UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

XXXII DOCTORAL PROGRAMME IN

PUBLIC HEALTH AND PREVENTIVE MEDICINE

Molecular epidemiology of antimicrobial resistance and virulence in epidemic *Klebsiella pneumoniae* clonal lineages.

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Abstract

Klebsiella pneumoniae is a Gram-negative, encapsulated, nonmotile bacterium belonging to the Enterobacteriaceae family. *K. pneumoniae* is one of the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) recognized as the most common opportunistic pathogens in nosocomial infections. However, over the past three decades, the notoriety of *K. pneumoniae* is due to the emergence of strains that have acquired additional genetic traits and become either antibiotic resistant or hypervirulent, associated with hospital outbreaks and severe community-acquired infections, respectively. The global dissemination of *K. pneumoniae* has been largely attributed to the increasing incidence of extended spectrum beta-lactamase (ESBL) and carbapenem-resistant (CRE) isolates, because of their capability to acquire antimicrobial resistance (AMR) genes primarily due to horizontal gene transfer (HGT) aided by plasmids and mobile genetic elements.

The first study of this thesis analyzes the mechanism of carbapenem resistance acquisition of multi-drug resistant *K. pneumoniae* isolates from 20 neonates in the neonatal intensive care unit (NICU) of the V. Monaldi Hospital in Naples, Italy. Genotype analysis by pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) identified PFGE type A and subtypes A1 and A2 in 17, 2, and 1 isolates, respectively, and assigned all isolates to sequence type (ST) 104. *K. pneumoniae* isolates were resistant to all classes of beta-lactams, including carbapenems, fosfomycin, gentamicin, and trimethoprim–sulfamethoxazole, but susceptible to quinolones, amikacin, and colistin. Conjugation experiments demonstrated that resistance to third-generation cephems and imipenem could be transferred along with an IncA/C plasmid containing the ESBL *bla*SHV-12 and carbapenem-hydrolyzing metallo-beta-lactamase *bla*VIM-1 genes. The plasmid that we called pIncAC_KP4898 was 156,252 bp in size and included a typical IncA/C backbone, which was assigned to ST12 and core genome (cg) ST12.1 using the IncA/C plasmid MLST (PMLST) scheme. pIncAC_KP4898 showed a mosaic structure with *bla*VIM-1 into a class I integron, *bla*SHV-12 flanked by IS6 elements, a mercury resistance and a macrolide 20-phosphotransferase clusters, *ant(3”), aph(3”), aacA4, qnrA1, sul1,* and *dfrA14* conferring resistance to aminoglycosides, quinolones, sulfonamides, and trimethoprim, respectively, and several genes predicted to encode transfer functions and proteins involved in DNA transposition. The acquisition of pIncAC_KP4898 carrying *bla*VIM-1 and *bla*SHV-12 contributed to the spread of ST104 *K. pneumoniae* in the NICU of V. Monaldi Hospital in Naples.

The growing epidemic of infections caused by multidrug-resistant (MDR) Gram-negative bacteria, including CRE strains, has led to the revival of polymyxins worldwide as the last-resort treatment option. Unfortunately, recent data reported a raising trend of colistin-resistant *K. pneumoniae*

isolates collected in the nosocomial setting worldwide. These data also showed how antibiotic resistant and hypervirulent phenotypes are overlapping in clinical isolates, with the increasing scarcity of effective treatments. The second study of the thesis focuses on the molecular epidemiology and virulence profiles of 25 colistin-resistant *K. pneumoniae* blood isolates from the Hospital Agency "Ospedale dei Colli," Naples, Italy. The inactivation of the *mgrB* gene, encoding a negative regulator of the PhoQ/PhoP signaling system, was the most frequent mechanism of colistin resistance found in 22 out of 25 isolates. Of these, ten isolates assigned to ST512 and PFGE types A and A4 showed identical frameshift mutation and premature termination of *mgrB* gene; four isolates assigned to ST258 and PFGE types A1 showed non-sense frameshift mutation and premature termination; three and one isolates assigned to ST258 and PFGE A2 and ST512 and PFGE A3, respectively, had insertional inactivation of *mgrB* gene due to IS5-like mobile element; two isolates assigned to ST101 and 1 to ST392 had missense mutations in the *mgrB* gene; one isolate assigned to ST45 showed insertional inactivation of *mgrB* gene due to IS903-like mobile element. *phoQ* missense mutations were found in 2 isolates assigned to ST629 and ST101, respectively, which also showed a missense mutation in *pmrA* gene. Colistin-resistant *K. pneumoniae* isolates showed variable virulence profiles in *Galleria mellonella* infection assays, with the infectivity of two isolates assigned to ST45 and ST629 being significantly higher than that of all other strains ($P < 0.001$). Interestingly, colistin MIC values proved to make a significant contribution at predicting lethal doses values (LD50 and LD90) of studied isolates in *G. mellonella*. Our data show that MgrB inactivation is a common mechanism of colistin resistance among *K. pneumoniae* in our clinical setting. The presence of identical mutations/insertions in isolates of the same ST and PFGE profile suggests the occurrence of clonal expansion and cross-transmission. Although virulence profiles differ among isolates irrespective of their genotypes, our results suggest that high colistin MIC could predict lower infectivity capability of the isolates.

The success of *K. pneumoniae* as a nosocomial pathogen correlates with high genome plasticity and understanding high-risk clones' ability to adapt and survive in the hospital environment is important to contain the spread of antibiotic resistance. The global dissemination of *K. pneumoniae* carbapenemase KPC-producing *K. pneumoniae* has been largely attributed to the spread of few high-risk STs (ST258, ST11, ST512) associated with human disease. ST101 is an emerging clone, which was identified in different parts of the world with the potential to become a global, persistent public health threat. Recent research suggests the ST101 lineage is associated with an 11% increase in mortality rate in comparison to non-ST101 infections. The third manuscript of the current thesis is a study on the genomic features of ST101 emerging clone to understand what allowed it to succeed and became an epidemic clone. A high-quality, near-finished genome assembly of a MDR

K. pneumoniae isolate from Italy (isolate 4743) was generated, that is a single locus variant of ST101 (ST1685). We demonstrated that the 4743 genome contains virulence features such as an integrative conjugative element carrying the yersiniabactin siderophore (ICEKp3), the mannose-resistant *Klebsiella*-like (type III) fimbriae cluster (*mrkABCDFHIJ*), the ferric uptake system (*kfuABC*), the yersiniabactin receptor gene *fyuA*, a capsular K type K17, and an O antigen type of O1. *K. pneumoniae* 4743 carries the *bla*KPC-2 carbapenemase gene along with genes conferring resistance to aminoglycosides, beta-lactams, fluoroquinolones, fosfomycin, macrolides, lincosamides, and streptogramin B. A comparative genomics analysis of 44 ST101 genomes as well as newly sequenced isolate 4743 identified variable AMR profiles and incompatibility plasmid types, but similar virulence factor profiles. Using Bayesian methodologies, we estimate that the common ancestor for the ST101 lineage emerged in 1990 (95% HPD: 1965 to 2007) and isolates within the lineage acquired *bla*KPC after the divergence from its parental clonal group and dissemination. The identification of virulence factors and antibiotic resistance genes acquired by this newly emerging clone provides insight into the reported increased mortality rates and highlights its potential success as a persistent nosocomial pathogen. With a combination of both colistin resistance, carbapenem resistance, and several known virulence factors, the ST101 genetic repertoire may be a “perfect storm” allowing for a newly emerging, high-risk, extensively antibiotic resistant clone. This high-risk clone appears adept at acquiring resistance and may perpetuate the dissemination of extensive AMR. Greater focus on the acquisition of virulence factors and antibiotic resistance genes is crucial for understanding the spread of antibiotic resistance in *K. pneumoniae*.

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Chapter 1

Introduction

1.1 *Klebsiella pneumoniae,* an emerging opportunistic bacterial pathogen

Klebsiella pneumoniae is a Gram-negative bacterium described for the first time by Carl Friedlander in 1882 and isolated from the lungs of patients who died from pneumonia (Friedlaender, 1882). This bacterium belongs to ESKAPE group that includes six nosocomial pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter spp.*) responsible for high morbidity and mortality in the clinical setting worldwide (Rice et al, 2008; Boucher et al., 2009). *Klebsiella* species cause a variety of opportunistic nosocomial infections in humans, including respiratory tract infections, urinary tract infections (UTIs), and bloodstream infections but are also found in the environment, including plants and animals (Podschun and Ullmann, 1998). Classically, *K. pneumoniae* infections occur in immunocompromised individuals, such as patients undergoing chemotherapy, neonates, the elderly, and are routinely treated with cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole (Tumbarello et al. 1994**).** However, there was an increase of community-acquired invasive infections, primarily in form of liver abscesses, due to the emergence and spread of hypervirulent (HV) strains. *K. pneumoniae* HV strains expanded the number of people susceptible to infections to include those who are healthy and immunocompetent (Chang and Chou, 1995). *K. pneumoniae* strains have become increasingly resistant to antibiotics, rendering infection by these strains very challenging to treat. Accordingly, the World Health Organization has listed *K. pneumoniae* as one of the global priority pathogens in critical need of next-generation antibiotics (World Health Organization, 2017, 2018). Two major types of antibiotic resistance are commonly reported in *K. pneumoniae*. One mechanism involves the expression of extended spectrum beta-lactamases (ESBLs), which render bacteria resistant to cephalosporins and monobactams. The other troubling mechanism of resistance is the expression of carbapenemases, which renders bacteria resistant to almost all available beta-lactams, including the carbapenems (CDC, 2015) with a drastic rise in the incidence of multidrug- resistant (MDR) and extremely drug-resistant (XDR) *K. pneumoniae* phenotypes .

