropenem disk (10 µg). The plates were incubated at 35 ± 2 °C under aerobic conditions for 24 h. The isolates were identified at the species level using an automated Vitek2 System (bioMerieux, Marcy l'Etoile, France). Resistance to carbapenems were interpreted according to the EUCAST criteria and confirmed with an immunochromato- graphic lateral flow assay Carba5 (NG Biotech, Guipry, France). A Vitek2 system (version 9.02, bioMérieux, Marcy l′Etoile, France) was used to confirm carbapenem resistance and to perform antimicrobial susceptibilities for other substances.

2.2.3 DNA Extraction and Whole-Genome Sequencing

Total genomic DNA was extracted from 1 mL of an overnight bacterial culture using a MagMAX Microbiome Ultra Nucleic Acid Isolation kit (Applied Biosystems and ThermoFisher Scientific, Monza, Italy) according to the manufacturer's instructions. The DNA concentrations were measured using a Qubit fluorometer (ThermoFisher Scientific) to determine DNA input. The genomic libraries were prepared using a Swift 2S Turbo DNA Library kit (Swift Biosciences, Ann Arbor, MI, USA) and the WGS was performed on an Illumina MiSeq platform using v3 reagent kits generating 2 × 300 bp paired-end reads (Illumina, San Diego, CA, USA).

2.2.4 Bioinformatic analysis

Quality control and sequences filtering were checked using DRAGEN FastQC + MultiQC tool and assembled with Velvet. In detail, FastQC tool v.0.11.6, BaseSpaceLabs, Illumina, San Diego, CA, USA and Velvet v.1.2.10, BaseSpaceLabs, Illumina, San Diego, CA, USA, for *K. pneumoniae* isolates [62];

Velvet is incorporated as an assembler in a multiple-tool workflow, the CGE Bacterial Analysis Pipeline (BAP) (BaseSpaceApps, Illumina, San Diego, CA, USA). The BAP application predicts the species of bacterial input genomes using a *k-mer*-based approach [63].

Acquired antimicrobial resistance genes and chromosomal point mutations were identified using ResFinder 4.1 (https://cge.cbs.dtu.dk/services/ ResFinder/, access date: 13 September 2021), a BLAST-based approach, where the nucleotide sequence of the input genome was compared to the genes in the ResFinder database [64].

Multilocus sequence typing (MLST) was performed also using a BLAST-based approach [65]. By the allele numeber of seven loci *gapA, infB, mdh, pgi, phoE, rpoB* and *tonB*, it was possible to determinate the Sequence Type (ST) of each *K.pneumonia.*

PlasmidFinder 2.1 and MobileElementFinder 1.0.3 were used to detect the incompatibility groups of plasmids. Identified plasmids of the IncF, IncH1, IncH2, IncI1, IncN or IncA/C type were subtyped by pMLST (plasmid Multilocus Sequence Typing) 2.0 (https://cge.cbs.dtu.dk/services/pMLST/) [66].

KmerFinder, ResFinder, MobileElementFinder 1.0.3, and PlasmidFinder databases were synchronized with databases from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org.).

2.3 Results

2.3.1 Antimicrobial susceptibility

1933 rectal swabs of 2890 residents were performed and, of 159 *K. pneumoniae* (*KPN),* isolated on a selective medium, only 68 of them were selected for their carbapenem resistance profile. A Vitek2 system used to confirm carbapenem resistance and perform antimicrobial susceptibilities for other molecules. All strains were resistant to at least two different class of antibiotics. Indeed, they exhibited a high resistance profile to β-lactams/β-lactamase inhibitors (amoxicillin-clavulanic acid and piperacillin-tazobactam), oxyiminocephalosporins (cefotaxime and ceftazidime), carbapenems (meropenem and ertapenem), and ciprofloxacin. Of 68 *KPN*, 36 (52.9%) were resistant to trimethoprim- sulfamethoxazole, respectively. Finally, all strains, analyzed were susceptible to colistin (Table 1)

LTCFs	No. Isolates	ST	Resistance Profile
CDS_RO	15	307, 2623	AMX,TZP,CTX,CAZ,ERT,MEM,CIP
POCS_VR	2	16	AMX,TZP,CTX,CAZ,ERT,MEM,CIP,SXT
	2	35	AMX,TZP,CTX,CAZ,CIP,MEM,SXT
	3	512	AMX,TZP,CTX,CAZ,ERT,MEM,CIP
POVSG_VR	2	512	AMX,TZP,CTX,CAZ,ERT,MEM,CIP
IPABMC_VI	2	253, 1519	AMX,TZP,CTX,CAZ,ERT,MEM,CIP,SXT
CAR_VI	2	512, 1519	AMX,TZP,CTX,CAZ,ERT,MEM,CIP,SXT
IPABRS_VI	11	37	AMX,TZP,CTX,CAZ,ERT,MEM,CIP,SXT
IPABRT_VI	5	512	AMX,TZP,CTX,CAZ,ERT,MEM,CIP,SXT
IPABSC_VI	3	416	AMX,TZP,CTX,CAZ,MEM,CIP,SXT
SSA_BL	5	321	AMX,TZP,CTX,CAZ,MEM,CIP,SXT
ISRAA_TV	7	307	AMX,TZP,CTX,CAZ,ERT,MEM,CIP,SXT
CRMC_VE	5	11, 273	AMX,TZP,CTX,CAZ,ERT,MEM,CIP,SXT
SAF_VE	4	307	AMX,TZP,CTX,CAZ,ERT,MEM,CIP,SXT

Table 1 Antimicrobial susceptibility of 68 *K. pneumoniae* isolated from rectal swabs. AMX, Amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CTX, cefotaxime; CAZ, ceftazidime; ERT, ertapenem; MEM, meropenem; CIP, ciprofloxacin; SXT, trimethoprim-sulfa- methoxazole. ST, sub type. For legal aspects, we used only the acronym of the twelve LCTFs.

2.3.2 Multilocus sequece typing

The MLST analysis, obtained from data by Whole Genome Sequencing, showed the presence of thirteen *KPN* lineages: ST11, ST16, ST35, ST37, ST253, ST273, ST307, ST321, ST416, ST512, ST1519, ST2623, and ST3227. The most widespread STs were ST307 (17 isolates), ST512 (11 isolates), and ST37 (11 isolates). The ST307, ST512, and ST37 were identified in four, three, and one LTCFs, respectively. The remaining STs were endemic of only one LTCF.

How data showed the most widespread clones were represented by ST307 and ST512 (in ISRAA_TV LCTF, this clone was the unique lineage found). *K. pneumoniae* 307 is responsible of a plethora of nosocomial infections and long term care center outbreaks [67]. Whereas, *K. pneumoniae* 512 has been endemic in Italy for many years (also ST258) and it is responsible of various and severe bloodstream infections. Nevertheless, other STs emerged in the last decade (Figure 7, Table 2).

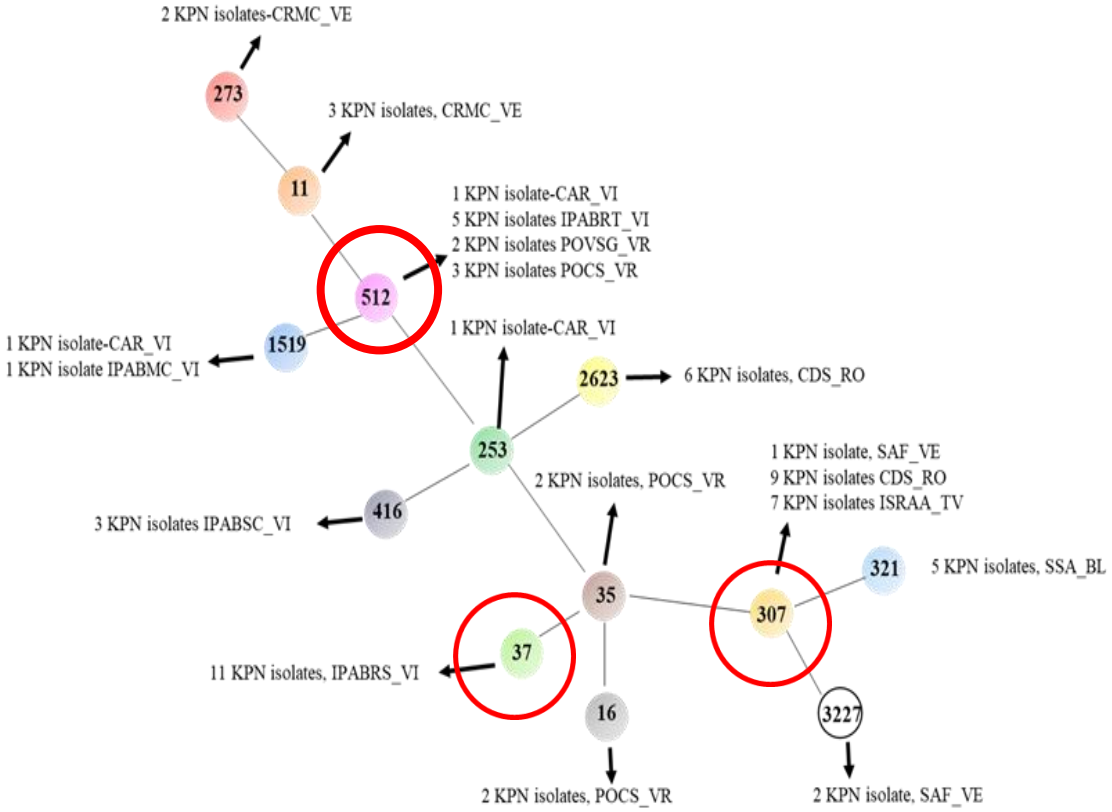

Figure 7 Phylogenetic tree of *K. pneumoniae* strains selected for MLST. The minimum spanning tree was obtained using PHYLOViZ online software (http://www.phyloviz.net/). For legal aspects, we used only the acronym of the twelve LTCFs.

LTCFs	No. Isolates	MLST	Genome SizE (bp)
SSA_BL	5	ST321	5.736.085
ISRAA_TV	2	ST307	5,597,503
	2	ST307	5,164,710
	2	ST307	5,578,258
	1	ST307	5,505,674
SAF_VE	1	ST307	5,545,376
	1	ST3227	5,330,546
	1	ST3227	5,545,568
	1	ST307	5,540,988
CRMC_VE	3	ST11	5,531,831
	2	ST273	5,387,384
CDS_RO	3	ST2623	5,424,562
	3	ST2623	5,666,417
	2	ST307	5,607,404
	37	ST307	5,496,232
	2	ST307	5,566,824
	2	ST307	5,634,072
CAR_VI	1	ST512	5,649,046

	1	ST1519	5,487,176
IPABMC_VI	1	ST1519	5,311,196
	1	ST253	5,651,358
IPABRS_VI	2	ST37	5,167,604
	4	ST37	5,451,323
	3	ST37	5,424,901
	2	ST37	5,444,858
IPABRT_VI	5	ST512	5,406,932
IPABSC_VI	1	ST416	5,634,812
	2	ST416	5,477,368
POVSG_VR	1	ST512	5,346,528
	1	ST512	5,101,877
POCS_VR	1	ST512	5,548,552
	2	ST512	5,589,5098
	2	ST16	5,604,773
	2	ST35	5,589,102

Table 2 Genome analysis of *K.pneumoniae* isolated from residents of twelve LTCFs; this table shows the STs found in the different LTCFs and their occurrences and genome sizes respectively.

The genome size carried out from whole genome sequencing of 68 *K. pneumoniae* analyzed, ranged from 5.1 to 5.74 Mb (Table 2).

2.3.3 Plasmids

A wide variety of plasmids were found in each whole *K.pneumoniae* genome. Incompatibility plasmids IncFII(K), IncFIB(K), IncFIA(HI1), IncN, IncF, IncFIB (pQil), IncL/M, IncX3, IncX4, Col (MG828), ColpVC, and ColRNAI were detected in all *KPN* analysed.. Overall, IncF was the predominant plasmid found in 100% of the KPN, followed by Col (38 out of 68), IncN (18 out of 68 KPN), IncX (4 out of 68 KPN), IncR (1 out of 68 KPN), and IncL/M (1 out of 68 KPN) type plasmids. Among IncF plasmids, the predominant was IncFII(K) (found in 63 out of 68 KPN), followed by IncFIB(K) (found in 52 out of 68 KPN), and IncFIA (found in 18 out of 68 KPN). IncF plasmid family is widely diffused in clinically relevant *Enterobacterales*, especially IncFII(K), which is considered a virulent plasmid because of its ability to co-exist with other plasmids in a single cell [68]. Most of these plasmids are conjugative and this facilitates the dissemination of resistance genes among different strains and species.

Referring pMLST, the major plasmid lineage was fiik7 (25 KPN isolates), followed by fiik1 (11 isolates), fia19 (9 isolates), fia10 (8 isolates), fiik5 (4 isolates), fiik2 (2 isolates), fiik4 (1 isolate), fiik9 (1 iso- late), and fia18 (1 isolate). In most KPNs, the simultaneous presence of more than one plasmid was found (Figure 8, Table 3).