The spread of MDR or XDR *K. pneumoniae* phenotypes (Bialek- Davenet et al., 2014; Bradford et al., 2015; Holt et al., 2015; Pitout et al., 2015; Cerqueira et al., 2017; Logan and Weinstein, 2017; Otter et al., 2017) has favored the use of colistin as the last resort therapeutic option (van Duin et al., 2013). Unfortunately, *K. pneumoniae* clinical isolates are developing different mechanisms of colistin resistance worldwide. These mechanisms include: covalent modifications of lipid A due to

mutations in regulatory genes such as *mgrB*, efflux pumps and capsule hyperproduction and release (Esposito et al, 2018; Jeannot et al., 2017; Poirel et al., 2017; Padilla et al., 2010; Srinivasan et al., 2010; Campos et al., 2004; Lloblet et al., 2008). For these reasons, the spread of MDR *K. pneumoniae* strains has been an area of intensive investigation over the past decade (Pitout et al., 2015; Munoz-Price et al., 2013; Chen et al., 2014 A; Chen et al., 2014 B; Cuzon et al., 2010). Genotyping analysis of *K. pneumoniae* isolates using multilocus sequence typing (MLST) showed the occurrence of distinct clonal complexes (CCs) among bacterial population (Bialek- Davenet et al., 2014). *K. pneumoniae* "high-risk clones" were identified with a global distribution and showed an enhanced ability to colonize, spread, and persist in a variety of niches (Baquero et al., 2013; Bialek- Davenet et al., 2014; Roe et al., 2019). They had the tenacity and flexibility to accumulate and exchange resistance and virulence genes with other bacteria (Bialek- Davenet et al., 2014; Roe et al., 2019).

1.2 *Klebsiella pneumoniae* colonization: a reservoir for health-care associate infections

In healthcare settings, *Klebsiella* bacteria can be spread through person-to-person contact (for example, from patient to patient via the contaminated hands of healthcare personnel) or, less commonly, by contamination of either the environment or medical devices. Once acquired, *K. pneumoniae* readily colonizes human mucosal surfaces, including the gastrointestinal (GI) tract and oropharynx, where the effects of its colonization appear benign (Bagley et al., 1985; Rock et al., 2014, Dao et al., 2014). Colonization rates differ by body site and whether *K. pneumoniae* are community (CA) or hospital-acquired (HA). Specifically, CA *K. pneumoniae* colonize the nasopharynx and the gastrointestinal tract with a percentage from 3 to 15% (Davis and Matsen, 1974; Wolf et al., 2001; Farida et al., 2013, Dao et al., 2014) and 35%, respectively (Davis and Matsen, 1974). Instead, the HA nasopharyngeal and gastrointestinal colonizations are slightly higher, up to 19% (Pollack et al., 1972) and 77% (Podschun and Ullmann, 1998), respectively. Although, in recent studies, the gastrointestinal colonization is estimated around 20% (Martin et al., 2016; Gorrie et al., 2017). The gastrointestinal microbiota is a significant reservoir in terms of risk of transmission and HA infection (Dorman and Short, 2017). From these sites, *K. pneumoniae* strains can gain entry to other tissues and cause severe infections. The mechanism of progression from intestinal *K. pneumoniae* colonization to infection is not clearly understood, but there are some apparent risk factors. Intestinal domination by Proteobacteria, which taxonomically includes *K. pneumoniae*, increases five-fold the risk of bacteremia in allogenic hematopoietic stem cell transplant patients (Taur et al., 2012). Invasive procedures such as endoscopy are a potential further source of endogenous infection (Spach et al., 1993). Individuals with cancer, diabetes mellitus, and

alcoholism are susceptible to CA and HA *K. pneumoniae* infections from colonizing strains because of the impaired bacterial defenses (Tsay et al., 2002; Happel and Nelson, 2005; Tsai et al., 2010). However, also fluid and electrolyte disorders, neurologic disease, and prior hospital admissions have been identified as significantly and independently associated with infection in hospitalized patients (Martin et al., 2016).While various risk factors for infection with *K. pneumoniae* have been identified, risk factors specifically associated with progression from colonization to infection have not been elucidated.

1.3 Core and accessory genome of *Klebsiella pneumoniae*

Within a bacterial species, the core genome is a set of genes that is conserved amongst all members. *K. pneumoniae* has a significantly large genome (mean 5.7 Mbp and 5455 protein coding genes) and the core genome is currently estimated to be comprised of ~2,000 genes (Holt et al., 2015). This means that the majority of the genome is comprised of accessory genes. The accessory genome includes a wide range of genes involved in different cellular pathways such as nitrogen fixation (Fouts et al., 2008), virulence factors and AMR genes (Bi et al., 2015). Accessory genes can be acquired through HGT among bacterial species, as evidenced by the presence of genomic islands and mobile genetic elements in many isolates. The genes encoded in genomic islands can help isolates to adapt to specific sites of infection or colonization (Chen et al., 2010; Zhang et al., 2011; van Aartsen et al., 2012). Elements of the accessory genome can be identified or predicted using various in silico applications, including computation of GC content and comparative genomic analysis (Ou et al., 2007; Zhang et al., 2014). The mean G + C content of *K. pneumoniae* core genes is 58%, whereas that of accessory genes ranges from 20% to >70%, suggesting they originate from a taxonomically diverse array of donors including numerous other members of the Enterobacteriaceae but also diverse groups such as *Acinetobacter*, *Burkholderia*, *Streptomyces*, *Vibrio*, *Xanthomonas* and *Xylella* (Holt et al., 2015).

1.4 *Klebsiella pneumoniae* species

The improvement of DNA sequencing methods allows to identify three distinct *Klebsiella* species in all *K. pneumoniae* isolates (Brisse and Verhoef, 2001; Brisse et al., 2004, 2014; Maatallah et al., 2014; Berry et al., 2015). Primarily, the species identification was based on the core genome variations among *K. pneumoniae* isolates, but recently these species can also be separated by the content of their accessory genome (Holt et al., 2015). The major phylogroup KpI is the specie *K. pneumoniae* (sensu stricto) and includes the majority of opportunistic isolates. Phylogroups KpII and KpIII are identified as *K. quasipneumoniae* and *K. variicola*, respectively (Fig. 1). These three

distinct species vary in their epidemiology of colonization and infection. Approximately 94% of *K. quasipneumoniae* isolates have been recovered from humans with infections of the urinary and respiratory tracts, and some isolates are ESBL-producers (Holt et al., 2015). *K. variicola* was proposed as a new, distinct species based on both genetic and biochemical differences from *K. pneumoniae* (Rosenblueth et al., 2004; Brisse et al., 2014). This specie colonizes a wide range of plant, playing a role in nitrogen fixation and plant growth promotion (Lin L, 2015). The *nif* operon, responsible for nitrogen fixation, can be considered part of the core genome of *K. variicola*, but can also be found in related *Klebsiella* species as part of the inter-species accessory genome (Fouts et al., 2008). While colonization of plants is common, colonization rates in humans is unknown. A recent study reported a higher 30-day mortality rate (29.4%) in patients with bloodstream infections due to *K. variicola* compared to *K. pneumoniae* (13.5%) and *K. quasipneumoniae* (11.1%) species (Maatallah et al., 2014). This increased mortality was not due to any known virulence factors and was significant after controlling for patient co-morbidities, suggesting that *K. variicola* harbors undiscovered and potentially clinically relevant virulence genes. The AMR patterns of 420 *K. pneumoniae* belonging to KpI, KpII and KpIII phylogroups was investigated (Brisse et al., 2004). The data showed that *K. pneumoniae* (KpI) has the highest resistance levels, *K. quasipneumoniae* (KpII) has intermediate resistance levels, and *K. variicola* (KpIII) has the lowest resistance. These three distinct but related phylogroups vary in their host distribution and AMR, likely due to differences in accessory genome composition (Brisse et al., 2004). Furthermore, a genetic exchange across these three species can occur (Holt et al., 2015; Long et al., 2017).