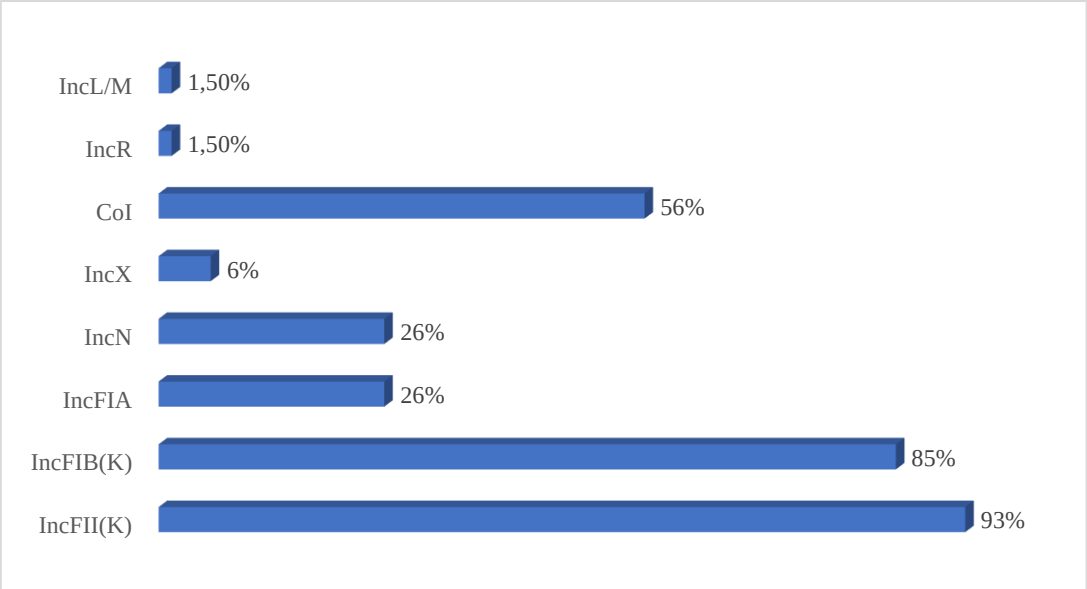

Figure 8 Occurrence of incompatibility plasmid groups in *K. pneumoniae*

Plasmid lineage	No isolates
fiik7	25
fiik1	11
fia19	9
fia10	8
fiik5	4
fiik2	2
fiik4	1
fiik9	1
fia18	1

Table 3 Plasmid Multilocus sequence typing (pMLST) of plasmids found in *K. pneumoniae* isolates

2.3.4 Antibiotic resistance genes

β-lactams resistance genes

The β-lactam resistance genes were found in all *K. pneumoniae* isolates with a moderate variability among the LTCFs. Class A,B and D β- lactamases were identified: the prototype genes $bla_{TEM\text{-}1A}$/$bla_{TEM\text{-}1B}$ were found in 65% of *KPN* isolates, whereas the bla_{SHV} variants ($bla_{SHV\text{-}1}$, $bla_{SHV\text{-}11}$, $bla_{SHV\text{-}14}$, $bla_{SHV\text{-}28}$, $bla_{SHV\text{-}33}$, $bla_{SHV\text{-}36}$, and $bla_{SHV\text{-}99}$) were found in 76% of *KPN* strains (52 out of 68) isolated in the twelve LTCFs with a prevalence of $bla_{SHV\text{-}11}$ (31% of isolates), followed by $bla_{SHV\text{-}28}$ (25% of isolates). The major carbapenemases were represented by KPC variants: $bla_{KPC\text{-}2}$ and $bla_{KPC\text{-}3}$ were found in 47 out of 68 isolates (69%) collected in 9 to 12 LTCFs; $bla_{KPC\text{-}2}$ was retrieved

in 17 isolates belonging to ST37 and ST307, whereas *bla*$_{KPC-3}$ was found in 30 *KPNs* belonging to seven different ST lineages. KPC-2 and KPC-3 differ from each other by a single amino acid at position 274, were a Tyr replaces an His. KPC-9 was found in 3% of isolates (two *K. pneumoniae*); in details in ST512 and ST3227 lineages carried this *KPN* variant, collected from two different LTCFs. KPC-9 is a variant of KPC-3, differing by an Ala (in KPN-9) which replaces a Val in KPC-3 (Figure 9).

The OXA variants were found in 93% of KPNs (63 out of 68 isolates). In detail, OXA-1, OXA-9, and OXA-1/OXA-9 associations were found in 20, 13, and 20 KPN isolates, respectively. *bla*$_{OXA-23}$ was identified in two ST512 isolated from two residents of the same LTCF. The *bla*$_{LEN-7}$ and *bla*$_{LEN-12}$ genes were found in 11 *KPN* isolates (ST512 and ST3227 lineages) in four of the LTCFs. CTX-M-15 is the most common ESBL in *K. pneumoniae*, in fact it was found in 35 out of 68 *KPNs*, belonging to eight different lineages (11,16,37,273,307,512,2623, and 3227).

The MBLs, VIM-1 and NDM-1, were detected in nine and three *KPN* isolates, respectively. The three NDM- 1-producing KPNs were identified only in one LTCF. In the present study, *K. pneumoniae* ST512, found in four LTCFs, harbored KPC-3 and KPC-9, CTX-M-15, SHV-11, OXA-9, OXA-23, and LEN-12 β-lactamases: this carbapenem resistant ST, is considered the predominant lineage causing severe bloodstream infections in a Northern Italian hospital [69]. Finally, the β-lactamases LEN and OKP-B are chromosomally encoded and are frequently found in *K. pneumoniae* as well as the oxacillinases (OXA-1 and OXA-9) (Table 4).

B-lactamases	Classes	Isolates No (%)	ST
TEM-1	A	44 (65)	16,35,37,273,307,512,1519,2623,3227
KPC-2	A	17 (25)	37,307
KPC-3	A	30 (44)	16,35,307,512,1519,2623,3227
KPC-9	A	2 (3)	512,3227
SHV-1	A	5 (7)	16,2623
SHV-11	A	21 (31)	11,37,273,307,512,1519
SHV-14	A	1 (1.5)	416
SHV-28	A	17 (25)	307
SHV-33	A	2 (3)	35
SHV-36	A	1 (1.5)	253
SHV-99	A	5 (7)	321
CTX-M-15	A	35 (51)	11,16,37,273,307,512,2623,3227
LEN-7	A	2 (3)	512,3227
LEN-12	A	9 (13)	512,3227

OKP-B3	A	2 (3)	416
VIM-1	B	9 (13)	253,321,416
NDM-1	B	3 (4.5)	11
OXA-1	D	40 (59)	16,37,273,307,512,2623,3227
OXA-9	D	33 (48)	35,307,512,1519,2623,3227
OXA-23	D	2 (3)	512

Table 4 distribution of β-lactamases among *K. pneumoniae* isolated from residents of 12 LTCFs

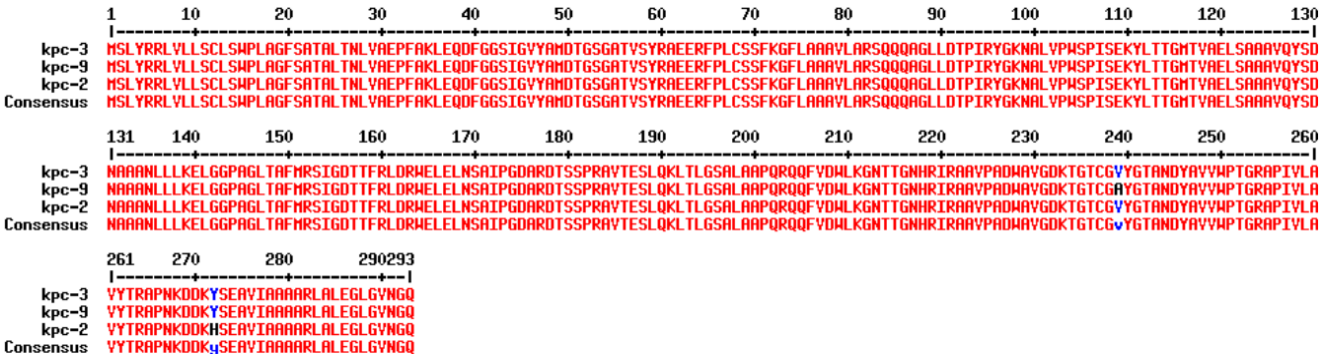

Figure 9 Alignment of KPC variants

Other Antimicrobial resistance genes

Fluoroquinolone Resistance Genes Plasmid-mediated resistance to fluoroquinolones was identified in all *K.pneumoniae* isolates. Different plasmid-mediated mechanisms implicated in quinolone resistance were detected:

i) *aac(6')Ib-cr found* in 100% of *KPNs* (aminoglycoside acetyltransferase is a bifunctional enzyme because it modifies aminoglycosides conferring resistance to these antibiotics but, simultaneously, it inhibits the activity of fluoroquinolones by modifying their targets such as DNA-gyrase and topoisomerase IV.

ii) *qnr* elements found in 58% of KPNs. The major *qnr* found were *qnrB66* (in 26 out of 68 *KPNs*), followed by *qnrS1* (9 out of 68 *KPNs)*, *qnrB6* (one isolate), and *qnrB19* (one isolate).

iii) *oqxAB* multidrug efflux pump that is detected in 53 *KPN* isolates disseminated in ten LTCFs.

Resistance to aminoglycosides was mediated by *aadA1, aadA2, aph(3')-XV, aacA4*, *aph(3')-Ia,* and *aph(3')-IIa*, and by the bi- functional gene *aac(6')Ib-cr*.

Other antimicrobial resistance genes were detected in *K. pneumoniae*. Concerning sulfamethoxazole/trimethoprim resistance, the most common mechanism is the acquisition of dihydrofolate reductase *dfr* gene. Indeed, 54 out of 68 strains that were analyzed showed the presence of *dfrA12, dfrA14, and dfrA30*. In detail, 4 *KPNs* showed *dfrA12*, 39 *KPNs* showed *dfrA14*, and 11 *KPNs* presented both *dfrA14* and *dfrA30*.

The *mphA* gene, involved in macrolide resistance, was detected in 8 *KPNs*; *sul1* and *sul2* engaged in sulfonamide resistance and detected in 22 and 20 *KPNs*, respectively; *catA1*, *catB2,* and *catB4* for chloramphenicol resistance, detected in 50 *KPNs*; *strA* and *strB* involved in streptomycin resistance

and detected in 23 *KPNs*; *tet(A)*, *tet(B)*, and *tet(D)* for tetracycline resistance, detected in 13 *KPNs*; and, finally, *fosA* gene that confer fosfomycin resistance, was detected in 61 KPNs (Figure 10).

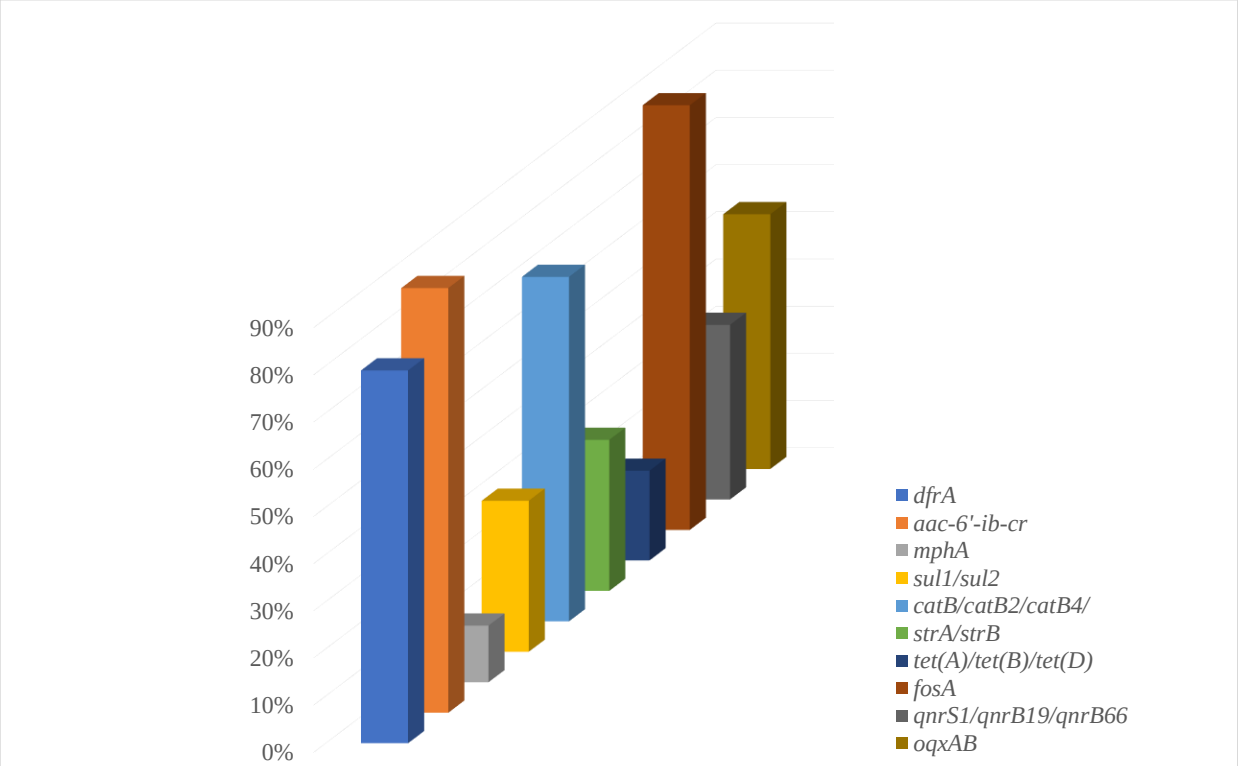

Figure 10 Antimicrobial resistance genes in *K. pneumoniae*

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2.4 Discussion

In Italy, an epidemic spread of *K. pneumoniae* ST258, as a major contributor of carbapenem-resistant *Enterobacterales*, has been observed since 2010 [70] and it is associated to severe community-acquired infections. The present study investigated the genome of carbapenem-resistant *KPN* in twelve LTCFs in a Northern Italy region. Among the 68 carbapenem-resistant *KPNs*, the most widespread clones were represented by ST307 and ST512. In ISRAA_TV LTCF, the ST307 was the unique lineage found, and it harbours the same plasmids but different resistance genes. *K. pneumoniae* ST307 has been reported from many countries, and it has been responsible for several global nosocomial [71] and long-term care centre outbreaks [67].

Whole-genome sequencing performed by Wyres et al. on 95 *K. pneumoniae* ST307 revealed the presence of FIB-like plasmids harbouring the $bla_{CTX\text{-}M\text{-}15}$ gene adjacent to the ISECp1 element such as the other ST307 isolated in different geographical areas [71]. The ST307 harbouring $bla_{CTX\text{-}M\text{-}15}$ in association with *aac(6')-Ib-cr* and *qnrB6* genes, as well as in our strains, was also described in an Italian regional survey (Sicily, Southern Italy) [72]. In the present study, *K. pneumoniae* ST512, found in four LTCFs, harboured KPC-3 and KPC-9, CTX-M-15, SHV-11, OXA-9, OXA-23, and LEN-12 β-lactamases. The carbapenem-resistant *K. pneumoniae* ST512 has been considered the predominant lineage in isolates, causing severe bloodstream infections in a Northern Italian hospital [69]. For a long time, Italy has been an endemic country for *K. pneumoniae* ST258/ST512 lineages [73,74], but recently, other STs emerged. The *K. pneumoniae* ST37 was the third most spread lineage (11 isolates) but it was detected only in one LTCF (IPABRS_VI). The ST37 has been described in

several papers as an ertapenem-resistant *K. pneumoniae* with a modified outer membrane permeability [75]. A wide variety of plasmids were found in each *KPN* genome.

The IncF plasmids were predominant in the *KPN* analysed in this study. This plasmid family is widely diffused in clinically relevant Enterobacterales, especially IncFII(K), which is considered a virulent plasmid because of its ability to co-exist with other plasmids in a single cell [76]. Most of these plasmids are conjugative and this facilitates the dissemination of resistance genes among different strains and species. This is the case of IncFIA(pBK30683 plasmid found in ST512 (POCS_VR LTCF), co-harbouring bla_{OXA-23}, bla_{OXA-1}, $bla_{CTX-M-15}$, and bla_{LEN-12}. The OXA-23 class D carbapenemase is normally produced by *A. baumannii*, and it is very rare in *K. pneumoniae* [77]. However, pBK30683 is a plasmid of 139,941 bp that seems to originate from the cointegration of pBK30661 (belonging to IncFIA family plasmids) with a 68 Kb genetic element, harbouring a complete set of genes for plasmid replication, stability, and conjugation [78]. This could explain the "jump" of bla_{OXA-23} from one species to another. Class A, B, and D β-lactamases were identified in all the KPN strains. The major carbapenemases were the KPC variants (KPC-2, KPC-3, and KPC-9), VIM-1, NDM-1 metallo- β-lactamases, and OXA-23. KPC-9 is a KPC-3 variant with a V239A substitution. CTX-M- 15 is the most common ESBL in *K. pneumoniae*. In our strains, CTX-M-15 was identified in eight different ST lineages. The $bla_{CTX-M-15}$ gene is often flanked by a sequence insertion (IS) such as ISEcp1, which facilitates its mobility.