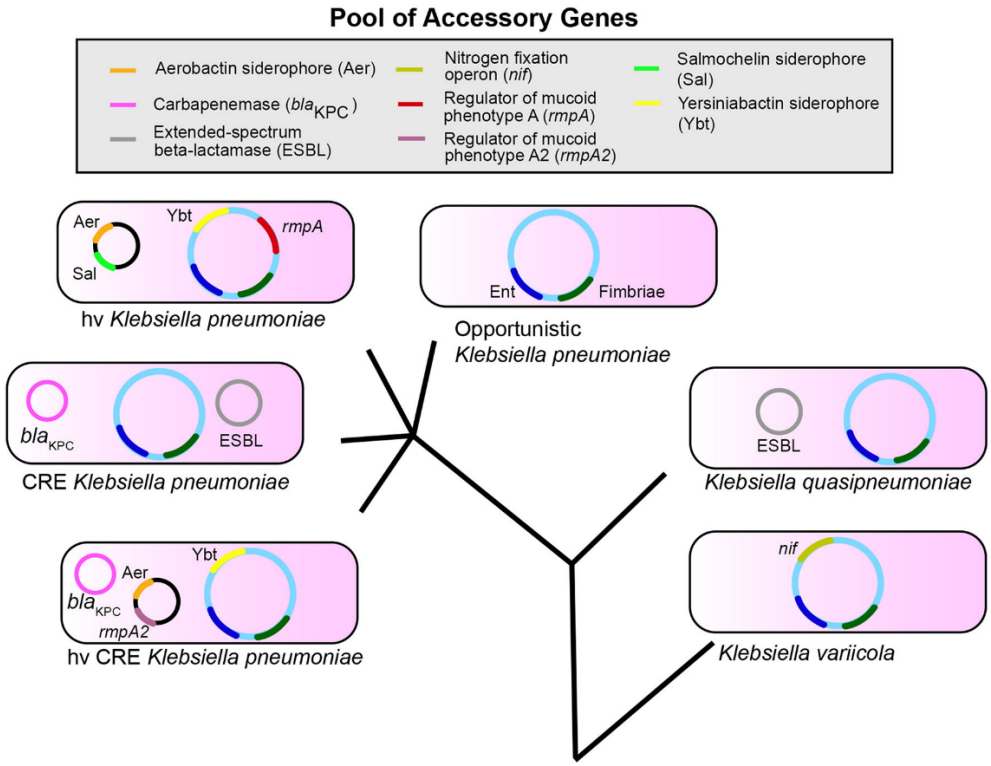

Figure 1. *K. pneumoniae* (KpI), *variicola* (KpII), and *quasipneumoniae* (KpIII) species and their pool of accessory genes. *K. pneumoniae* includes opportunistic, carbapenem-resistant (CRE), hypervirulent (hv) strains and new pathotype (hvCRE) (Martin and Bachman, 2018).

1.5 Antimicrobial resistances in *Klebsiella pneumoniae*

In the last three decades, *K. pneumoniae* has evolved several mechanisms to resist antibiotics. In comparison to *Escherichia coli*, *K. pneumoniae* has acquired double the number (more than 400) of AMR genes (Wyres and Holt, 2018), primarily through HGT aided by plasmids and mobile genetic elements (Pendleton et al., 2013) and occasionally through chromosomal mutations. Other antimicrobial mechanisms involve the over-expression of efflux pumps, reduced membrane permeability, enzymatic degradation of antibacterial drugs and alteration of bacterial proteins that are antimicrobial targets. Figure 2 shows the "Timeline of mobile AMR genes first detected in *K. pneumoniae*":

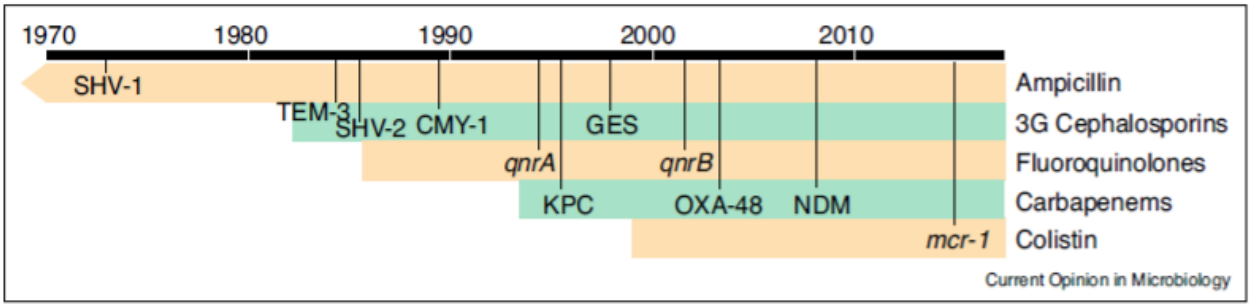

Figure 2. Timeline of mobile AMR genes first detected in *K. pneumoniae*. Shading indicates the period since which isolates of *K. pneumoniae* resistant to each drug class have been reported. Note ampicillin resistance is intrinsic to *K. pneumoniae* due to the chromosomal betalactamase gene SHV-1 (Wyres and Holt, 2018).

1.5.1 β-Lactamase producing *Klebsiella pneumoniae*

Resistance to beta-lactam antibiotics is achieved through hydrolysis of the antibiotic β-lactam ring by beta-lactamases. In *K. pneumoniae*, resistance to some beta-lactams is intrinsic since the enzyme is encoded in the core genome of the species. For example, SHV is consistently found in the chromosome, and corresponding ampicillin resistance is a hallmark of the species (Babini and Livermore, 2000; Bialek-Davenet et al., 2014). Other β -lactamases are part of the accessory genome. In the 1960s the first plasmid-mediated beta-lactamase, TEM-1, was discovered in *E. coli* (Datta and Kontomichalou, 1965). *K. pneumoniae* is also known to harbor plasmid-mediated beta-lactamases, such as AmpC enzymes which confer resistance to most penicillin antibiotics (Jacoby, 2009).

1.5.2 Extended-Spectrum beta-Lactamases

Extended-spectrum beta-lactamases (ESBL) are plasmid based resistance enzymes, able to hydrolyze the third-generation cephalosporins and aztreonam, but are inhibited by clavulanic acid (Bush et al., 1995). The most widely dispersed ESBL gene is CTX-M, variants of which were detected in *Escherichia coli* and *K. pneumoniae* in the late 1980s and early 1990s, having been mobilized out of environmental Enterobacteriaceae (D'Andrea et al., 2013; Canton et al., 2012). CTX-M is present in diverse plasmid backgrounds, resulting in broad dissemination amongst hospital, human commensal, and animal associated microbial populations (Mathers et al., 2015; Woerther et al., 2013).

1.5.3 Resistance to Fluoroquinolones

The introduction of fluoroquinolones in 1990 was met by rapid appearance of associated resistance genes. Fluoroquinolone resistance in *K. pneumoniae* has been associated with mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* (gyrase) and *parC* (topoisomerase IV) genes, acquisition of mobile quinolone resistance genes *qnrA* and *qnrB,* altered permeability (porin loss), and lower uptake of quinolones because of efflux overexpression (Martinez- Martinez et al., 1998; Jacoby et al., 2006; Mazzariol et al., 2002).

1.5.4 Carbapenem-resistant *Klebsiella pneumoniae*

Perhaps due to the selective pressure of treating ESBL infections with carbapenems, carbapenem resistance rendered *K. pneumoniae* the most common carbapenem-resistant *Enterobacteriaceae* (CDC, 2015). Carbapenemases hydrolyze beta-lactams and cephalosporins, but also monobactams and carbapenems, so that no beta-lactam can remain effective. Carbapenemase are assigned to group A, B or D according to the Ambler classification based on analogies of the peptide sequence (Jacoby 2009). The most common carbapenemase in *K. pneumoniae* is KPC (*K. pneumoniae* carbapenemase), but there are others, such as *Serratia marcescens* (SME), non-metallo carbapanemase (NMC), imipenemase (IMI), Guyana extended-spectrum-lactamase (GES), all belonging to group A serine-beta-lactamases. First identified in the 1980s, they are enzymes the activity spectrum of which extends to carbapenems. They remain inhibited, at least in vitro, by beta-lactamase inhibitors, especially clavulanic acid. The second group, the class B metallo-proteins, is represented mainly by NDM-, VIM- and IMP-type enzymes. These metalloproteins hydrolyze all beta-lactam antibiotics, except aztreonam. The third group, class D, is that of oxacillinases, OXA-48 derivatives. They hydrolyze penicillins, 1st generation cephalosporins, and carbapenems; they are resistant to beta-lactamase inhibitors, and weakly active against 2nd and 3rd generation cephalosporins such as cefotaxime or ceftazidime, and hydrolyze only partly carbapenems (Logan and Weinstein, 2017) (Fig. 3).

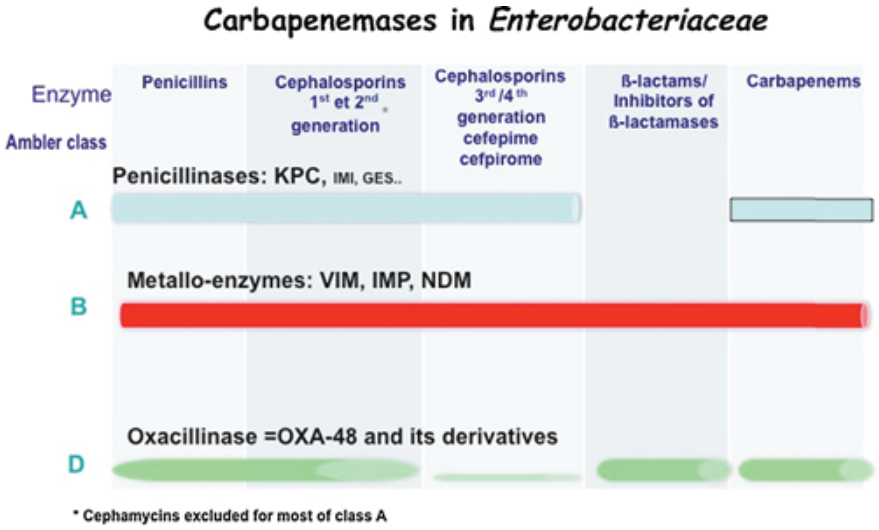

Figure 3. Activity spectrum of carbapenemases in *Enterobacteriaceae* (Nordmann, 2014).

1.5.5 Colistin resistance

Acquired resistance to polymyxins has been identified in several genera of the *Enterobacteriaceae*, such as *Klebsiella*, *Escherichia*, *Enterobacter*, and *Salmonella*. The most common mechanism of polymyxin-resistance consists in modification of the LPS via cationic substitution. Similar to what is observed in strains that are naturally resistant to colistin, addition of cationic groups phosphoethanolamine (pEtN) and/or 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the LPS is responsible for acquisition of colistin resistance in *Enterobacteriaceae*. A large panel of genes are involved in modification of the LPS (Fig. 4), including genes and operons coding for enzymes that are directly involved in LPS modifications (genes responsible for synthesis of cationic groups and/or their addition to the LPS), i.e., the *pmrC* gene, the *pmrE* gene, and the *pmrHFIJKLM* operon; regulatory genes, such as those encoding proteins involved in the *PmrAB* and *PhoPQ* two-component systems and the regulators of these two-component systems, i.e., the *mgrB* gene, which negatively regulates the *PhoPQ* system, and the newly described *crrAB* two-component regulatory system, which regulates the *PmrAB* system (Jeannot et al., 2017; Poirel et al., 2017). A single transferable mechanism of resistance was identified due to the acquisition of *mcr-1* gene. The plasmid-mediated *mcr-1* gene was described first for *E. coli* and *K. pneumoniae* isolates recovered in China between 2011 and 2014 (Liu et al., 2016). The encoded MCR-1 protein is a member of the phosphoethanolamine transferase enzyme family. Its acquisition results in the addition of phosphoethanolamine to lipid A, and consequently in a more cationic LPS, similarly to the chromosomal mutations mentioned above.

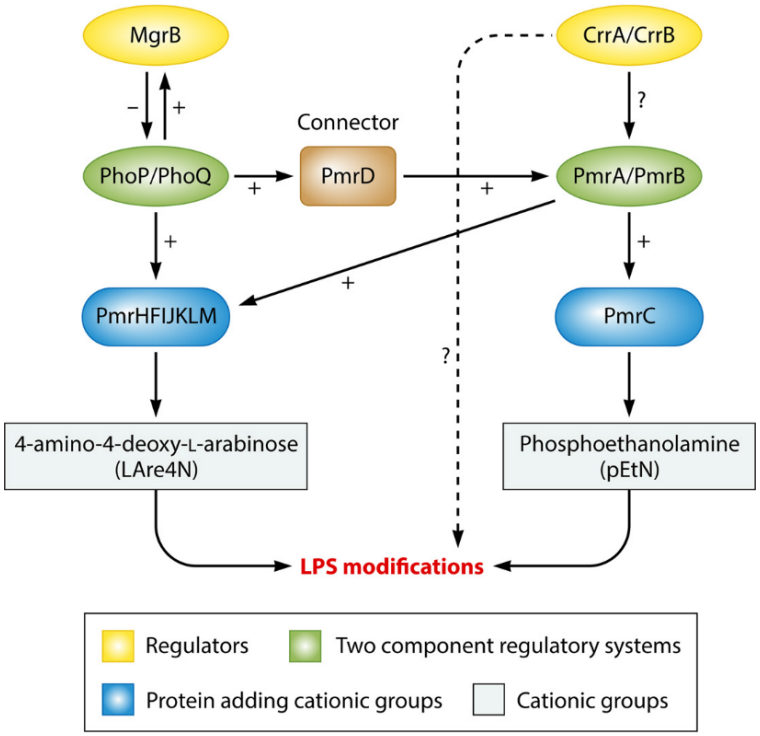

Figure 4. Regulatory pathways of LPS modifications in *K. pneumoniae* (Poirel L., et al., 2017)

1. 6 Virulence factors in *Klebsiella pneumoniae*

During the infective process, *K. pneumoniae* must overcome mechanical and chemical barriers and escape host humoral and cellular innate defenses. After gaining access to the host, the invading organisms are recognized by the immune cells through the pattern recognition receptors that trigger production of various immune mediators.

To date, there are four major classes of virulence factors that have been well characterized in *K. pneumoniae* and consist of capsule, lipopolysaccharide (LPS), siderophores, and fimbriae, also known as pili (Fig. 5).

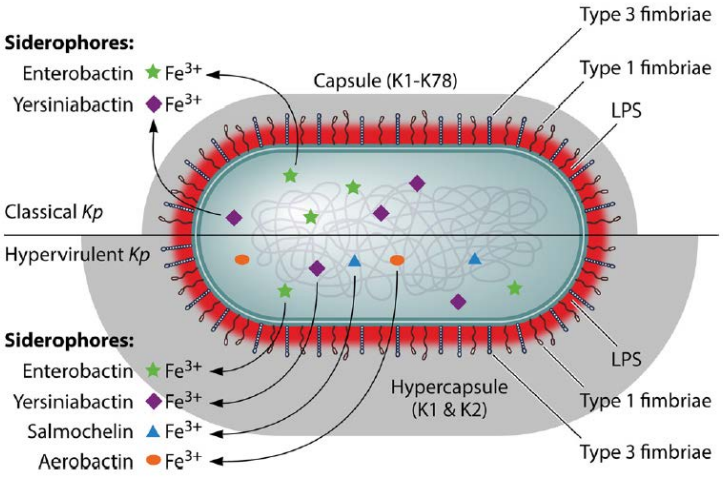

Figure 5. Virulence factors in non- and HV *K. pneumoniae* strains. There are four well-characterized virulence factors for pathogenic *K. pneumoniae*: capsule, LPS, fimbriae (type 1 and type 3), and siderophores (Paczosa et al., 2016).

1.6.1 Capsule

During *K. pneumoniae* infection, capsule protects against the host immune response through multiple mechanisms, including inhibiting phagocytosis by immune cells, preventing activation of the early immune response, and abrogating lysis by complement and antimicrobial peptides. The HV *K. pneumoniae* strains produce a hypercapsule, also known as being hypermucoviscous, which consists of a mucoviscous exopolysaccharide bacterial coating that is more robust than that of the typical capsule and may contribute significantly to the pathogenicity of HV *K. pneumoniae* (Yu et al., 2006; Fang et al., 2004; Yeh et al., 2007). Based on the composition of capsular polysaccharides (CPs) of *K. pneumoniae*, 78 distinct capsular serotypes (K1 to K78) have been recognized. Of note, the vast majority of HV *K. pneumoniae* strains belong to serotypes K1 and K2. Particularly, the serotype K1 strains belong to clonal complex 23 (CC23) in which the major of HV strains converge.

1.6.2 Lipopolysaccharide

LPS, also known as endotoxin, is a major and necessary component of the outer leaflet of the outer membrane of all Gram-negative bacteria and it is typically comprised of an O antigen, a core oligosaccharide, and lipid A. There have been only 9 different O-antigen types identified in *K. pneumoniae* isolates, and O1 is the most common (Hansen et al., 1999). Certain *K. pneumoniae* strains may modify the LPS to a degree that is not recognized by the host cells and others may use capsule to mask LPS from detection by toll-like receptor (TLR4) receptors (Llobet E, 2015).