Chapter 3: Resistome and virulome of Multi-Drug Resistant Escherichia coli ST131 isolated from residents of Long-Term Care Facilities in the Northern Italian Region

3.1 Introduction

The number of infection due to multidrug-resistant *E. coli* has increased dramatically in the last 20 years. The emergence of community-acquired infections due to extended-spectrum β-lactamases began in the late 1990s and has since spread worldwide [79].

The global emergence of antimicrobial-resistant sequence type 131 (ST131) *Escherichia coli* has been well documented across the entire spectrum of health-care setting. It has widespread as a global endemic and multidrug-resistant clone causing extra-intestinal infections, including bacteriemia, pneumoniae, intrabdominal infections, meningitis, epididymo-orchitis, prostatitis, musculo-skeletal infections, wound infections and most commonly urinary tract infections. [80]

Humans are likely the primary reservoir of ST131 but its prevalence among human clinical isolates varies by geographic region and host population, ranging from 12.5% to nearly 40%. Furthermore, ST131 and, more specifically, its H30 subclone (so named for containing allele 30 of *fimH*, the type-1 fimbriae adhesin gene) is now highly prevalent among fluoroquinolone-resistant and CTX-M ESBL-producing *E. coli* isolates worldwide [81] . In fact, fluoroquinolones and trimethoprim-sulfamethoxazole are no longer adequate options for empiric therapy when *E. coli* ST131 is the primary suspect of an infection. Extended-spectrum cephalosporins, piperacillin-tazobactam and carbapenems are options to treat serious non-ESBL-producing *E. coli* ST131 infections, while carbapenems are indicated for ESBL-producing infections. Moreover there is a growing interest in reevaluating oral agents including fosfomycin and pivmecillinam for less serious infections such as uncomplicated cystitis [82].
It is not so clear what has enabled ST131 to disseminate so widely and rapidly, but it is apparently more extensively antimicrobial-resistant then other *E.coli* and contains an abundance of virulence factors that facilitate its dissemination [83].
Emerging evidence from the United States and Europe suggests that long-term care facilities (LTCFs) may be one of the biggest reservoir of ST131 and the principal risk factor of "sharing" of genetic material and then, receipt of new antimicrobial agents[84].
In this part of the study we characterized, from a molecular point of view, 43 *E. coli* strains collected from residents of LTCFs in Northern Italy.

3.2 Materials and methods

3.2.1 Setting and strains selection of Multi-Drug resistant *E. coli*

Joined by samples collection of carbapenem-resistant *K. pneumoniae*, in the same timeframe (between July 2018 and June 2019) we conducted a point prevalence of multidrug-resistant *E. coli* in the population of ederly residents of the same 27 LTCFs in Veneto Region. Joining the survey was still voluntary, but at least one facility for each province of the region was enrolled. Even here, the survey was proposed to all patients who were hospitalized for at least 48 h and physically present in the facility at 8:00 a.m. of the survey day. For each enrolled patient, 118 variables were collected, including the type and etiology of concurrent infections and respective antibiotic therapy, any antibiotic treatments in the previous 3 months, hospital admissions and surgery in the previous 12 months and invasive medical devices in situ. For each enrolled host, a rectal swab was performed to

assess the status of colonization by Gram negative MDR (*Enterobacterales* and non-fermenter Gram negatives producing ESBL and/or resistant to carbapenems).

3.2.2 Antimicrobial Susceptibility of *E. coli*

The strain selection was performed by inoculating the rectal swabs onto ChromID ESBL agar (bioMérieux, Marcy l'Etoile, France) and on Mac Conkey agar. Identification of the isolates was carried out in an automated Vitek2 System (bioMérieux, Marcy l'Etoile, France). Antimicrobial susceptibilities were performed on a Vitek2 system (version 9.02, bioMérieux, Marcy l'Etoile, France). The strains that showed resistance to carbapenems were also analyzed with an immunochromatographic lateral flow assay Carba5 (NG Biotech, Guipry, France). Resistance to antibiotics was interpreted according to the EUCAST criteria. The E. coli that showed susceptibility to third-generation cephalosporins and carbapenems were excluded from the study.

3.2.3 Dna Extration and Whole-Genome Sequencing

See paragraph 2.2.3

3.2.4 Bioinformatic analysis

See paragraph 2.2.4

DRAGEN FastQC + MultiQC v3.6.3 (https://basespace.illumina.com/apps/10562553/DRAGEN-FastQC-MultiQC, access date: 9 September 2021) and Velvet v1.2.10 (https://basespace.illumina.com/apps/8556549/Velvet-de-novo-Assembly) were used *E. coli* sequences analysis.

The allele number of the seven housekeeping genes, *adk, fumC, gyrB, icd, mdh, purA* and *recA*, and the Sequence Type of each *E. coli* isolate was determined by combining seven allelic profiles in MLST *E. coli* Atchman database (https://pubmlst.org/bigsdb?db=pubmlst_mlst_sqdef&page=schemeInfo&scheme_id=4).
To individuate virulence genes detected, VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/VirulenceFinder/) and Virulence Factor Database *(VFDB,* http://www.mgc.ac.cn/VFs/ *)* were used.
Morover, Serotypes and fimH types were determined using SerotypeFinder 2.0 and FimTyper 1.0 (http://genomicepidemiology.org/services/) tools, respectively.

3.3 Results

3.3.1 Antimicrobial suscepibility

Mostly of *E. coli* analyzed showed the resistance to amoxicillin/clavulanic acid and piperacillin/tazobactam. Nevertheless, these strains were resistance to ciprofloxacin and trimetoprim and, just one isolate was sensible to carbapenem (Table 5).

LTCFs	**No isolates**	**ST**	**Resistance profile**
IBABRS_VI	1	131	AMX,CTX,CAZ,CIP
	1		CTX,CAZ
	1		AMX,TZP,CTX,CAZ,ERT,SXT
	1	1193	AMX,TZP,CAZ,ERT,CIP,SXT
IPABSC_VI	1	131	AMX,TZP,CTX,CAZ,CIP,SXT
	1		AMX,CTX,CAZ,GMC,CIP,SXT
	1	131	AMX,CTX,CAZ,CIP,SXT

POCS_VA	1	410	AMX,TZP,CTX,CAZ,ERT,MEM,CIP
	1	131	AMX,TZP,CTX,CAZ,ERT,MEM,CIP
SSL_BL	2	69	AMX,TZP,CTX,CAZ,ERT,CIP,SXT
	1		AMX,TZP,CTX,CAZ,ERT,MEM,GMC,CIP,FOF,SXT
	1		AMX,TZP,CTX,CAZ,ERT,MEM,SXT
	1		AMX,TZP,CTX,CIP,SXT
	1		AMX,TZP,CTX,CAZ,SXT
	1		AMX,TZP,CTX,CAZ,CIP,SXT
	1	131	AMX,CTX,GMC,CIP,FOF,NFT,CS
	1	69	AMX,TZP,CTX,CAZ,ERTG,GMC,CIP,SXT
	1	131	AMX,CTX,GMC,CIP
ISRAA_TV	1	131	AMX,TZP,CTX,CAZ,CIP,SXT
CRMC_VE	2	131	CTX,CAZ,CIP
CDS_RO	1		AMX,CTX,CAZ,CIP
	1		AMX,CTX,CAZ,GMC,CIP
	1		AMX,TZP,CTX,CAZ,ERT,MEM,GMC,CIP
	1	131	AMX,CTX,CAZ,GMC,CIP,NFT
IPABMC_VI	2	131	AMX,CTX,CAZ,GMC,CIP,SXT
	1		AMX,TZP,CTX,CAZ,CIP
	1		AMX,TZP,CTX,CAZ,GMC,CIP,SXT
IPABRT_VI	2	131	AMX,CTX,CAZ,CIP
	2	131	CTX,CAZ,CIP,SXT
	1		AMX,TZP,CTX,CAZ,ERT,GMC,CIP
POSC_VR	1		AMX,TZP,CTX,CAZ,CIP,SXT
	1		CAZ
IPABPC_VI	1		AMX,TZP,CTX,CAZ,CIP,SXT
ISRAARZ_TV	1		AMX,CTX,CAZ,SXT
SAF_VE	1		CAZ
POBB_VR	1	131	AMX,CTX,CAZ,GMC,CIP,SXT
POVI_VR	1		CTX,CIP,SXT

Table 5 Antimicrobial suscepibility of *E. coli* isolated from LTCFs. Amoxicillin-clavulanic acid, AMX; piperacillin-tazobactam, TZP; cefotaxime,CTX; ceftazidime, CAZ; ertapenem, ERT; meropenem, MEM; ciprofloxacin, CIP; fosfomycin, FOF; trimethoprim-sulf

3.3.2 MLST and Serotype

The whole-genome size of the 43 *E. coli* ranged from 4.9 to 10 Mb (table 7). On the basis of the Achtman scheme, which considers *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* housekeeping genes, the predominant ST found was ST131 (74% of isolates) followed by ST12 (5% isolates), ST69 (7% of isolates), ST48 (2% isolates), ST95 (2% isolates), ST410 (2% isolates) and ST1193 (7% isolates) (Figure 11). The serotype of each *E. coli* was also determined. Thirty ST131 isolates showed a O25b:H4 serotype with the fimbria variant fimH30 and two O61:H4 serotypes with fimH94. The ST12 belonged to the O4:H5 serotype with fimH5 and fimH240, the ST48 belonged to the O137:H4 with fimH54, the ST69 belonged to O15:H18 with fimH27 and O44-O77:H18 with fimH27, the ST95 belonged to O18:H7 with fimH15, the ST410 belonged to H21 with fimH24 and the ST1193 belonged to O75:H5 with fimH64 (Table 6).

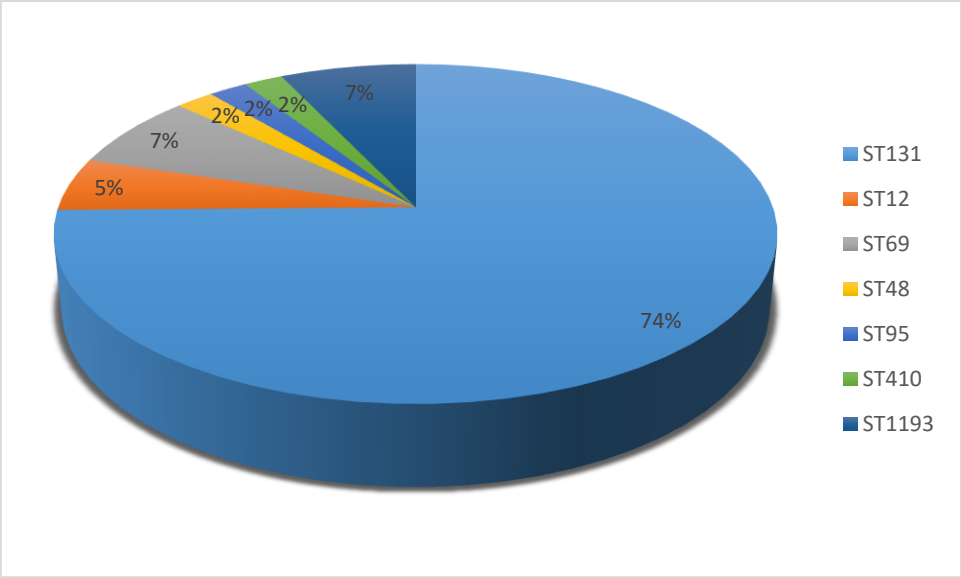

Figure 11 On the basis of the Achtman scheme the predominant ST found was ST131 (74% of isolates) followed by ST12 (5% isolates), ST69 (7% of isolates), ST48 (2% isolates), ST95 (2% isolates), ST410 (2% isolates) and ST1193 (7% isolates).

LTCFs	No.isolates	MLST	Serotype	fimH	Genome size (bp)
SSL_BL	2	ST69	O15:H18	27	5.360.264
	1	ST131	O25b:H4	30	5.064.257
	3	ST131	O25b:H4	30	5.318.811
	1	ST69	O17/O44-	27	5.428.542
	1	ST131	O77:H18	30	5.506.897
	1	ST131	O25b:H4	30	5.102.820
	1	ST95	O25b:H4	15	5.257.199
	1	ST12	O18:H7	204	5.278.074
	1	ST12	O4:H5	5	5.110.437
			O4:H5		
ISRAA_TV	1	ST131	O25b:H4	30	5.117.808
SAF_VE	1	ST48	O137:H4	54	5.003.689
CRMC_VE	1	ST131	O25b:H4	30	5.143.042
	1	ST131	O25b:H4	30	5.126.0556
CDS_RO	2	ST131	O61:H4	94	10.087.318
	1	ST1193	O75:H5	64	5.158.809
	1	ST131	O25b:H4	30	5.151.322
IPABMC_Vi	1	ST131	O25b:H4	30	5.362.937
	2	ST131	O25b:H4	30	5.045.886
	1	ST131	O25b:H4	30	5.277.401
IPABRS_VI	2	ST131	O25b:H4	30	5.238.706
	2	ST1193	O75:H5	64	5.131.308
IPABRT_VI	2	ST131	O25b:H4	30	4.999.937
	2	ST131	O25b:H4	30	5.120.568
	1	ST131	O25b:H4	30	4.865.293
	1	ST131	O25b:H4	30	5.080.670

IPABSC_VI	2	ST131	O25b:H4	30	5.165.353
	1	ST131	O25b:H4	30	5.052.997
POBB_VR	1	ST131	O25b:H4	30	5.254.036
POVI_VR	1	ST131	O25b:H4	30	5.353.306
POSC_VR	2	ST131	O25b:H4	30	3.813.125
POCS_VR	1	ST410	H21	24	4.907.147
	1	ST131	O25b:H4	30	5.230.571

Table 6 Genome analysis of *E. coli* isolated from residents of 13 LTCFs (Northern Italian Region)

3.3.3 Plasmids

The different incompatibility plasmids IncI1, IncFII(29), IncFII(pRSB107), IncFIB, IncFIA, IncN, IncF, IncB/O/K/Z, IncQ1, IncX1, IncX3, IncX4, Col(MG828), Col156, Col8282 and ColRNAI were detected in all *E. coli* analyzed. Each *E. coli* isolate harbored more than one type of plasmid. Overall, IncFII was the predominant plasmid, found in 84% of *E. coli*, followed by Col (Col(MG828), Col156, Col8282) in 76%, IncFIA in 64%, IncFIB in 60%, ColRNAI in 40%, IncN in 28%, IncI1 in 24%, IncX (IncX4, IncX3 and IncX1) in 24% and IncB/O/K/Z in 12% of *E. coli*. (Figure 12) The pMLST showed that IncF was represented by several lineages such as fii_2, fii_24, fii_29, fia_1, fib_1, fib_10, F1:A2:B20, F1:A20:B31, F29:B10, F31:A20:B1, F1:A2, F4:A19, F2:A1, F1:A1:B10 and F46:A6:B47, whereas IncN plasmid was represented by the ST7 lineage. In detail, the pMLST of ST131 isolates revealed the presence of F1:A2:B20 (13 isolates), F1:A20:B31 (3 isolates), F29:B10 (7 isolates), F31:A20:B1 (3 isolates), F1:A2 (1 isolate), F2:A1 (5 isolates) and F1:A1:B10 (3 isolates) (Table 7).