1.6.3 Type 1 and 3 Fimbriae

Fimbriae represent another class of *K. pneumoniae* virulence factors and are important mediators of *K. pneumoniae* adhesion to epithelial and immune cells as well as to abiotic surfaces. In *K. pneumoniae*, type 1 and 3 fimbriae are the major adhesive structures that have been characterized as pathogenicity factors and are encoded by the *fim* gene cluster and the *mrkABCD* gene cluster, respectively (Struve et al., 2008, Tarkkanen et al., 1998). Four other adhesive structures have been identified in *K. pneumoniae*, including another fimbria called KPF-28, a non fimbrial factor called CF29K, and a capsule-like material (Di Martino et al., 1996, Darfeuille-Michaud et al., 1992; Favre-Bonte et al., 1995). These structures were found to confer to *K. pneumoniae* the capability to bind to human carcinoma or intestinal cell lines, suggesting an involvement of these genes in GI tract colonization (Di Martino et al., 1996). The most important clinically significant roles for fimbriae may be in biofilm formation and binding to abiotic surfaces, as the ability of *K. pneumoniae* to bind to surfaces, such as indwelling catheters or other devices, provides it with an ability to seed vulnerable sites and to persist in patients (Struve et al., 2009).

1.6.4 Siderophores

The ability of *K. pneumoniae* to steal iron from the host is critical for its growth and replication. Four iron-acquisition molecules were identified as siderophores in *K. pneumoniae*: yersiniabactin, enterobactin, salmochelin and aerobactin. Yersiniabactin was originally discovered in the Gram-negative bacterial pathogen Yersinia as part of a Yersinia high-pathogenicity island, but this siderophore has since been identified in other bacteria, including *K. pneumoniae* (Bach et al., 2000). The *irp* gene cluster encode the proteins for yersiniabactin synthesis and *ybt*, *fyu* and *ybtQ* genes encode the transporters required for its secretion (Hsieh et al., 2008; Lawlor et al., 2007; Bach et al., 2000). Interestingly, yersiniabactin has been observed in only ~18% of classical but 90% of HV *K. pneumoniae* clinical isolates (Hsieh et al., 2008, Bachman et al., 2011). In *K. pneumoniae*, enterobactin is ubiquitous expressed among both classical and HV *K. pneumoniae* strains and is therefore considered to be the primary iron uptake system utilized by these bacteria. The genes that are required for enterobactin biosynthesis are carried on the chromosome in the *entABCDEF* gene cluster, while the *fepABCDG* gene cluster encodes the proteins that mediate its transport, with *fepA* specifically encoding its uptake receptor (Hsieh et al., 2008; Müller et al., 2009). Aerobactin and salmochelin are acquired via HGT by *K. pneumoniae*. The encoding genes of aerobactin and salmochelin are co-located on the so-called ‘virulence plasmids’ of *K. pneumoniaea*. The best characterized virulence plasmids are the 224 kbp plasmid pK2044 from serotype K1, sequence type

(ST) 23 strain NTUH-K2044; the 219 kbp plasmid pLVPK from K2, ST86 strain CG43; and the 121 kbp plasmid Kp52.145pII from serotype K2, ST66 strain Kp52.145 (strain also known as 52145 or B5055; plasmid also known as pKP100) (Naffis et al., 1986; Wu et al., 2009; Chen et al., 2004; Lery et al., 2014). Aerobactin is a citrate-hydroxamate siderophore present in 93 to 100% of HV *K. pneumoniae* isolates (Yu et al., 2008, Podschun et al.,1993; El Fertas-Aissani et al., 2013; Vernet et al., 1995). The presence of aerobactin is always associated with elevated expression of the capsule, although not all hyper-capsulated *K. pneumoniae* strains possess aerobactin siderophore (Yu et al., 2008; Miethke et al., 2007; Vernet et al., 1995). This association was explained by the localization of the aerobactin gene cluster, *iucABCD*, on the same virulence plasmids that carry *rmpA*, an enhancer of capsule production (Hsieh et al., 2008; Chen et al., 2004; Tang et al., 2010). Salmochelin is a c-glucosylated form of enterobactin (Fischbach et al., 2005; Fischbach et al., 2006). The synthesis, excretion, and uptake of salmochelin requires five genes, *iroBCDE* operon and the adjacent, convergently oriented iroN gene, an outer membrane receptor through which ferric salmochelin is internalized (Bäumler et al., 1998). Importantly, this modification prevents the binding of salmochelin by lipocalin-2, thus preventing siderophore neutralization and lipocalin-2-dependent induction of inflammation (Fischbach et al., 2006). Recent data suggest that HV *K. pneumoniae* strains have the ability to produce larger amounts and biologically more active iron-acquisition molecules than nonvirulent strains, a mechanism that may contribute to virulence and pathogenesis (Russo et al. 2011).

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Chapter 2

A novel IncA/C1 group conjugative plasmid, encoding VIM-1 Metallo-Beta-Lactamase, mediates the acquisition of carbapenem resistance in ST104 *Klebsiella pneumoniae* isolates from neonates in the Intensive Care Unit of V. Monaldi Hospital in Naples.

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Front Microbiol. 2017 Nov 3;8:2135.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 03 November 2017
doi: 10.3389/fmicb.2017.02135

A Novel IncA/C1 Group Conjugative Plasmid, Encoding VIM-1 Metallo-Beta-Lactamase, Mediates the Acquisition of Carbapenem Resistance in ST104 *Klebsiella pneumoniae* Isolates from Neonates in the Intensive Care Unit of V. Monaldi Hospital in Naples

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OPEN ACCESS

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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 26 July 2017
Accepted: 19 October 2017
Published: 03 November 2017

Citation:
Esposito EP, Gaiarsa S, Del Franco M, Crivaro V, Bernardo M, Cuccurullo S, Pennino F, Triassi M, Marone P, Sassera D and Zarrilli R (2017) A Novel IncA/C1 Group Conjugative Plasmid, Encoding VIM-1 Metallo-Beta-Lactamase, Mediates the Acquisition of Carbapenem Resistance in ST104 Klebsiella pneumoniae Isolates from Neonates in the Intensive Care Unit of V. Monaldi Hospital in Naples. Front. Microbiol. 8:2135. doi: 10.3389/fmicb.2017.02135

The emergence of carbapenemase producing Enterobacteriaceae has raised major public health concern. The aim of this study was to investigate the molecular epidemiology and the mechanism of carbapenem resistance acquisition of multidrug-resistant *Klebsiella pneumoniae* isolates from 20 neonates in the neonatal intensive care unit (NICU) of the V. Monaldi Hospital in Naples, Italy, from April 2015 to March 2016. Genotype analysis by pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) identified PFGE type A and subtypes A1 and A2 in 17, 2, and 1 isolates, respectively, and assigned all isolates to sequence type (ST) 104. *K. pneumoniae* isolates were resistant to all classes of β-lactams including carbapenems, fosfomycin, gentamicin, and trimethoprim–sulfamethoxazole, but susceptible to quinolones, amikacin, and colistin. Conjugation experiments demonstrated that resistance to third-generation cephems and imipenem could be transferred along with an IncA/C plasmid containing the extended spectrum β-lactamase bla_{SHV-12} and carbapenem-hydrolyzing metallo-β-lactamase bla_{VIM-1} genes. The plasmid that we called pIncAC_KP4898 was 156,252 bp in size and included a typical IncA/C backbone, which was assigned to ST12 and core genome (cg) ST12.1 using the IncA/C plasmid MLST (PMLST) scheme. pIncAC_KP4898 showed a mosaic structure with bla_{VIM-1} into a class I integron, bla_{SHV-12} flanked by IS6 elements, a mercury resistance and a macrolide 2′-phosphotransferase clusters, *ant(3″)*,

aph(3″), *aacA4*, *qnrA1*, *sul1*, and *dfrA14* conferring resistance to aminoglycosides, quinolones, sulfonamides, and trimethoprim, respectively, several genes predicted to encode transfer functions and proteins involved in DNA transposition. The acquisition of pIncAC_KP4898 carrying bla_{VIM-1} and bla_{SHV-12} contributed to the spread of ST104 *K. pneumoniae* in the NICU of V. Monaldi Hospital in Naples.