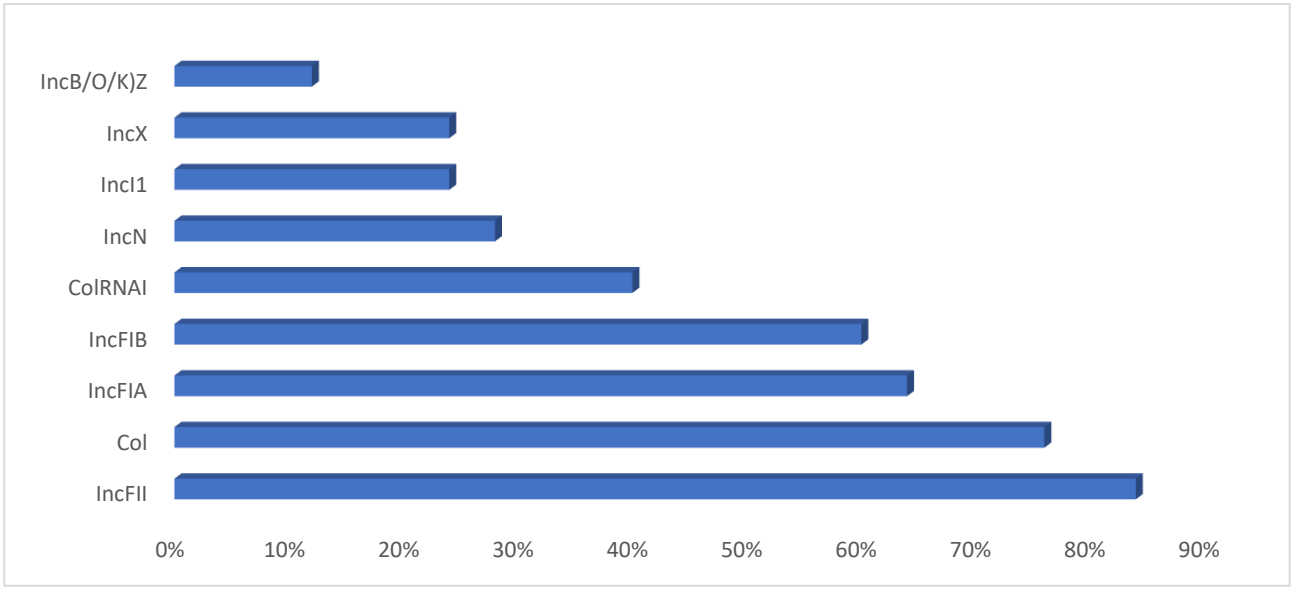

Figure 12 Occurrence of incompatibility plasmid group in *E.coli*

Inc group	pMLST	No isolates
	fii_2	-
	fii_24	1
	fii_29	2
	fia_1	-
	fib_1	1
	fib_10	2
	F1:A2:B20	13
IncF	F1:A20:B31	3
	F29:B10	7
	F31:A20:B1	3
	F1:A2	1
	F4:A19	1
	F2:A1	5
	F1:A1:B10	3
	F46:A6:B47	1
	F36:A20:B1	2
IncN	ST7	5

Table 7 Plasmid Multilocus sequence typing (pMLST) of plasmids found in *E. coli* isolates.

It is noteworthy how many mobile genetic elements have been found both in *K. pneumoniae* and *E. coli* collected from the same facility, demonstrating that plasmids can "jump" from one bacterial species to another carrying with them the entire assortment of resistance and/or virulence genes. These genes, joining those already present naturally in the strain, increase the rate of antimicrobial resistance (Table 8).

LTCFs	Plasmids found at the same time in *Eco* and *Kpn* from the same LTCF
SSL_BL	IncN
CRMC_VE	Col(MG828)
CDS_RO	ColRNAI
IPABMC_VI	ColRNAI
IPABSC_VI	ColRNAI
POCS_VR	ColRNAI, IncX3, FIA(pBK30683), IncFIB(pQil)

Table 8 Shared plasmids between *K. pneumoniae* and *E. coli* collected from the same LTCFs

3.3.4 Antibiotic resistance genes

β-lactam resistance genes

All *E. coli* strains produced one or more β-lactamases belonging to molecular classes A, B, C and D. The ESBLs were the most widespread enzymes, especially CTX-M-type, that, in the last decade, have been increasigly detected in *E. coli* worldwide. They included enzymes of the CTX-M-1 group (CTX-M-1, CTX-M-3 and CTX-M-15) in 21 isolates, CTX-M-27 (belonging to CTX-M-9 group) in 12 isolates and SHV-12 in 3 isolates. The CTX-M enzymes were found only in ST131 strains (94% of isolates). As previously reported, CTX-M-15 and CTX-M-27 alleles are associated with the ST131

clade C2 and ST131 clade C1 [19–21]. CTX-M-27 belongs to CTX-M-9 group, differing from each other by a single amino-acid substitution. It also differs from CTX-M-14 by one amino-acid (Figure 13). Overall, 37 out 43 *E. coli* (87%) were positive to ESBLs.

The VIM-1 enzyme was found in three ST69, one ST12 and one ST95. The *E. coli* ST410 produced the metallo-β-lactamase NDM-4 and CMY-42 class C enzyme. However other metallo- β-lactamases were not found in ST131, and this is in agreement with results reported in different countries [85].

The OXA-1 and OXA-9 were found in 12 and 3 isolates, respectively (47% of isolates), and in association with other β-lactamase genes.

The KPC-3 carbapenemase was identified in three isolates, specifically two ST131 and one ST1193 collected in CDS_RO, IPABRT_VI and PCS_VR LTCFs (Table 9).

β-lactamases	Classes	%	ST (No isolates)
TEM-1	A	35	ST12(1),ST48(1),ST69(1),ST131(9),ST1193(3)
CTX-M-1	A	2	ST12(1)
CTX-M-3	A	7	ST131(3)
CTX-M-15	A	39	ST131(17)
CTX-M-27	A	28	ST131(12)
SHV-12	A	7	ST48(1),ST1193(2)
KPC-3	A	7	ST131(2),ST1193(1)
NDM-4	B	21	ST410(1)
VIM-1	B	9	ST12(1),ST69(3),ST95(1)
CMY-42	C	2	ST410(1)
OXA-1	D	30	ST131(13)
OXA-9	D	7	ST131(2),ST1193(1)

Table 9 Distribution of β-lactamase genes among *E. coli* strains

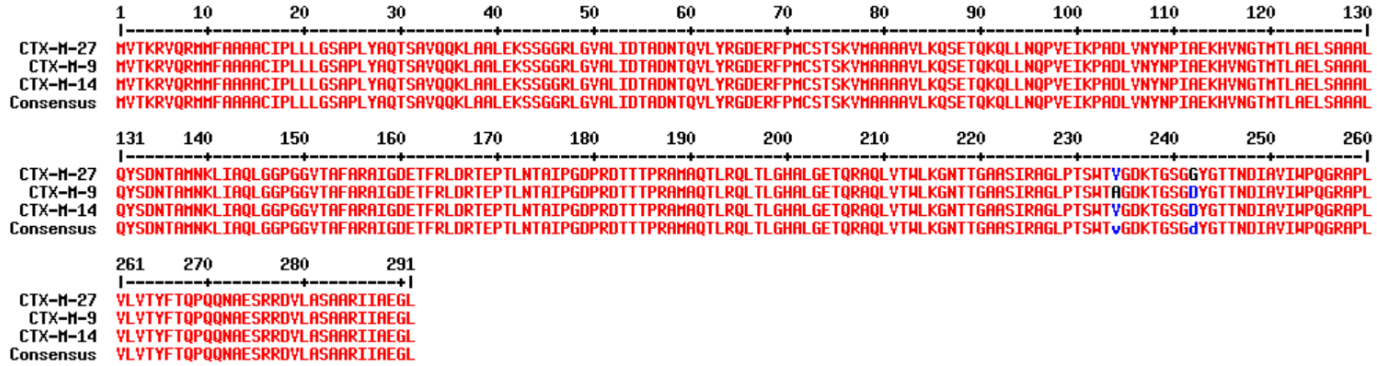

Figure 13 Alignment of CTX-M-variants

Other Antimicrobial resistance genes

The aminoglycoside resistance genes *aadA1, aadA2, aadA5, aacA1, aacA4, aac(3)-IId, aph(30)-XV, aph(30)-Ia* and *aph(30)-IIa* and the bi-functional gene *aac(60)Ib-cr* were found in all *E. coli* analyzed (Table 3). The most common mechanism of sulfamethoxazole/trimethoprim resistance was represented by the acquisition of dihydrofolate reductase *dfrA1*(1 isolate), *dfrA14* (8 isolates) and *dfrA17* (19 isolates). Other antibiotic resistance genes were the following: *mphA* (macrolide resistance) detected in 23 *E. coli*, *sul1/sul2* (suphonamide resistance) detected in 26 isolates, *catB2/catB4* (chloramphenicol resistance) detected in 17 isolates, *strA/strB* (streptomycin resistance) detected in 17 isolates and *tet(A)/tet(B)* (tetracycline resistance) detected in 22 isolates. The lincosamide nucleotidyltransferase gene (*lnu(F))* was found in one ST48 isolate (Figure 14).

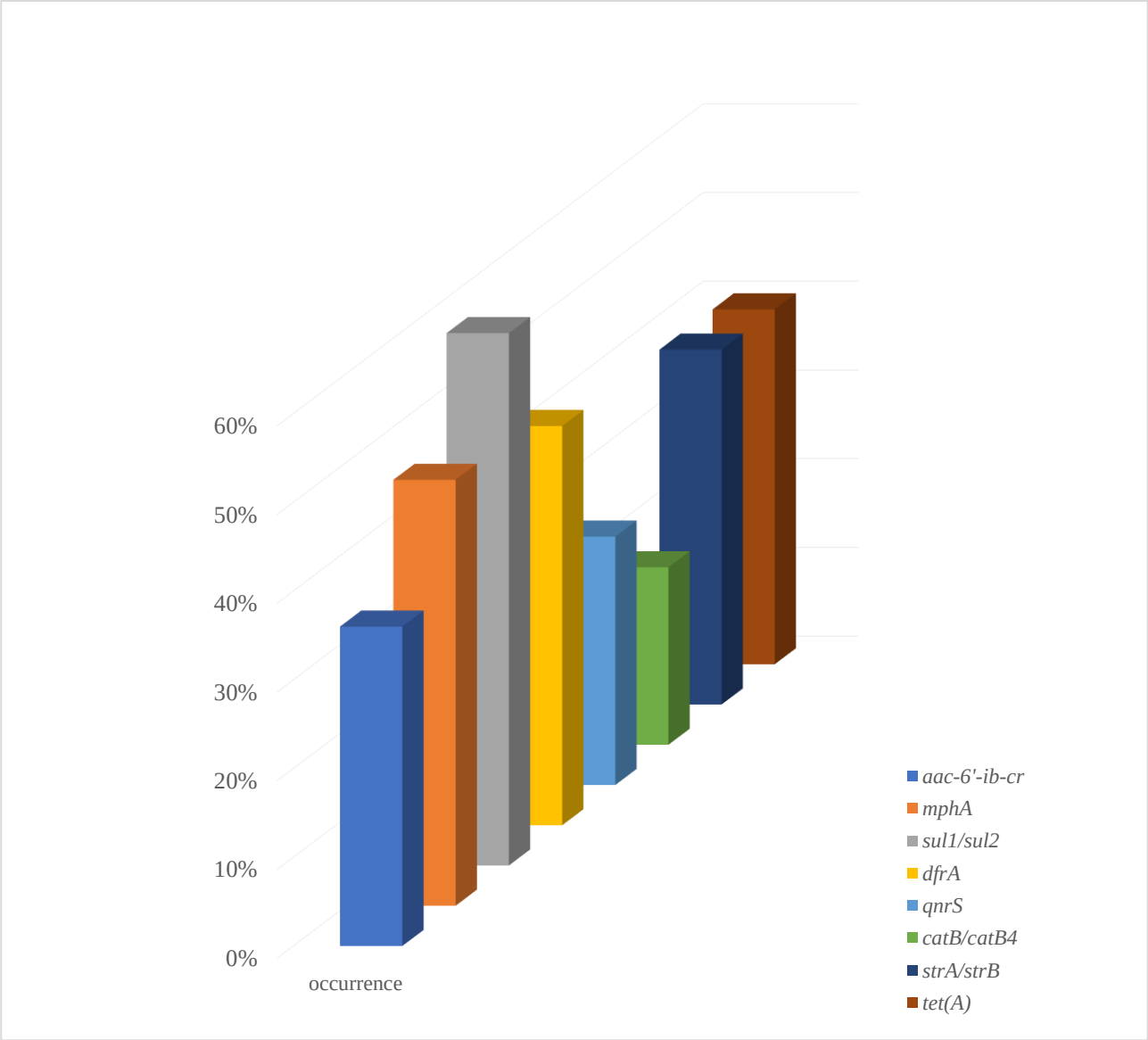

Figure 14 Antimicrobial resistance genes in *E. coli*

Fluoroquinolone resistance was plasmid-mediated by the presence of *qnr* elements. In particular, the *qnrS1* was identified in ten *E. coli*, whereas *qnrB19* and *qnrB66* were found in two and one isolates, respectively. In one isolate of E. coli, the simultaneous presence of *qnrS1* and *qnrB19* was identified. Resistance to fluoroquinolones was also mediated by mutations in *gyrA*, *parC* and *parE*. The S83L/D87N in *gyrA*, S80I/E84V in *parC* and I529L in *parE* were the most common substitutions found in twenty-nine ST131. The S83L/D87N in *gyrA*, S80I in *parC* and L416F in parE mutations were found in three ST1193. The S83L/D87N in *gyrA*, S80I in *parC* and S458A in *parE* mutations were identified in one ST410. Two isolates of E. coli ST69 showed the S83L and D87N mutations in *gyrA* and S80I in *parC*.

ST131 is composed by five clades (A, B, C0, C1 and C2), and clade C seems to be more fit than the others. The clade C showed resistance to fluoroquinolones and was able to acquire IncF plasmids, giving to *E. coli* a rapid and continual adaptation to different environments.

In the present study, the ST410-H24 strain showed resistance to carbapenems, producing NDM-4 metallo-β-lactamase, and to fluoroquinolones via plasmid qnrS1 and to chromosomes by mutations in *gyrA, parC* and *parE*. The ST131 isolates were found to be associated with IncF plasmids, ESBLs (CTXM-1-group, CTX-M-27) and chromosomal resistance to fluoroquinolone (gyrA/parC/parE mutations). They also possessed *aac(60)-Ib-cr*, catB4, OXA-1/OXA-9 and other ARGs which reduced susceptibility to aminoglycoside, chloramphenicol, oxacillin and other classes of antibiotics.