Keywords: carbapenemase producing *Klebsiella pneumoniae*, VIM-1 carbapenemase, IncA/C plasmid, horizontal gene transfer, neonatal intensive care unit

INTRODUCTION

The spread of carbapenem-resistant Enterobacteriaceae (CRE) has increased globally and these strains have become endemic in several countries including Italy. CRE may colonize or infect patients both in the hospital and in the community setting (Nordmann and Poirel, 2014; Del Franco et al., 2015; Onori et al., 2015; Pitout et al., 2015; Conte et al., 2016; Logan and Weinstein, 2017). The prevalence of CRE infections is increasing among children and neonates also (Logan, 2012; Logan et al., 2015; Zhu et al., 2015).

The international spread of CRE is primarily due to clonal expansion of isolates belonging to *Klebsiella pneumoniae* and *Escherichia coli* epidemic clonal lineages (Bialek-Davenet et al., 2014; Nordmann and Poirel, 2014; Del Franco et al., 2015; Gaiarsa et al., 2015; Pitout et al., 2015; Conte et al., 2016; Logan and Weinstein, 2017).

Additionally, CRE dissemination is contributed by horizontal gene transfer of carbapenemase genes carried by transposons and plasmids (Pitout et al., 2015; Logan and Weinstein, 2017). Class B metallo-β-lactamases (MBLs) (IMP, VIM, NDM), class A (KPC) or class D (OXA-48) serine carbapenemases have been isolated worldwide (Nordmann and Poirel, 2014; Del Franco et al., 2015; Pitout et al., 2015; Conte et al., 2016; Giani et al., 2017; Grundmann et al., 2017; Khan et al., 2017; Logan and Weinstein, 2017; Matsumura et al., 2017). Of these, MBLs show increasing clinical relevance because they cannot be neutralized by the available β-lactamase inhibitors and are able to horizontally disseminate via mobile genetic elements. Among acquired MBLs, VIM- and NDM-type enzymes are those having the widest geographical distribution and range of bacterial hosts (Di Pilato et al., 2014; Peirano et al., 2014; Zurfluh et al., 2015; Khan et al., 2017; Matsumura et al., 2017).

Because intestinal carriage of CRE is an important source of transmission, guidelines have been established worldwide to monitor and isolate CRE carriers in health care facilities (Nordmann and Poirel, 2014; Viau et al., 2016).

The aim of this study was to analyze the molecular epidemiology of VIM-1 producing *K. pneumoniae* isolates from intestinal carriers in neonatal intensive care unit (NICU) of an Italian hospital in Naples and to characterize the structure of the conjugative plasmid, which mediates the horizontal transfer of carbapenem resistance.

MATERIALS AND METHODS

Setting

The NICU of V. Monaldi Hospital in Naples is a tertiary care level NICU and consists of three rooms and 16 cot spaces with a 1:2–1:3 nurses/neonates ratio. The NICU serves approximately 260 admissions per year and admits exclusively babies from the regional Newborn Emergency Transport Service or Territorial Emergency Service and through the transfer from the internal departments of Pediatric Cardiology and Pediatric Heart Surgery. In case of necessity, the department performs repeated hospitalizations for some particular types of newborns (lower birth weight, heart disease, etc.). Active patient-based surveillance of healthcare-associated infections on neonates with >2 days NICU stay is performed as previously described (Horan et al., 2008; Crivaro et al., 2015). Surveillance of CRE in V. Monaldi Hospital, based on rectal swabs at hospital admission, is performed according to the guidelines of European Centre for Disease Prevention and Control [ECDC] (2012) and the Ministero della Salute (2013), Italy.

Bacterial Strains and Microbiological Methods

Klebsiella pneumoniae isolates were identified using the Vitek 2 automatic system and the ID-GNB card according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France) as previously described (Del Franco et al., 2015).

Antimicrobial Susceptibility Testing

Carbapenem resistance of Enterobacteriaceae was screened using the meropenem disk alone as previously described (Pournaras et al., 2013). Identification of MBL activity was performed using imipenem + EDTA 15 + 750 μg combined disk method (ROSCO Diagnostica A/S, Taastrup, Denmark). Susceptibility tests were performed using the Vitek 2 system and the AST-GN card (bioMérieux, Marcy l'Etoile, France); carbapenem and colistin susceptibility were evaluated by broth microdilution in Mueller–Hinton broth II (MHBII) according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2015). Breakpoints values were those recommended by the EUCAST (2016).

Molecular Analysis of Antimicrobial Resistance Genes

Characterization of β-lactamase genes was performed as previously described (Poirel et al., 2011). Two multiplex PCRs were set up, the reaction no. 1 including detection of bla_{KPC}

and $bla_{OXA-48-like}$ and the reaction no. 2 including detection of bla_{IMP}, bla_{VIM}, and bla_{NDM}. The following thermal cycling conditions were used: 3 min at 94°C and 35 cycles of amplification consisting of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°c with 5 min at 72°C for the final extension. PCR products were analyzed by electrophoresis in a 1.8% agarose gel stained with ethidium bromide. The following strains were used as positive quality controls: *Acinetobacter baumannii* AC 54/97 (Riccio et al., 2000), *A. baumannii* 161/07 (Bonnin et al., 2012), *K. pneumoniae* D001 (Pournaras et al., 2013), *K. pneumoniae* 1, *K. pneumoniae* 2, and *E. coli* 6 (Del Franco et al., 2015) for bla_{IMP-2}, bla_{NDM-1}, bla_{OXA-48}, bla_{KPC-2}, bla_{KPC-3}, and bla_{VIM-1} carbapenemase genes, respectively. The full-length alleles of bla_{VIM} were amplified using primers 5′VIM1 and 3′VIM1 shown in Supplementary Table S1. Sanger DNA sequencing and identification of deduced amino acid sequences were performed as previously described (Del Franco et al., 2015).

PFGE Typing and Dendrogram Analysis

Klebsiella pneumoniae isolates were genotyped by *Xba*I DNA macrorestriction, pulsed-field gel electrophoresis (PFGE) and dendrogram analysis as described previously (Del Franco et al., 2015).

MLST Analysis

Klebsiella pneumoniae isolates were typed using the Institut Pasteur's MLST (multi-locus sequence typing) scheme (Diancourt et al., 2005) and primes and PCR conditions available at http://bigsdb.pasteur.fr/klebsiella/primers_used.html eBURST analysis of profiles available at http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public&page=profiles was performed as described previously (Feil et al., 2004). Minimum spanning trees of sequence types (STs) were built by Phyloviz using the goeBURST algorithm (Francisco et al., 2012).

Conjugative Transfer of Carbapenem Resistance and Plasmid Typing

Filter mating experiments were performed using sodium azide resistant *E. coli* J53 (Jacoby and Han, 1996) as recipient strain in the presence of either 5 μg/ml imipenem and 100 mg/l sodium azide or 50 μg/ml ampicillin and 100 μg/ml sodium azide. The frequency of transfer was calculated as the number of transconjugants divided by the number of surviving recipients as previously described (Zarrilli et al., 2005). Plasmids typing was performed by PCR-based replicon typing (PBRT) kit (Diatheva s.r.l., Fano, Italy) using previously described primers and conditions (Carattoli et al., 2005, 2006).

Whole-Genome Sequencing and Plasmid Reconstruction

DNA was extracted from the *K. pneumoniae* parental strain, and from the *E. coli* strain before and after transconjugation using a DNeasy Blood & Tissue Kit according to the manufacturer's instructions (Qiagen, Milan, Italy). Whole genome sequencing was performed using an Illumina Miseq platform with a 2 by 250 paired-end run after Nextera XT paired-end library preparation. Sequencing reads from the *K. pneumoniae* parental strain and from the *E. coli* strain before the transconjugation obtained in this study were assembled using the software SPAdes (Bankevich et al., 2012) with accurate settings.

The BIGSdb genome database software (Jolley and Maiden, 2010) was used in the BIGSdb-Kp database[1] to identify genes associated with virulence, heavy metal and drug resistance in *K. pneumoniae* 4898 genome sequence. *In Silico* detection of plasmids was performed using PlasmidFinder Web tool at https://cge.cbs.dtu.dk/services/PlasmidFinder/ as previously described (Carattoli et al., 2014). Sequencing reads of the *E. coli* transconjugant strain containing the plasmid were mapped to the assembled genome of the same *E. coli* sequenced prior to transconjugation, using the mapping software Bowtie2 (Langmead and Salzberg, 2012). All non-mapping reads were extracted and assembled using SPAdes (Bankevich et al., 2012) with accurate settings. Presence of the obtained contigs in the original *K. pneumoniae* genome was verified by blast-searching followed by manual analysis, i.e., all the newly obtained contigs that aligned with the *K. pneumoniae* assembly with an identity over 95% were kept. The co-linearity of the contigs was assessed using Bandage tool for visualizing assembly graphs (Wick et al., 2015). The connections indicated by Bandage were used as starting point for finishing experiments by PCR and Sanger sequencing to bridge the ends of contigs (see Supplementary Table S1 for a complete list of PCR primers used).