3.3.5 Virulence factors

E. coli isolates analyzed in this study detected the following virulence genes: *sat* (secreted autotransporter toxin), *iha* (adhesion-siderophore receptor), *iss* (increased serum survival), *senB* (plasmid encoded enterotoxin), *gad* (glutamate decarboxylase), *astA* (east-1 heat-stable toxin), *lpfA* (long polar fimbriae), *air* (enteroaggregative immunoglobulin repeat protein), *eilA* (Salmonella HilA homolog), *cnf1* (cytotoxic necrotizing factor), *vat* (vacuolating autotransporter toxin), *ireA* (iron-regulated outer membrane virulence protein), *iroN* (salmochelin siderophore receptor), *sfaS* (S fimbriae), *nfaE* (non fimbrial adhesion) and *mcmA, mchB, mchC* and *mchF*, which are the microcin H47 system virulence genes . The increased serum survival (*iss*), secreted autotransporter toxin (*sat*), adhesion-siderophore receptor (*iha*) and plasmid encoded enterotoxin (senB) were found in 84%, 72%, 63% and 51% of E. *coli*, respectively. Finally, in the *E. coli* ST12, ST69 and ST95, different virulence genes were found; on the contrary, only two virulence genes were identified in ST410 and ST1193 (Table 10).

Virulence gene	Target Class	Total n. 43/(%)
air	Enteroaggregative immunoglobulin repeat protein	2 (5%)
astA	East-1 heat stable toxin	7 (16%)
cma	Colicin M	2 (5%)
cnf1	Cytotoxic necrotizing factor	9 (21%)
eilA	Salmonella HilA homolog)	3 (7%)
gad	Glutamate decarboxylase	10 (23%)
iha	Adhesion-siderophore receptor	27 (63%)
ireA	Iron-regulated outer membrane virulence protein	3 (7%)
iroN	Salmochelin siderophore receptor	7 (16%)
iss	Increased serum survival	36 (84%)
lpfA	Long polar fimbriae	5 (12%)
mchB	Microcin H47 system	3 (7%)
mchC	Microcin H47 system	5 (12%)
mchF	Microcin H47 system	7 (16%)
mcmA	Microcin H47 system	3 (7%)
nfaE	non-fimbrial adhesion	2 (5%)
sat	Secreted autotransporter toxin	31 (72%)
senB	Plasmid encoded enterotoxin	22 (51%)
sfaS	S fimbriae	2 (5%)
vat	Vacuolating autotransporter toxin	7 (16%)

Table 10 Virulence factors found in E. *coli* isolates analyzed

3.4 Discussion

Whole genome of 43 multidrug resistant *E. coli* collected from 13 LTCFs in the Veneto region were analyzed. The majority of *E. coli* analyzed belongs to the ST131 lineage, an extra-intestinal pathogenic bacterium which lives in the digestive and urinary tract [86]. Almost all ST131 (30 out of 32) analyzed were of the O25b:H4 serotype and H30 subclone.

The *fimH* allele, which encodes fimbriae has been used to phylogenetically classify the ST131 isolates in three clades (A, B, C), being also based on antibiotic resistance genes [85]. As reported by Pitout et al., ST131 clades C1 and C2 are fluoroquinolone resistant, and C2 has a strong association with CTX-M production [85]. Several studies have proved that the most prevalent subclonal lineage of *E. coli* ST131 is fimH30, which is also associated with a specific mutation in *gyrA* and *parC*, conferring chromosomal resistance to fluoroquinolones [87]. Some *E. coli* lineages were identified only in one LTCF; this is the case for ST12, ST69 and ST95, found only in SSL_BL, the ST48 in SAF_VE and ST410 in POCS_VR. The ST410 lineage seems to be phylogenetically older than ST131, but because of its virulence profile, ST131 is globally distributed in several environments over the world. The feature of ST410 strains, in contrast to ST131, is the resistance to carbapenems [88].

In the present study, the ST410-H24 strain showed resistance to carbapenems, producing NDM-4 metallo-β-lactamase, and to fluoroquinolones via plasmid *qnrS1* and to chromosomes by mutations in *gyrA, parC* and *parE*. In ST131, we found the predominance of CTX-M variants (94% of isolates) followed by OXA-1/OXA-9 (47% of isolates). As previously reported, CTX-M-15 and CTX-M-27 alleles are associated with the ST131 clade C2 and ST131 clade C1 [89]. The CTX-M-27 is predominantly associated with the F1:A2:B20 replicon, as reported in other ST131 strains isolated worldwide [90]. The CTX-M-27 belongs to subgroup CTX-M-9, and it showed two amino acid substitutions from CTX-M-9 (A231V and D240G) and one from CTX-M-14 (D240G). CTX-M-27 was found, not only in clinical strains, but also in bacteria isolated from food-producing animals, livestock and environment [91,92,93] (Figure 13). Metallo β-lactamases were not found in ST131, and this is in agreement with results reported in different countries [87]. Similarly, the KPC-3 carbapenemase was found only in three *E. coli* strains (ST131 and ST1193) collected in CDS_RO, IPABRT_VI and PCS_VR LTCFs. In a previous paper where we characterized the resistome of *K. pneumoniae* isolates, we found, in the same LTCFs, KPC-3 producing *K. pneumoniae* [55]. The *E. coli* and *K. pneumoniae* strains harbored the same IncFIB(pQil) plasmid. We speculate that, in the same LTCF, a transfer of the bla_{KPC-3} gene, presumably located in IncFIB(pQil) plasmid, may have occurred between *E. coli* and *K. pneumoniae*.

Chapter 4: Whole-Genome Sequencing of ST2 *A. baumannii*

4.1 Introduction

Acinetobacter baumannii has emerged in the last two decades as one the major causes of nosocomial infections associated with significant morbidity and mortality. It belongs to a group of nosocomial pathogens, designed by the acronym ESKAPE and it has been recognized by World Health Organization (WHO) as a "critical priority pathogen". *A. baumannii* is ubiquitous and it can be found in various environmental sources (soil, water, food) but, especially, in hospital setting, mainly in intensive care units, where it can cause bacteremia, endocarditis, urinary tract infections, meningitis, gastrointestinal and skin/wound infections, ventilator-associated pneumonia and bloodstream infections [94]. However, community acquired *A.baumannii* infections have been described, in particular, in people with comorbidities [96]. Infact, prolonged hospitalization in the intensive care unit (ICU) may be linked with increased possibility of developing bacterial co-infection, especially with multi-drug resistant (MDR). Moreover, several studies have reported that respiratory viral infections, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), predispose patients to bacterial co-infections and secondary infections [96].

Antibiotic resistance in *A. baumannii* is due to different mechanisms: (a) inactivation of antibiotics by enzymatic hydrolysis (i.e., β-lactamases), (b) reducing membrane permeability, (c) increasing efflux of antibiotics (i.e., overexpression of drug efflux pumps) and (d) mutations in antibiotic binding targets by genetic insertion sequences. Additionally, *A. baumannii* is also able to promote antibiotic resistance by virulence factors such as outer membrane protein modification, cell envelope factors, enzymes, quorum sensing, biofilm formation, motility and micronutrient acquisition systems [97]. Since 1990, carbapenems have been the mainstay antibiotics in the treatment of A. baumannii infections. carbapenem-resistant *A. baumannii* (CRAb) poses a global threat to human health. CRAb is emerging worldwide, and the majority of these isolates often show multidrug-resistant, extensively drug-resistant and pandrug-resistant phenotypes . So, in 2018, the World Health Organization (WHO) considered *A. baumannii* a priority for the research and development of new antibiotics [98].

Currently, few therapeutic options are available for *CRAb* treatment [99]. Generally, colistin (CST), tigecycline and aminoglycosides are used against MDR *A. baumannii*; above all CST has been successfully used to treat pneumonia and, bloodstream and meningitis infections caused by CRAb. Unfortunately, colistin-resistant isolates are emerging worldwide [99]. Fosfomycin is also used in combination with colistin or tigecycline or aminoglycoside for the treatment of hospital-acquired pneumonia caused by *CRAb* [100].

Cefiderocol, a novel siderophore cephalosporin, has recently been approved for the treatment of MDR *A. baumannii* [101]. Durlobactam (DUR), previously called ETX2514, is a non-β-lactam diazabicyclooctane (DBO) inhibitor with activity against Ambler class A, C and D β-lactamases [102]. Recently, some studies have shown that sulbactam in combination with durlobactam is active

against MDR *A. baumannii* . Sulbactam (SUL) is one of the first β-lactamase inhibitors used in combination with ampicillin for the treatment of class A β-lactamase-producing pathogens. In *A. baumannii*, SUL also has antibacterial activity by targeting PBPs (i.e., PBP1a/b and PBP3), enzymes required for cell wall synthesis. DUR inactivates serine-β-lactamases by forming a reversible covalent bond with the active site serine. This potent activity of durlobactam allows the susceptibility of CRAb to sulbactam to be restored [103].

The first part of the study on the *A. baumannii* isolates was to explore, by whole-genome sequencing (WGS), antibiotic resistance genes (ARGs) and virulence factors of *A. baumannii* strains isolated from COVID-19 patients affected by sepsis and admitted to the ICU of Spirito Santo Hospital, Pescara, Central Italy.

4.2 Materials and methods

4.2.1 Setting and strains selection of *A.baumannii*

A total of 43 *A. baumannii* strains were isolated from bloodstream infection of 43 COVID-19 patients with evident clinical signs of bacterial sepsis. The patients were admitted to the ICU ward of Spirito Santo Hospital (Pescara, Central Italy). Overall, 32 (74%) patients were male and 11 (26%) were female, with an age ranging from 31 to 82 years old. These patients had comorbidities such as kidney disease, diabetes, hypertension, or heart disease, and in some of them a co-presence of E. faecalis, S. marcescens and *K. pneumoniae* was also ascertained. The blood sample collection was repeated with an interval of 2 days for each patient. All samples were transferred to clinical molecular laboratory and analyzed following methods according to Clinical and Laboratory Standards Institute (CLSI) guidelines [77]. One *A. baumannii* strain per patient was included in the study. The strains, selected from positive cultures, were grown in Cled agar plates (Oxoid, UK) at 37 ◦C, overnight, and identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Billerica, MA, USA).

4.2.2 Antimicrobial Susceptibility of *A. baumannii*

Antimicrobial susceptibility was determined by the Phoenix system (Becton, Dickinson and Company, Sparks, MD, USA) at Clinical Microbiology and Virology Unit, Spirito Santo Hospital, Pescara, Italy. The MICs for amikacin, gentamicin, ciprofloxacin, meropenem, colistin and trimethoprim–sulfamethoxazole association were also performed by conventional broth microdilution procedures in Mueller–Hinton broth (Biolife Italiana, Milan, Italy) supplemented with calcium and magnesium (CAMHB), using an inoculum of 5 × 105 CFU/mL according to CLSI [78].

4.2.3 DNA Extraction and Whole-Genome Sequencing

See paragraph 2.2.3

4.2.4 Bioinformatic analysis

See paragraph 2.2.4

Quality control and sequences filtering of were checked using DRAGEN FastQC + MultiQC v3.6.3 (https://basespace.illumina.com/apps/10562553/DRAGEN-FastQC-MultiQC) and Velvet v1.2.10 (https://basespace.illumina.com/apps/85 56549/Velvet-de-novo-Assembly).
To individuate virulence genes detected, VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/VirulenceFinder/) and Virulence Factor Database (VFDB, http://www.mgc.ac.cn/VFs/) were used.

MLST by *A. baumannii* genomes was performed utilizing the Pasteur scheme that includes the identification of seven internal housekeeping genes: citrate synthase (gltA), homologous recombination factor (recA), 60-kDa chaperonin (cpn60), elongation factor EF-G (fusA), CTP synthase (pyrG), 50S ribosomal protein L2 (rplB) and RNA polymerase subunit B (rpoB) [85].

4.3 Results

4.3.1 Minimal Inhibitory Concentrations (MICs)

A total of 43 *A. baumannnii* strains, isolated from positive blood cultures, were analyzed against a panel of antibiotics: amikacin, ciprofloxacin, colistin, gentamicin, trimethoprim-sulfamethoxazole and meropenem. All the strains showed the same antimicrobial susceptibility profile with a remarkable resistance to amikacin (MIC ≥ 64 mg/L), gentamicin (MIC ≥ 16 mg/L), ciprofloxacin (MIC ≥ 4 mg/L), meropenem (MIC > 8 mg/L), colistin (MIC > 8 mg/L) and trimethoprim–sulfamethoxazole association (MIC > 160 mg/L) (Table 11).

	MIC (MG/L)					
STRAINS	**AMK**	**CIP**	**CST**	**GEN**	**SXT**	**MEM**
***A. baumannii* 491575**	> 32	>2	>8	>8	>160	>8
***A. baumannii* 489091**	> 32	>2	>8	>8	>160	>8
***A. baumannii* 491641**	> 32	>2	>8	>8	>160	>8
***A. baumannii* 491662**	> 32	>2	>8	>8	>160	>8
***A. baumannii* 510518**	> 32	>2	>8	>8	>160	>8
***A. baumannii* 510941**	> 32	>2	>8	>8	>160	>8

Table 11 Antimicrobial susceptibility of *Acinetobacter baumannii* isolated from ICU of Santo Spirito Hospital, Pescara

4.3.2 Multilocus Sequence Typing (MLST)

The WGS was performed on eight non-replicative *A. baumannii* strains isolated from patients admitted to ICU in different time periods. By molecular analysis we identified the sequence type (MLST) and the genome size of that ranged from 3.8 to 4.0 Mb. The MLST analysis using the Pasteur scheme (based on seven housekeeping genes, *cpn60, fusA, gltA, pyrG, recA, rplB, rpoB*)revealed that all isolates belonged to the ST2 sequence type (Table 12).

Strain	Serotypes	Genome Size (bp)
A. baumannii PE1	ST2	4.023.584
A. baumannii PE2	ST2	4.023.635
A. baumannii PE3	ST2	3.850.309
A. baumannii PE4	ST2	3.965.839
A. baumannii PE5	ST2	3.828.825
A. baumannii PE6	ST2	4.041.529
A. baumannii PE7	ST2	3.841.128
A. baumannii PE8	ST2	3.852.243

Table 12 MLST and genome size of *A. baumannii*

4.3.3 Mobile genetic elements (MGEs)

A wide variety of sequence insertions (ISs), belonging to different IS families such as IS3 (ISAba2), IS4 (ISEc29), IS5 (IS17 and ISAba13), IS6 (IS26 and IS6100), IS30 (ISAba125), IS91 (ISVsa3) and IS256 (ISAba26), and transposon elements were identified in all analyzed strains. In detail, transposon Tn6207 was found in 4 *A. baumannii* isolates. Sequence insertion elements such as ISAba2 of 1308bp length (3 isolates), ISAba13 of 1039 bp length (4 isolates), ISAba26 of 1318 bp length (4 isolates), IS26 of 820 bp length (1 isolate), ISVsa3 of 977 bp (3 isolates), ISEc29 of 1325 bp (7 isolates), IS6100 of 880 bp (4 isolates) and IS17 of 1040 bp (4 isolates) were identified in the genome of *A. baumannii* (Table 13)

ISAba13 acts through an upstream insertion that decreases the transcription of *adeN* (a deaminase) conferring resistance to erythromycin, tetracycline and azithromycin; ISAba125 acts on promoter sequences increased the expression of bla_{ampC} and bla_{NDM-1} conferring resistance to cephalosporins and carbapenems. Some carbapenem-resistant *A. baumannii* isolates containing the acquired β-lactamase bla_{OXA-58}. Bla_{OXA-58} was typically located downstream of promoters introduced by IS elements, such asISAba1, ISAba2, an ISAba3-like element, and IS18 [104]. In all analyzed strains we also found gene structures indicating the presence of the composite transposon Tn6207.