Plasmid *in Silico* Typing and Annotation

Plasmid MLST (PMLST) and core genome PMLST (cgPMLST) analysis of Inc A/C plasmid profiles available at https://pubmlst.org/bigsdb?db=pubmlst_plasmid_seqdef was performed as previously described (Hancock et al., 2017). The 28 IncA/C conserved genes from each PMLST profile were aligned using Muscle (Edgar, 2004). Unreliable positions were removed from each alignment using Gblocks (Castresana, 2000). All alignments were concatenated and used as input for a maximum likelihood phylogenetic analysis, which was performed with the software PhyML 3.0 (Guindon et al., 2010) using the GTR substitution model. PMLST minimum spanning trees were built by Phyloviz (Francisco et al., 2012). The gene annotation of the plasmid was performed using the software Prokka (Seemann, 2014) followed by accurate manual control, based on blast-searches against the nr-protein database. Inverted repeats were identified manually, based on the sequence and patterns found in Hancock (AAC 2017).

Nucleotide Sequence Accession Numbers

The genome sequences of *K. pneumoniae* 4898 and plasmid pIncAC-KP4898 have been deposited in the GenBank nucleotide database under accession numbers FWYI00000000.1 and KY882285, respectively.

[1]http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public&page=sequenceQuery

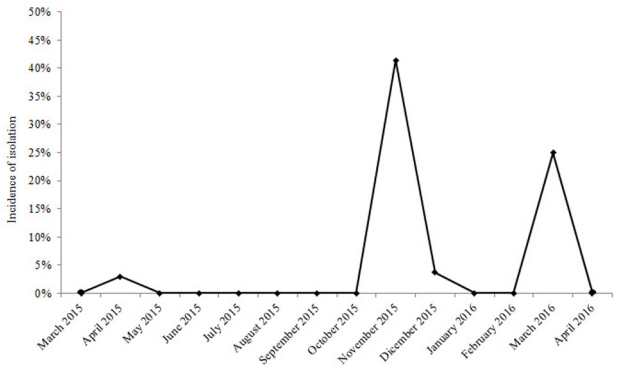


FIGURE 1 | Incidence of isolation of VIM-1 carbapenemase producing ST104 *Klebsiella pneumoniae* from neonates in the NICU of V. Monaldi Hospital in Naples, Italy, during 2015 and 2016.

Ethics Statement

The study has been evaluated by the local Ethics committee (Comitato Etico Università degli Studi della Campania "Luigi Vanvitelli" AOU "Luigi Vanvitelli" – AORN "Ospedali dei Colli") (protocol number 421/2017). Because the patients included in the study were anonymized, no written informed consent was required.

RESULTS

Epidemiology of CR *K. pneumoniae* in the NICU and Infection Control Measures

The emergence of carbapenem-resistant (CR) *K. pneumoniae* was observed in the NICU of V. Monaldi Hospital from October 2015 to March 2016, when CR *K. pneumoniae* were isolated from rectal swabs of 19 neonates into two consecutive clusters. Only one sporadic CR *K. pneumoniae* isolate was obtained from a rectal swab of a neonate in the NICU of the hospital during the previous 22 months, while CRE were occasionally isolated from rectal swabs and other clinical specimens of adult patients in other wards of the hospital (**Figure 1** and data not shown). Immediately following the first isolation of CR *K. pneumoniae*, a multimodal infection control program was implemented in the NICU, which included: weekly or biweekly screening for CRE at rectal swab of hospitalized neonates, increased frequency of environmental cleaning using chloride derivatives at 1100 ppm, reinforce handwashing compliance before and after patient contact, and daily visits to the ward by the hospital Infection Control Nurse. Colonized neonates were isolated either structurally or following strict adherence to contact precautions and staff cohorting was performed. During the two clusters, the ward was temporarily closed to external admissions. Environmental microbiological investigation of room surfaces, equipment, and staff hands failed to identify sources or reservoirs of CR *K. pneumoniae*. Neonates' mothers were not screened for the presence of CR *K. pneumoniae*. Because all neonates with CR *K. pneumoniae* isolation did not show signs of infection, they did not receive antimicrobial therapy against CR *K. pneumoniae* and were not eradicated before discharge. The cluster ended on March 2016 when the last CR *K. pneumoniae* intestinal carrier neonate was discharged from the ward (**Figure 1**).

Antimicrobial Susceptibility Testing and Characterization of Carbapenemase Genes in CR *K. pneumoniae* Isolates

All CR *K. pneumoniae* isolates from neonates in the NICU showed a MDR phenotype. In fact, they exhibited resistance or intermediate resistance to carbapenem (imipenem, meropenem, ertapenem), resistance to aminopenicillins, ureidopenicillins, third and fourth generation of cephalosporins (ceftazidime, cefotaxime, cefepime), gentamicin, fosfomycin, and trimethoprim–sulfamethoxazole, but were susceptible to amikacin, ciprofloxacin, and colistin (**Table 1**). All *K. pneumoniae* isolates gave a positive result in the MBL-assay performed using the imipenem–EDTA combined disk method. PCR and sequence analysis identified the presence of bla_{VIM-1} but not any of the other carbapenemase genes tested in all CR *K. pneumoniae* isolates from NICU (**Figure 2**).

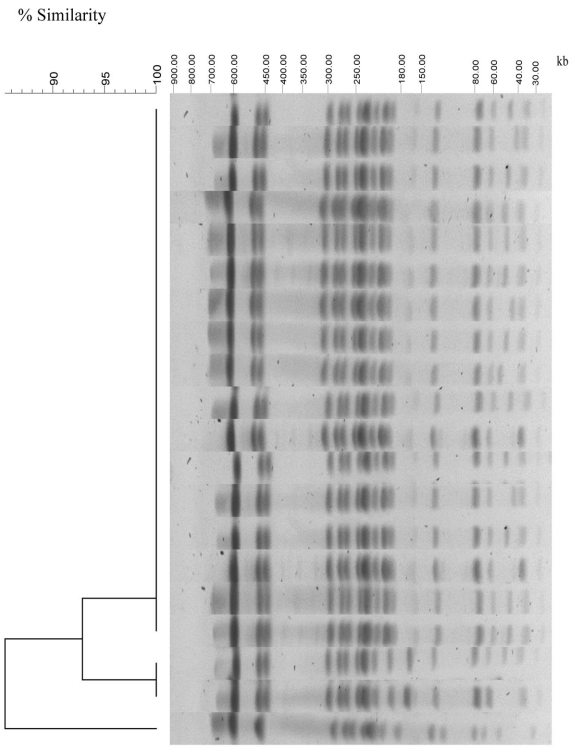
Molecular Epidemiology of CR *K. pneumoniae*

To assess whether the increase of CR *K. pneumoniae* isolation in the NICU was caused by the spread of epidemic strains, all 20 CR *K. pneumoniae* isolates from 20 neonates were genotyped by *Xba*I digestion, PFGE, and dendrogram analysis. Molecular analysis identified an identical macrorestriction pattern in 17 isolates, which we named PFGE type A, whereas two isolates showing difference in the migration of one band and one isolate

TABLE 1 | Antibiotic susceptibility profiles of MDR *K. pneumoniae* in the NICU.

	MIC* (mg/l)		
Antibiotic	***K. pneumoniae* (20 total stains)**		
	MIC_{50}	**MIC_{90}**	**Range**
Amoxicillin	>32	>32	>32
Piperacillin–tazobactam	>128	>128	>128
Ceftazidime	>64	>64	>64
Cefotaxime	>64	>64	>64
Cefepime	>64	>64	32 to >64
Imipenem	8	>16	8 to >16
Meropenem	>16	>16	>16
Ertapenem	4	4	4
Fosfomycin	>128	>128	>128
Amikacin	≤2	≤2	≤2
Gentamicin	4	4	4
Ciprofloxacin	0.5	1	0.5–1
Trimethoprim–sulfamethoxazole	>320	>320	>320
Tigecycline	≤0.5	≤0.5	≤0.5
Colistin	≤0.5	≤0.5	≤0.5