Strain	Mobile Genetic Elements
A. baumannii PE1	Tn6207 ISEc29 ISAba26 IS26 ISAba125
A. baumannii PE2	Tn6207

	ISAba125
	ISAba26
	ISVsa3
A. baumannii PE3	ISEc29
	ISAba125
	ISAba2
	ISAba13
	IS17
	IS6100
A. baumannii PE4	Tn6207
	ISVsa3
	ISEc29
	ISAba125
	ISAba26
A. baumannii PE5	ISEc29
	ISAba125
	ISAba2
	ISAba13
	IS17
	IS6100
A. baumannii PE6	Tn6207
	ISVsa3
	ISEc29
	ISAba125
	ISAba26
A. baumannii PE7	ISEc29
	ISAba125
	ISAba2
	ISAba13
	IS17
	IS6100
A. baumannii PE8	ISEc29
	ISAba125
	ISAba2
	ISAba13
	IS17
	IS6100

Table 13 Mobile Genetic Elements found in *A.baumannii*

4.3.4 Antimicrobial resistance genes (ARGs)

β-lactamase genes

The *A. baumannii* strains analyzed in this study harbored molecular class C and class D β-lactamases. Overall, bla_{ADC-25} and bla_{OXA-23} genes were found in all isolates with a co-presence of bla_{OXA-66} (four out of eight isolates) or bla_{OXA-82} (four out of eight isolates) (Figure 15). Both bla_{OXA-66} and bla_{OXA-82} belong to the constitutive bla_{OXA-51}-type genes that is intrinsically over-expressed and it is able to confer high resistance to carbapenems in *A. baumannii* isolates [105]. The OXA-82 enzyme differs from OXA-66 only by one amino acid residue: the leucine 157 (L157) in OXA-66 is replaced by valine (V157) in OXA-82. Also bla_{ADC-25} gene, was found in the totally strains (Figure 16).

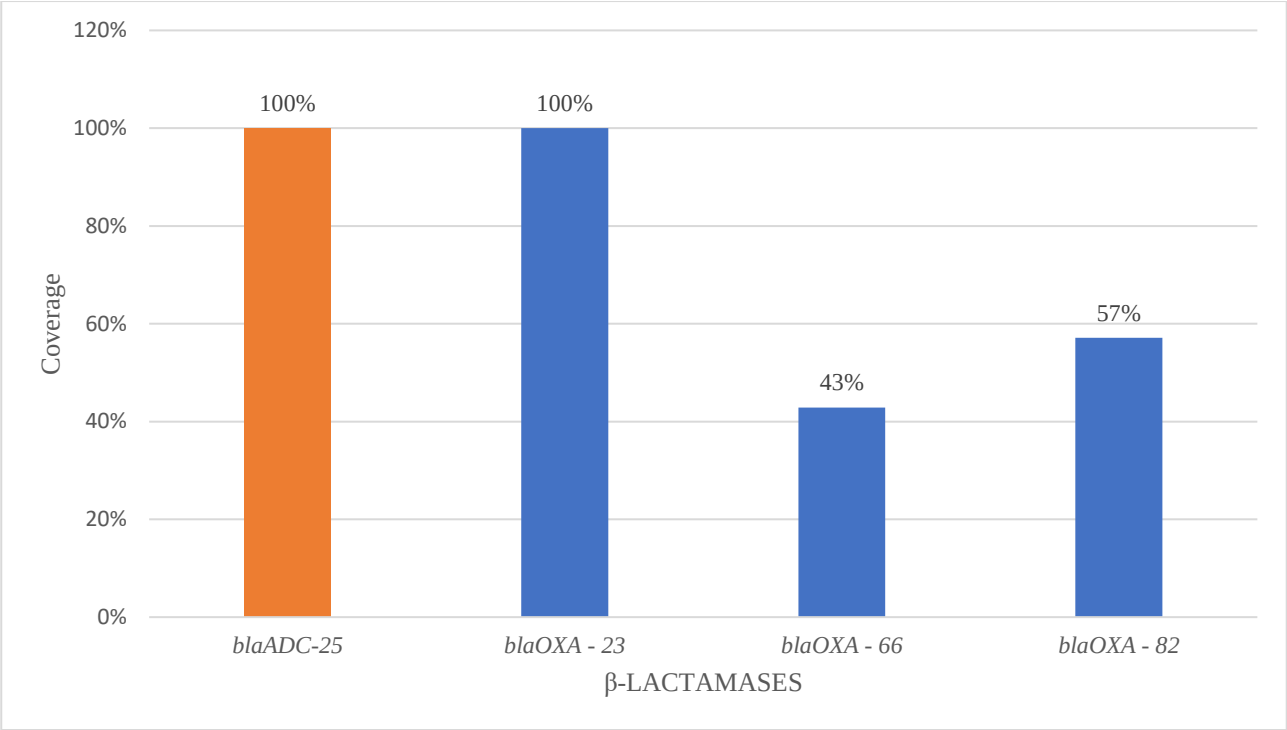

Figure 15 Distribution of β-lactamases genes in *A. baumannii*

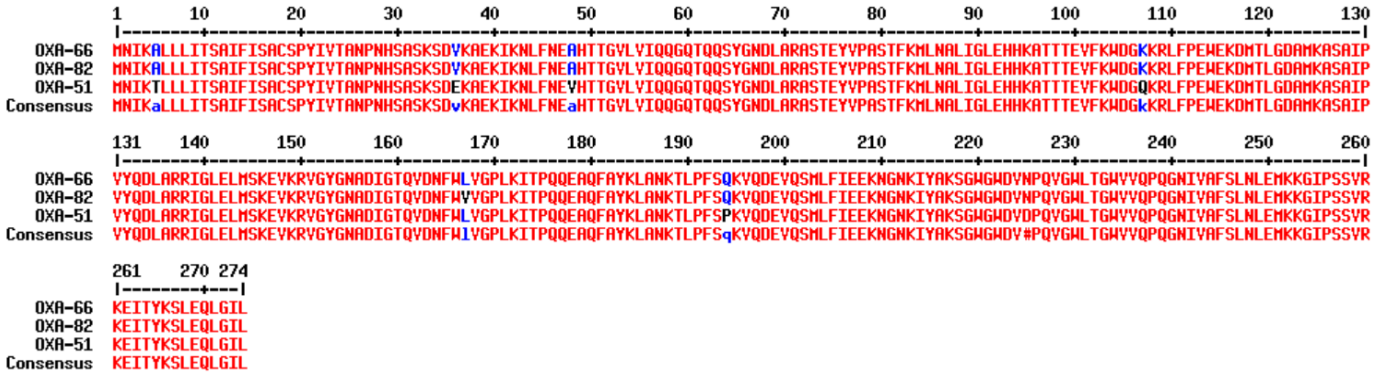

Figure 16 Alignment of OXA-51-like enzymes found in *A.baumannii*

Other Antimicrobial Resistance Genes

Resistance to aminoglycosides was mediated by *aadA1*, *aacA4*, *armA* and bi-functional gene *aac(6')Ib-cr*, founded in all *A.baumanii* analyzed. Gentamicin and amikacin resistance is generally plasmid-mediated by *armA* gene. Other aminoglycoside resistance genes such as *aph(30)Iva*, *aacA2*, *aadB*, *aac(30)-Ic* and *aac(3)Ia*, were found in four isolates (table 15).

Resistance to fluoroquinolones is mediated by the *aac(60)Ib-cr* gene and bi-functional gene which confers resistance to aminoglycosides simultaneously. Other genes that confer resistance to other

classes of antibiotics were found in every isolates analyzed: *mph(E)* and *msr(E)* (macrolide resistance), *sul1* (sulphonamide resistance), *tet(B)* (tetracycline resistance) and *catB8* (chloramphenicol resistance) genes. The *strA/strB* genes, which confer resistance to streptomycin, were identified in 50% of the analyzed strains (Table 14).

Resistance	Genes	No isolates
Amynoglicoside	*aadA1*	7
	aadB	4
	aacA2	4
	aacA4	7
	aph(3')VIa	4
	aph(3')-Ic	4
	aac(3)Ia	4
	aac(6')Ibcr	2
	strB	3
	strA	3
	armA	7
Macrolide	*mph(E)*	7
	msr(E)	7
Fluoroquinolone	*aac(6')Ibcr*	7
Others	*sul1*	7
	tet(B)	3
	catB8	7

Table 14 Antimicrobial resistance genes (ARGs) and relative occurrences in *A. baumannii* analyzed by WGS

4.3.5 Virulence factors

Virulome analysis identified, in 100% of *A. baumannii* isolates, various genes involved in different virulence mechanisms: biofilm (*bap* and *pgaABCD* locus) and pili (*csu*) formation; *ompA* porin production; lipid A synthesis (*lpxABCDLM* locus); biosynthesis of the LPS core (*lpsB*); serum resistance (*pbpG*);synthesis of phospholipase C and D (*plc* and *plcD*); synthesis of *LysR*-type transcriptional regulator as well as acinetobactin genes cluster (*bau, bas, bar, ent*). Efflux pump genes (*RND: adeFGH*) were also found in the totality of the analyzed strains. The gene *hemO*, related to iron uptake, and the catalase gene (*katA*), which protects bacteria from superoxidants produced by leukocytes as a host defense mechanism, were also present in all sequenced strains (Table 15).

Category	Virulence factors	Related genes
Adherence	Outer membrane protein	*ompA*
Biofilm formation	AdeFGH efflux pump	*adeF; adeG; adeH;*
	Biofilm-associated protein	*bap*

	Csu fimbriae	*csuA; csuB; csuC; csuD; csuE;*
	Polysaccharide poly-N-acetylglucosamine	*pgaA; pgaB; pgaC; pgaD;*
Enzyme	Phospholipase C	*plc*
	Phospholipase D	*plcD*
Immune evasion	LPS	*lpsB; lpxA; lpxB; lpxD; lpxL; lpxM;*
Iron uptake	Acinetobactin	*barA; barB; basA; basB; basC; basD; basF; basG; basH; basI; basJ; bauA; bauB; bauC; bauD; bauE; bauF; entE;*
	Heme utilization	*hemO*
Regulation	Quorum sensing	*abaI; abaR;*
	Two-component system (BfmRS)	*bfmR; bfmS;*
Serum resistance	PbpG (Penicillin-binding protein)	*pbpG*
Stress adaption	Catalase	*katA*

Table 15 Virulence factors of the eight *A. baumannii* analyzed by WGS

4.4 Discussion

In this study, we used WGS to explore the resistance mechanisms in carbapenem- and colistin-resistant *A. baumannii* isolated by COVID-19 patients admitted to the ICU of Spirito Santo Hospital, Pescara. All isolates belonged to ST2 , which is the most representative ST type clone over the world [106]. Several studies showed the dissemination of CRAb isolates harboring the bla_{OXA-23}-like and belonging to ST2 from different countries [107].

In our strains, the OXA-23 class D carbapenemase was identified in association with OXA-66 or OXA-82 (bla_{OXA-23} was the most widely reported gene, and bla_{OXA-66} was the most common variant of OXA enzymes). The OXA-66 and OXA-82 differ each other by L167V substitution and belong to the OXA-51-like enzymes. The OXA-51 is intrinsically over-expressed and it is able to confer high resistance to carbapenems in *A. baumannii* isolates [105,107].

In our study we found the presence of an AmpC enzyme, the ADC-25 cephalosporinase, and the AdeFGH RND efflux pumps in all *A. baumannii* strains. To our knowledge, the AdeFGH RND efflux pumps seem to play a major role in acquired resistance and may also be associated with carbapenem non-susceptibility in *A. baumannii* [108].

The pathogenicity of *A. baumannii* isolates analyzed in the present study is exacerbated by the presence of virulence factors such as the abaI/abaR quorum sensing system which is involved in morphology, growth characteristics, biofilm formation, motility, resistance and virulence of these microorganisms [109]. In *A. baumannii* we found also two-component of signal transduction system BfmRS, which seem to be implicated in the control of various virulence related traits and acting as a global modulator of *A. baumannii* physiology [110].

The main types of mobile elements involved in the capture and mobilization of ARGs in *A. baumannii*, is represented by gene cassettes, transposons and sequence insertions (ISs). In the present study, we found a wide variety of ISs belonging to different IS families such as IS3 (ISAba2), IS4 (ISEc29), IS5 (IS17 and ISAba13), IS6 (IS26 and IS6100), IS30 (ISAba125), IS91 (ISVsa3) and

IS256 (ISAba26). The ISAba1 was first identified in *A. baumannii* upstream of bla_{OXA-23}, bla_{OXA-51}, bla_{OXA-58} and bla_{ADC} genes, and it seems to be an important factor in the genetic plasticity of this microorganism. The presence of ISAba1 upstream of bla_{OXA-51}-like genes might represent a real mechanism of carbapenem resistance. Insertion of ISAba125 in the bla_{OXA-23} promoter sequence has been reported to be associated with overexpression of bla_{OXA-23}, bla_{OXA-51} and carbapenem resistance phenotypes in *A. baumannii*. In all analyzed strains we also found Tn6207 transposon, a composite transposon containing a tetracycline efflux pump and its regulator genes *tetB* and aminoglycoside resistance genes *strB* and *strA* [111,112].

Respiratory viral infections, such as SARS-CoV-2, predispose patients to bacterial co-infections and secondary infections [113] representing a high risk for hospitalized patients. However, bloodstream *CRAb* and *K. pneumoniae* remain the most common ICU-acquired infections [114].

Chapter 5: Identification and biochemical analysis of a new class A β-lactamase, the KPC-53 enzyme

5.1 Introduction

Carbapenem antibiotics are generally considered the most effective antibacterial agents for the treatment of multidrug-resistant bacterial infections, such as *E. coli* and *K. pneumoniae*. However, with the widespread use of these antibiotics, carbapenem-resistant *Enterobacterales* have increased rapidly, becoming a global threat to public health. The production of carbapenemases, including KPC, NDM and OXA-48-like is the most common resistance mechanism among carbapenem-resistant Enterobacteriaceae clinical isolates [115]. KPC carbapenemases are class A serine-β-lactamases which have a large substrate specificity. These enzymes are usually encoded by trasferable plasmids and that contributes to a global dissemination of carbapenemase-producing *Enterobacterales*. To date, 108 KPC variants which differ from each other by one to five aminoacids substitutions have been known. However KPC-2 and KPC-3 are the most widespread, differing from each other by a single amino acid substitution at position 274 where a Tyr in KPC-3 replaces an His.

The structure of KPC is typically of class A β-lactamases, consisting in two domains: one of the subdomains has an a-helical structure, whereas the other subdomain is represented by five β-sheets flanked by a-helical structures [116]. The active site of KPC-2 includes several conserved residues which appear to be directly or indirectly involved in the substrate recognition and catalytic process. The active site contains the catalytic residue S70, typical of all class A β-lactamases, and some conserved residues such as K73, W105, N132, E166, R220, and K234. Residue E166 is involved in the deacylation step of catalysis, while residues W105 and R220, located on opposite sides of the active site, are involved with the recognition of substrates and inhibitors [117] (Figure 17).

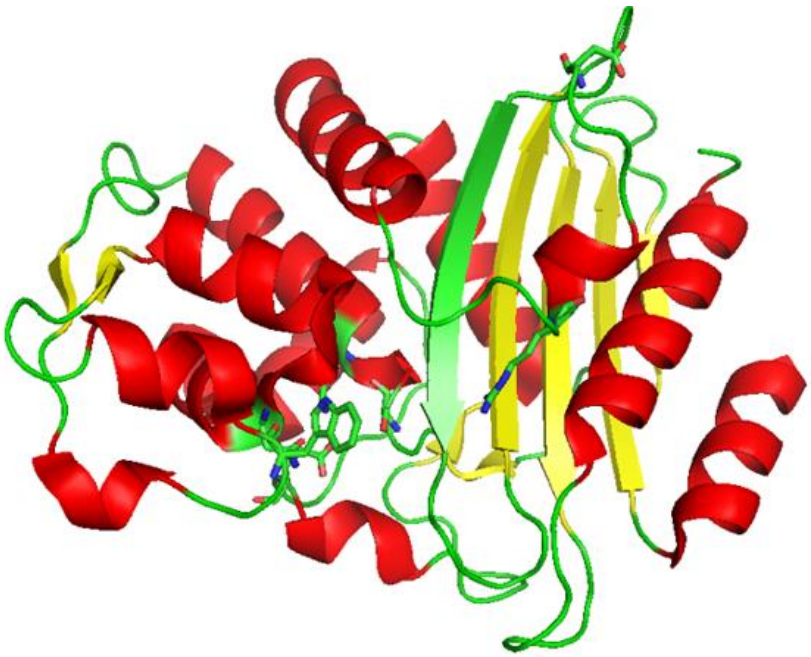

Figure 17 Molecular structure of KPC enzymes

Among the resistome determinants of analyzed clinical strains, a new variant of KPC enzyme was identified, the β-lactamase KPC-53. In detail it was found in LC-1825/18 *K. pneumoniae* strains.

KPC-53 is a recently described natural KPC variant, detected in a *K. pneumoniae* strain resistant to CZA, which exhibits a L167E168 duplication in the Ω-loop of the enzyme [118]. The duplication of these two amino acids residues was described also in KPC-25, KPC-31 and KPC-40 (Figure 18) [119]. In this part of the project we have characterize by molecular point of view, the kinetic profile of KPC-53 and investigated by molecular modeling the structure function relationships of this KPC variant.

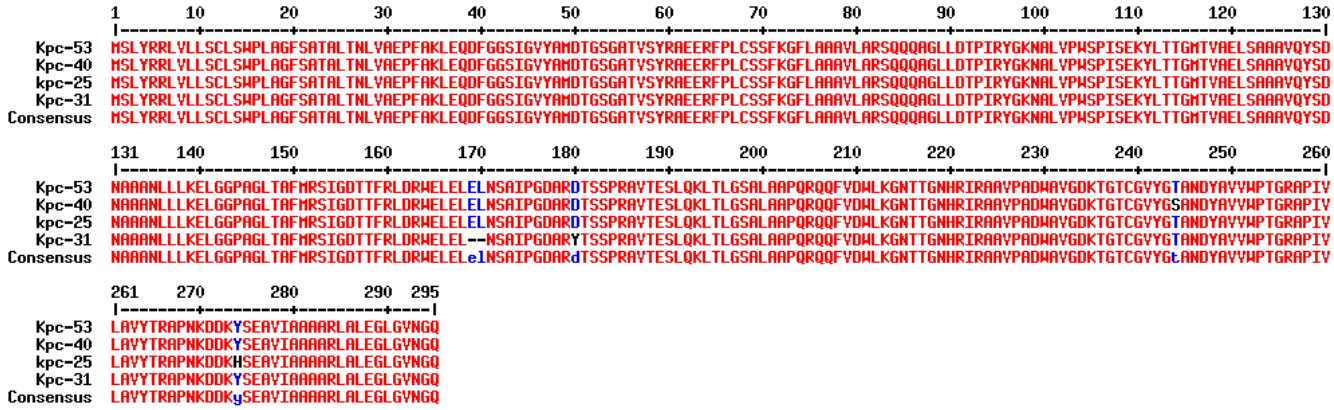

Figure 18 KPC-31 has an aspartic acid-to-tyrosine (D179Y) substitution at position 179 in the Ω-loop of KPC-3, which is known to confer resistance to CZA. In addition to L167E168 duplication, the KPC-25 and KPC-40 showed Y274H and Y244S substitutions, respectively

5.2 Materials and methods

5.2.1 Organisms

The strains examined included *E. coli* Nova Blue (endA1 hsdR17 [r_{K12}^{-} m_{K12}^{+}] supE44 thi-1 recA1 gyrA96 relA1 lac F' [proA^{+}B^{+} lacI^{q} ZΔM15::Tn10]; Tetr), *E. coli* BL21(DE3) [B F^{-} ompT gal dcm lon hsdSB(r_B^{-} m_B^{-}) λDE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB^{+}]K-12(λS)], and *E. coli* XL-1 (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI^{q} ZΔM15 Tn10; Tet^{r}]).

5.2.2 Antibiotics

Meropenem was from AstraZeneca (Milan, Italy). Imipenem and ertapenem were from Merck Sharp & Dohme (Rome, Italy). Nitrocefin was kindly provided by Shariar Mobashery (Notre Dame University, South Bend, IN). Tazobactam was from Wyeth-Lederle (Catania, Italy), and clavulanic acid was from GlaxoSmithKline (Verona, Italy). Avibactam and other antimicrobial agents were purchased MedChemExpress (DBA, Italy) and Sigma-Aldrich (Milan, Italy), respectively. The molar extinction coefficients and the wavelengths of the β-lactams (Δε) used in the assay were as follows: benzylpenicillin ($\Delta\varepsilon_{235}$ = 2775 M^{-1} cm^{-1}) carbenicillin ($\Delta\varepsilon_{235}$= –780 M^{-1} cm^{-1}), cefazolin ($\Delta\varepsilon_{260}$= 27,400 M^{-1} cm^{-1}), ceftazidime ($\Delta\varepsilon_{260}$= 29,000 M^{-1} cm^{-1}), nitrocefin ($\Delta\varepsilon_{482}$= 15,000 M^{-1} cm^{-1}), imipenem ($\Delta\varepsilon_{300}$= 29,000 M^{-1} cm^{-1}), meropenem ($\Delta\varepsilon_{297}$= 26,500 M^{-1} cm^{-1}), and ertapenem ($\Delta\varepsilon_{298}$= 27,500 M^{-1} cm^{-1}).

5.2.3 Cloning

The *bla*$_{KPC-53}$ gene was amplified from *K. pneumoniae* LC-1825/18 clinical isolate [118] using primers reported below and was cloned into pET-24(a) and pBC-SK vectors. Transformation in *E. coli* competent cells was performed by using heat shock. The plasmid transfer was confirmed by PCR and automated sequencing (ABI Prism 3500; Life Technologies, Monza, Italy).

KPC_F ATGTCACTGTATCGCCGTCTAGTT

KPC_R TTACTGCCCGTTGACGCCCAAT

5.2.4 Overexpression and purification

E. coli BL21(DE3) cells carrying the recombinant plasmid pET24-blaKPC-53 were grown in 2 L of Luria-Bertani (LB) medium with 50 mg/L kanamycin at 37°C in an orbital shaker (180 rpm). The bacteria were grown at 37°C until reaching an optical density at 600 nm (OD600) of 0.8. Then, 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added, and the culture was incubated at 22°C for 18 h. Cells were harvested by centrifugation at 10,000 xg for 10 min at 4°C and washed twice with 25 mM sodium phosphate buffer (pH 7.0). The crude extract was obtained by sonication on ice (five cycles at 60 W with a 2-min break). The lysate was centrifuged at 100,000 xg for 30 min, and the supernatant was dialyzed overnight at 4°C in 25 mM sodium acetate buffer (pH 5.2) and loaded onto a SP Sepharose FF equilibrated with the same buffer progressing with a linear gradient of NaCl (0 to 1 M) in the same buffer to eluate β-lactamases. Active fractions were pooled, dialyzed in 25 mM sodium phosphate buffer (pH 7.0), and loaded onto a Sephacryl S-100 column (XK16/70; bed volume, 130 mL) equilibrated with 25 mM sodium phosphate buffer (pH 7.0) and 0.15 M NaCl. The active fractions were pooled and stirred at -40°C. KPC-53 purified by two chromatographic steps, has a purity grade >95%, estimated by SDS-PAGE. The isoelectric points (pIs) of purified KPC-53 and KPC-3 were 6.3 and 6.7, respectively.

5.2.5 Kinetic characterization

Kinetic experiments were performed following the hydrolysis of each substrate at 25°C in 25 mM sodium phosphate buffer (pH 7.0). Data were collected with a Perkin-Elmer Lambda 25 spectrophotometer (Perkin-Elmer Italia, Monza, Italy). Steady-state kinetic experiments were determined under initial-rate conditions using the Hanes linearization method [120]. Kinetic parameters were determined under initial-rate conditions using the Origin Pro 8.5.1 to generate Michaelis-Menten curves. For K_m values higher than 1 mM, the K_m was determined as K_i using nitrocefin as the reporter substrate [121]. Competitive inhibition assays were performed using the following equation:

$$v_0/v_i = 1 + [(K_m \times I)/(K_m + S) \times K_i]$$

(v_i and v_0 represent the initial rates of hydrolysis of nitrocefin with and without inhibitor, respectively; I is the concentration of inhibitor or poor substrate; K_i is the inhibition constant; K_m is the Michaelis constant; S is the concentration of the reporter substrate).
The plot of v0/vi versus I yielded a straight-line slope: $K_m/(K_m + S) \times K_i$ [121].

In compounds behaving as transient inhibitors, the accumulation and slow hydrolysis of EC* were studied based on the following model:

$$E + C \overset{K}{\rightleftarrows} EC \overset{k_{+2}}{\longrightarrow} EC \overset{k_{+3}}{*\longrightarrow} E + P$$

(E is the enzyme; C is the substrate; EC is the Henri-Michaelis complex; EC* is the acyl-enzyme complex; P is the hydrolysis product; k_{+2} and k_{+3} are the first-order acylation and deacylation constants, respectively; K is the dissociation constant of the Henry-Michaelis complex)

In the case of poor substrates, the values of k_i (first-order rate constant characterizing the EC* accumulation) were obtained by time course hydrolysis of nitrocefin according to the following equations:

$$(v_t - v_{ss})/(v_0 - v_{ss}) = e^{-kit}$$

v_0, v_t , and v_{ss} are the rate transformation of substrate at time zero, t, and steady state, respectively:

$$k_i = k_{+3} + \frac{K_{+2}[C]}{[C] + K\left(1 + \frac{[S]}{K_m S}\right)}$$

([S] is the concentration of reporter substrate; K_mS is the K_m of the reporter substrate).

The condition [S] ≈ K_m, where [S] is the concentration of substrate reporter, was respected. If k_i varies linearly with [C] (indicating that the range of [C] = K), the k_{+2}/K value is calculated from the slope of the line, and k_{+3} is obtained from the extrapolation at [C] = 0 [119]. In the case of avibactam and tazobactam for KPC-3, the plot k_i versus [C] was not linear; k_{+3} = 0, k_{+2}, and K were calculated by plotting [C]/k_i versus [C] [121,122]. Each kinetic value is the mean of three different measurements; the error was below 10%.

5.2.6 Antimicrobial susceptibility

The MICs were performed by conventional microdilution procedure, as suggested by the Clinical and Laboratory Standard Institute, using a bacterial inoculum of 5 x 105 CFU/mL [123]. IPTG was added to the cation-adjusted Mueller-Hinton broth at a concentration of 0.4 mM. The experiments were performed in triplicate using *E. coli* XL-1/pBC/KPC-53, *E. coli* XL-1/pBC/KPC-3, and *E. coli* XL-1/pBC-SK. Tazobactam and avibactam were used at a fixed concentration of 4 mg/mL. Clavulanic acid was used in association with amoxicillin at a ratio of 2:1 (amoxicillin-clavulanic acid).

5.2.7 Thermo Fluor assay

The thermal stabilities of KPC-53 and KPC-3 were determined using a fluoresmeltcence-based thermal stability assay (protein thermal shift kit; Thermo Fisher Scientific, Monza, Italy) in a 7500 Fast real-time PCR system (Applied Biosystems, Monza Italy). The protein melt reaction mix (20 mL total) was added to the 96-well PCR plate. The melt reaction mix included 1 mg of each enzyme, protein shift dye (8x), and 25 mM sodium phosphate buffer (pH 7.0). The plate was heated from 25°C (2 min) to 99°C (2 min) with a heating rate of 1°C/min. The fluorescence intensity was measured with Ex/Em value of 490/530 nm. Analysis of Boltzmann T_m (T_mB) was carried out by using Protein Thermal Shift software, version 1.4. The melting temperature, T_mB, was calculated by fitting data in the region of analysis to the Boltzmann equation (Protein Thermal Shift software).

5.2.8 Fluorescence emission spectra

Fluorescence studies were carried out on a Perkin-Elmer LS-50B spectrofluorometer using the buffer 25 mM sodium phosphate (pH 7.0). The protein concentrations were 8 mg/mL; the excitation

wavelength was 280 nm, and the emission spectra, in the range of 300 to 500 nm, were recorded at 25°C.

5.2.9 Molecular modeling

The 3D structure of KPC-53 was obtained by in silico comparative protein structure modeling using Modeller 10.1 (release 12156; https://salilab.org/modeller/) [124]. Loop region refinement has been performed by generating the five most plausible loop models and ,finally, the generated structures were visually inspected using ChimeraX 1.2.5, which was also used for figure generation [125,126].

5.3 Results

The *bla*$_{KPC-53}$ gene, identified in *K. pneumoniae* LC-1825/18 [118], was cloned both in pBC-SK and pET-24a(1) vectors. The recombinant plasmid (pET24-KPC-53) was introduced into Escherichia coli BL21(DE3) and used to overexpress the KPC-53 enzyme, whereas pBC/ KPC-53 was inserted by transformation in *E. coli* XL-1 to evaluate the impact of KPC-53 on antimicrobial susceptibility.

5.3.1 MIC

E. coli XL-1/pBC/KPC-53 and *E. coli* XL-1/pBC/KPC-3 showed low MIC values for carbapenems (0.25 and 0.125 mg/L for imipenem and meropenem, respectively), but these strains were resistant to ceftazidime (Table 1). Avibactam reduced the MIC of ceftazidime from 128 to 1 mg/L and from 16 to 4 mg/L in *E. coli* XL-1/pBC/KPC-3 and *E. coli* XL-1/pBC/KPC-53, respectively,acting as an inactivator for KPC-3, unlike KPC-53. Tazobactam was unable to restore the susceptibility of piperacillin in *E. coli* XL-1/pBC/KPC-3. The *E. coli* XL-1/pBC/KPC-53 showed MICs for piperacillin and piperacillin-tazobactam of 8 and 2 mg/L, respectively (Table 16). Thus, CZA association worked better on *E. coli* XL-1/pBC/KPC-3 than *E. coli* XL-1/pBC/KPC-53 with MIC reductions of 128- and 4-fold, respectively, showing that KPC-53 was more resistant to avibactam than KPC-3. This results were comparable with kinetic data plus inhibitors.

β- Lactam	XL-1/pBC/KPC-53	XL-1/pBC/KPC-3	XL-1/pBC-SK
PIP	8	256	0.25
TZP	2	64	0.25
AMX	8	64	0.25
AMC	4	16	0.25
CAZ	16	128	<0.06
CZA	4	1	<0.06
IMP	0.25	0.25	<0.06
MEM	0.125	0.125	<0.06

Table 16 Antibiotic MICs (mg/L) against *E. coli* XL-1/pBC/KPC-53 compared to that of *E. coli* XL-1/ pBC/KPC-3 (5 × 105 CFU/mL). PIP, piperacillin; TZP, piperacillin-tazobactam; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CZA, ceftazidime-

5.3.2 Kinetic characterization

The kinetic constants of KPC-53 with several substrates were determined and compared to those previously reported for KPC-3 [119]. A reduction in k_{cat} was observed with all substrates, while K_m was variably affected and k_{cat}/K_m values were generally decreased compared to KPC-3, except for

ceftazidime. Imipenem and meropenem were poor substrates of KPC-53, and to determine their K_m values the reporter substrate method was used [121]; the MIC values for both KPC-3 and KPC-53 against imipenem and meropenem were very low and this is in contrast to the k_{cat} values determinated. (Table 17).

Substrate	Parameter	Mean±SD		KPC-3/KPC-53 ratio
		KPC-3[a]	KPC-53	
Benzylpenicillin	K_m(μM)	144±22	23±2	6.3
	k_{cat}(s^{-1})	708±15	1±0.1	708
	k_{cat}/K_m(μM^{-1s-1})	4.92	0.04	123
Carbenicillin	K_m(μM)	108±10	3±0.2	36
	k_{cat}(s^{-1})	18±1	0.18±0.014	100
	k_{cat}/K_m(μM^{-1s-1})	0.17	0.06	2.8
Cefazolin	K_m(μM)	189±25	383±34	0.49
	k_{cat}(s^{-1})	351±9	21±2	16.7
	k_{cat}/K_m(μM^{-1s-1})	1.86	0.05	37.2
Ceftazidime	K_m(μM)	100±15	31±2	3.2
	k_{cat}(s^{-1})	1.4±0.1	0.35±0.02	4
	k_{cat}/K_m(μM^{-1s-1})	0.01	0.01	1
Imipenem	K_m(μM)	88±6	0.3±0.02	293
	k_{cat}(s^{-1})	41±2	0.015±0.001	2733
	k_{cat}/K_m(μM^{-1s-1})	0.46	0.05	9.2
Meropenem	K_m(μM)	68±3	0.76±0.06	89.5
	k_{cat}(s^{-1})	51±0.2	0.024±0.001	2125
	k_{cat}/K_m(μM^{-1s-1})	0.75	0.03	25
Tazobactam[b]	K_m(μM)	277±12	PS[c]	ND[d]
	k_{cat}(s^{-1})	6±1	PS	ND
	k_{cat}/K_m(μM^{-1s-1})	0.022	PS	ND
Nitrocefin	K_m(μM)	138±10	106±10	1.3
	k_{cat}(s^{-1})	72±4	4±0.4	18
	k_{cat}/K_m(μM^{-1s-1})	0.52	0.04	13

Table 17 Kinetic costants of KPC-53 and KPC-3 toward some β-lactamases. [a]Values are expressed as means ± the standard deviations where applicable.
[b]KPC-3 kinetic data were obtained from reference 153. The kinetic constants of KPC-3 against tazobactam were determined in this study.
[c]PS, poor substrate.
[d]ND, not determined.

KPC-53 and KPC-3 were also tested against some inhibitors including clavulanic acid, tazobactam, and avibactam . Clavulanic acid behaved as a competitive inhibitor for KPC-3, with a K_i value of 41 mM, whereas it behaved as a transient inactivator for KPC-53. In this case, the plot of *ki*AQ versus clavulanic acid concentration was linear, and the second-order acylation rate constants were $k_{+2}/K = 3 \times 10^2$ M^{-1} s^{-1} and $k_{+3} = 0.0016$ s^{-1} . Tazobactam was hydrolyzed by KPC-3 with a k_{cat} of 6 s^{-1} and a k_{cat}/K_m value of 0.022 μM^{-1} s^{-1}.Instead, tazobactam acted as a transient inactivator for KPC-53 with a k_{+2}/K of 2×10^2 M^{-1} s^{-1} and a k_{+3} of 0.0004 s^{-1} . Avibactam behaved as a transient

inactivator of KPC-53, with a k_{+2}/K of 6.3 M^{-1} s^{-1} and a k_{+3} of 0.0008 s^{-1} . In contrast, the KPC-3 plot of *ki* versus the avibactam concentration was not linear, and the inhibition constants were k_{+3} = 0, k_{+2} = 74 s^{-1} , and K = 1.1 x 10^{-2} M (Table 18).

	KPC-53		KPC-3[a]				
Inhibitor	**k_{+3}(s^{-1})**	**k_{+2}/K($M^{-1}s^{-1}$)**	**k_{+2}(s^{-1})**	**k_{+3}(s^{-1})**	**k_{+2}/K($M^{-1}s^{-1}$)**	**K(M)**	**K_i(μM)**
Avibactam	0.0008	6.3	74	0	$6.8x10^3$	$1.1x10^{-2}$	ND
Tazobactam	0.0004	$2x10^2$	-	-	-	-	-
Clavulanic acid	0.0016	$3x10^2$	ND	ND	ND	ND	41

Table 18 Inactivation of KPC-53 and KPC-3 by avibactam, tazobactam, and clavulanic acid. [a]– , acted as a substrate; ND, not determined

5.3.3 Molecular modelling

Molecular modeling was performed to explain the changes in the catalytic parameters observed in KPC-53 versus KPC-3. The three-dimensional (3D) structure of KPC-53 was obtained by comparing its aminoacidic sequence with the known 3D structures KPC-2 (PDB 5MGI) and KPC-3 (PDB 6QWD). The preliminary 3D structure of KPC-53 obtained by the satisfaction of spatial restraints, including the catalytic water molecule, was subsequently optimized for the loop regions, generating five plausible structures.

Every structures showed a good score of the reliability: the model score GA341, which assesses the quality of the model based on the percentage of sequence identity between the known 3D structure and the target, was 1 for all generated structures (a value higher than 0.7 is suggestive of a good prediction);the statistical potential z-DOPE (expected value lower than 0) for all structures were about 21.5 (ranging from 21.47 to 21.69); the last score is represented by the predicted root-mean-squared deviation (RMSD) between the spatial coordinates of the backbone carbon atoms in the model and the native structure that was, for every structures, below 1 (ranging from 0.95 to 0.96).

All simulated KPC-53 structures are characterized by an α-helix, composed of five amino acids extending from E168 to N172 (N170 in KPC-2 and KPC-3), which partially overlays the short α-helix (3 amino acids from E168 to N170) commonly found in KPC-2 and KPC-3. The distance between the Od of N170 (N172 in KPC-53) for all simulated structures and the catalytic water ranges between 2.63 and 2.99 Å, allowing the formation of a hydrogen bond, as observed in all class A serine-β-lactamases. The L167E168 insertion distorts the carbon backbone between the W165 and the E166 (E168 in KPC-53), located in KPC-53, just before the ordered α-helix (L167-S171).

The Cα of this glutamic residue lays from 0.463 to 0.831 Å apart and in any direction if compared to the Cα of E166 in KPC-2 crystallographic structure. The distance between the same carbon atom in KPC-2 and KPC-3 structures is only 0.06 Å. The distance between the W165 Ca is 0.05 Å between KPC-2 and KPC-3 but ranges from 0.30 to 0.90 Å in KPC-53 compared to KPC-2. In all simulated models, E166 Oε is unable to interact via hydrogen-bond with the Nδ of N170 (N172 in KPC-53) except for one model where the distance is 2.79 Å, conceivable with a hydrogen-bond. Finally, the spatial coordinates of the backbone positions of all the residues involved in catalysis—S70, K73, S130, and K234 (K236 in KPC-53) were found to be invariant (Figure 19).

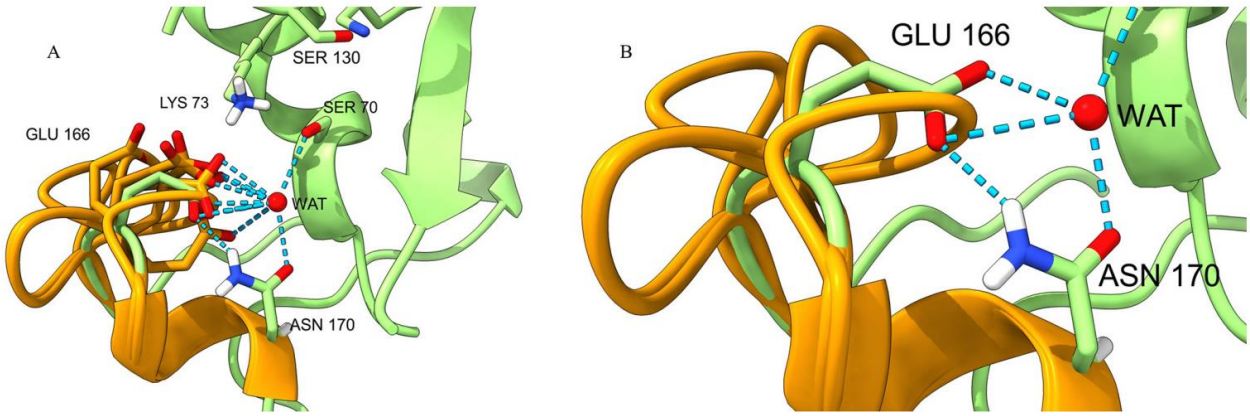

Figure 19 Superimposition of the five modeling-generated models of KPC-53 (orange) with the known crystallographic structure of KPC-2 (green) (PDB 5MGI). (A) Detail of the interactions between E166 of KPC-2 and the five models generated for KPC-53 in relation to the catalytic water molecule. (B) Detail of the deformation of the region between residues R164 and N170 of KPC-2 (green) and R164 and N172 of KPC-53 (orange).

5.4 Discussion

The KPC-53 enzyme has the peculiarity to have the duplication of L167E168 residue in the Ω-loop element. The duplication of these two amino acid residues was described also in KPC-25 (GenBank accession no. NG_051167.1), KPC-31 (GenBank accession no. NG_055494.1), and KPC-40 variants [127]. KPC-31 has an aspartic acid-to-tyrosine (D179Y) substitution at position 179 in the Ω-loop of KPC-3, which is known to confer resistance to CZA [128]. In addition to L167E168 duplication, the KPC-25 and KPC-40 showed Y274H and Y244S substitutions, respectively.

Antimicrobial susceptibility test showed that KPC-53 was more resistant to avibactam than KPC-3: CZA association worked better on *E. coli* XL-1/pBC/KPC-3 than *E. coli* XL-1/pBC/KPC-53 with MIC reductions of 128- and 4-fold, respectively; avibactam acted as an inactivator for KPC-3, unlike KPC-53. The MIC results agree with kinetic data obtained for inhibitors even if MIC values for both KPC-3 and KPC-53 against imipenem and meropenem were low in contrast to the k_{cat} values determined. Low MICs for carbapenems in carbapenemase-producing *E. coli* recombinant strains are not unusual [129].

Comparing the 3D structure of KPC-53 to that of KPC-2/KPC-3, we demonstrated that L167E168 duplication seems to induce the elongation of the short α-helix E168-N170, which in KPC-53 spans from E168 to N172 (N170 in KPC-2 and KPC-3). The Cα backbone of the catalytic residues was found to be invariant with respect to the model templates KPC-2 and KPC-3, although E166 displays in each of the five simulated models for the optimized Ω-loop region, a movement of the backbone carbon. The E166 in KPC-53 is located at the end of the highly ordered region of the new α-helix and the beginning of the highly disordered region of the Ω-loop The L167E168 duplication in KPC-53 destabilized the hydrogen bonding network inside the Ω-loop structure, resulting in a substantial reduction in the catalytic efficiencies toward most of the tested β-lactams. Moreover, the elongation of the α-helix might decrease the flexibility of the loop during the deacylation step and this effect is much more evident for small substrates as carbapenems.

In KPC-53, the distance observed between E166 and N170 seems to affect the deacylation step of the β-lactams hydrolysis with a dramatic reduction in k_{cat} values for most β-lactams. This behavior is remarkable for carbapenems: compared to KPC-3, KPC-53 exhibited 2,700- and 2,125-fold decreases in k_{cat} values for imipenem and meropenem, respectively.

In KPC-53, ceftazidime hydrolysis was 4-fold lower than that of KPC-3, even if their k_{cat}/K_m values were similar to each other. In “in vitro” inhibition experiments, avibactam, tazobactam, and clavulanic acid acted as transient inactivators for KPC-53 with a low deacylation constant. To investigate structural changes in this variant, tryptophan fluorescence measurements and molecular modeling were carried out. Since the fluorescence of tryptophan is affected by the polarity of its microenvironment, the decrease in the intrinsic fluorescence intensity of KPC-53 with respect to KPC-3 indicates that the L167E168 duplication in the Ω-loop led to some local conformational changes. In particular, W165, adjacent to the duplication in the Ω-loop, could be involved in the modulation of intrinsic fluorescence.

Conclusions

In these studies, we confirmed the spread of extended-spectrum β-lactamases and carbapenemases in *Enterobacterale*s such as *K. pneumoniae, E. coli* and *A. baumannii*. A detailed molecular characterization of resistome, including ARGs and MGEs, and virulome were performed on all clinical strains analyzed. To conclude, carbapenem-resistant *Enterobacterales* are a significant concern for patients in health and hospital facilities. Some bacteria of this family are resistant to nearly all β-lactams and other classes of antimicrobial agents. Among *E. coli* and *K. pneumoniae* some ARGs, in particular bla_{KPC-3} gene, were found within strains in the same plasmid and in the same LTCF. So we have ascertained the circulation of these resistance determinants between *K. pneumoniae* and *E. coli*. In Italy, in the last decade, the percentage of carbapenem resistant Gram-negative, increased from 1% to 34%, and hospitals and residential or community facilities represent an important reservoir for class A, B and D carbapenemases, ESBLs, other antibiotic resistance genes and several virulence factors.

So, it is important for all these facilities, including hospital units, to develop effective control measures to prevent outbreaks of MDR strains.

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