**Minimal inhibitory concentration.*



Isolate nr.	Taxonomic identification	Isolation date	PFGE	MLST	Carbapenemase
10	*K. pneumoniae*	10-12-15	A	104	VIM-1
11	*K. pneumoniae*	10-11-15	A	104	VIM-1
12	*K. pneumoniae*	18-11-15	A	104	VIM-1
14	*K. pneumoniae*	16-11-15	A	104	VIM-1
15	*K. pneumoniae*	09-03-16	A	104	VIM-1
16	*K. pneumoniae*	22-03-16	A	104	VIM-1
17	*K. pneumoniae*	22-03-16	A	104	VIM-1
18	*K. pneumoniae*	22-03-16	A	104	VIM-1
19	*K. pneumoniae*	22-03-16	A	104	VIM-1
2	*K. pneumoniae*	16-11-15	A	104	VIM-1
20	*K. pneumoniae*	22-03-16	A	104	VIM-1
3	*K. pneumoniae*	16-11-15	A	104	VIM-1
4	*K. pneumoniae*	18-11-15	A	104	VIM-1
5	*K. pneumoniae*	16-11-15	A	104	VIM-1
7	*K. pneumoniae*	18-11-15	A	104	VIM-1
8	*K. pneumoniae*	16-11-15	A	104	VIM-1
9	*K. pneumoniae*	18-04-15	A	104	VIM-1
1	*K. pneumoniae*	18-11-15	A1	104	VIM-1
6	*K. pneumoniae*	18-11-15	A1	104	VIM-1
13	*K. pneumoniae*	18-11-15	A2	104	VIM-1

FIGURE 2 | Genotypic analysis of VIM-1 carbapenemase producing *K. pneumoniae* isolates in the NICU. Dendrogram analysis of *Xba*I PFGE profiles of VIM-1 carbapenemase producing *K. pneumoniae* isolates from the NICU. Percentage of similarity and sizes in kilobase (kb) of lambda DNA molecular mass markers are indicated. Isolate number, taxonomic identification, isolation date, PFGE type, MLST, and carbapenemase are shown also.

in the migration of four bands and a similarity of >85% at dendrogram analysis were classified into subtypes A1 and A2, respectively (**Figure 2**). The above data indicated that the increase of CR *K. pneumoniae* isolation in the NICU was caused by the spread of a single VIM-1-producing *K. pneumoniae* epidemic genotype. MLST analysis assigned CR *K. pneumoniae* isolates with PFGE type A and subtypes A1 and A2 to ST104 (**Figure 2**). *K. pneumoniae* ST104 isolate from milk during bovine mastitis and *K. pneumoniae* ST1923 and ST1942 isolates from human blood and human feces, respectively, which were single-locus variants of ST104, and 21 other STs, which were double-locus variants of ST104, were reported worldwide and found in Klebsiella PubMLST isolates database[2] (**Figure 3**).

Genomic Features of VIM-1-Producing *K. pneumoniae* ST104 KP4898

Additional epidemiological information was provided by genome sequence of representative *K. pneumoniae* KP4898 isolate. Genome sequence confirmed MLST assignment of *K. pneumoniae* KP4898 isolate to ST104. Capsular typing through sequencing of CD1–VR2–CD2 region of wzc and outer membrane protein wzi genes of locus identified wzc-32 and wzi-102 alleles, respectively, which are associated with K31 capsular type. In the KP4898, an array of virulence-associated genes was found, which includes the type-3 fimbriae cluster *mrkABCDF* and transcription regulators *mrkHIJ*, the yersiniabactin siderophore cluster *ybtAPQSTUX*, the yersiniabactin receptor *fyuA*, yersiniabactin biosynthetic protein genes *irp1* and *irp2* and *allB* and *allD* genes of allantoinase cluster. The analysis of drug-associated resistance genes in KP4898 genome identified bla_{SHV-5} and bla_{SHV-12} extended-spectrum β-lactamases, bla_{VIM-1} carbapenemase, *aadA1*, *aphA15*, and *aacA4* aminoglycoside resistance genes, a macrolide 2′-phosphotransferase cluster, other antimicrobial resistance genes, heavy metal resistance genes, efflux system, and regulators genes (Supplementary Table S2). The PlasmidFinder web tool identified A/C, FII(K) and FIB(K) replicons, thus suggesting the presence of at least three plasmids in KP4898 genome.

Conjugal Transfer of Carbapenem Resistance

The transfer of carbapenem resistance from VIM-1-producing ST104 *K. pneumoniae* isolates with PFGE type A and subtypes A1 and A2 was evaluated by filter mating experiments. Resistance or intermediate resistance to aminopenicillins, ureidopenicillins, third and fourth generation cephems, imipenem, but not meropenem and ertapenem, was transferred from ST104 *K. pneumoniae* isolates with PFGE type A and subtypes A1 and A2 to *E. coli* J53 aziR at frequency ranging from 6.5×10^{-3}

[2]http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_isolates_public&page=profiles

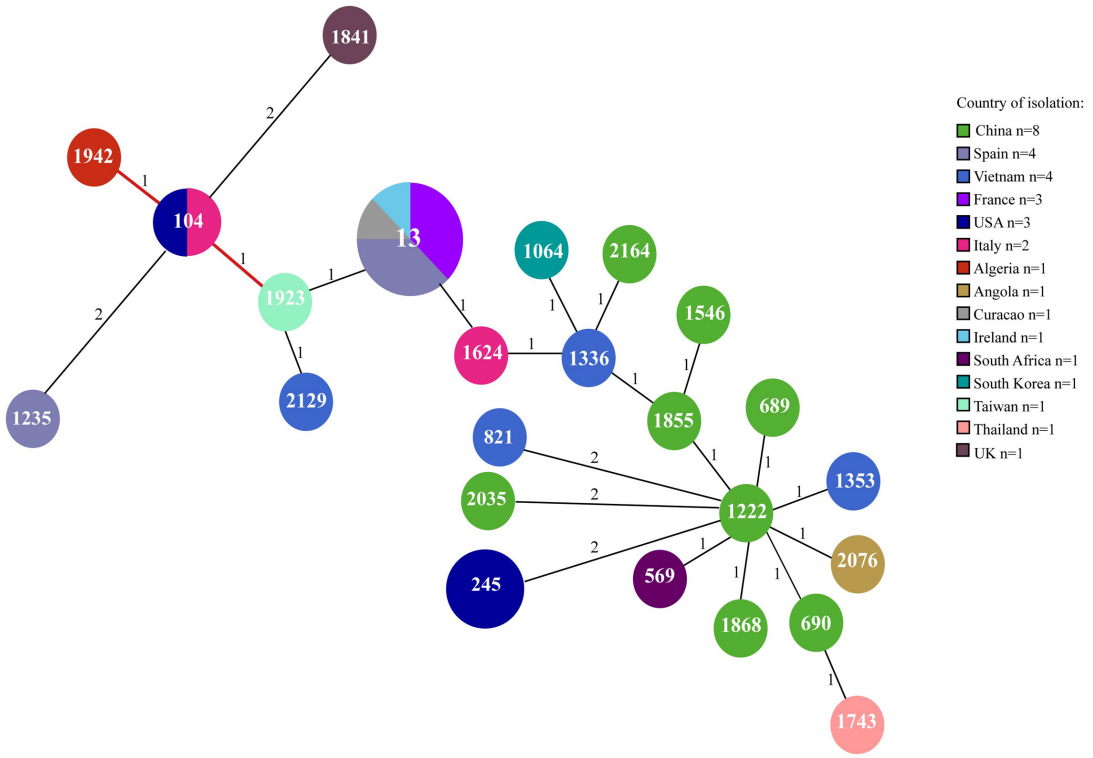


FIGURE 3 | Minimum spanning tree of ST104 and single-locus and double-locus variant isolates at Klebsiella PubMLST (http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_isolates_public&page=profiles). ST1942 and ST1923 are single-locus variant of ST104. The other STs are ST104 double-locus variant. Numbers inside each circle indicate the ST types. The number on the branch indicates the different alleles between STs. Circle size is proportional to the number of isolates belonging to the same ST type. The pie charts for each ST indicate the country of isolation.

to 1.5×10^{-3} cfu/recipient cells. The frequency of transfer of imipenem resistance from *K. pneumoniae* ST104/PFGE A, A1, and A2 isolates to *E. coli* J53 aziR did not change if transconjugants were selected in the presence of imipenem and sodium azide or ampicillin and sodium azide. All transconjugants showed identical antimicrobial susceptibility profile and resistance to sodium azide (**Table 2**). Moreover, all transconjugants showed a PFGE profile identical with that of the recipient strain. The presence of bla_{VIM-1} and bla_{SHV-12} genes was demonstrated in all transconjugants. Furthermore, PBRT identified A/C replicon and IncA/C incompatibility group plasmid/s in ESBL positive and CR *K. pneumoniae* ST104 donor isolates and *E. coli* transconjugants expressing bla_{VIM-1} and bla_{SHV-12} genes. The above data suggested that conjugative plasmid/s mediated the horizontal transfer of carbapenem resistance.

Genetic Structure of pIncAC-KP4898

In the genome sequence of *E. coli* transconjugants, one single plasmid was identified, which was present in *K. pneumoniae* KP4898 donor strain, but not in *E. coli* J53 recipient strain, and was designated pIncAC-KP4898, for plasmid of IncA/C incompatibility group from *K. pneumoniae* 4898 isolate. The pIncAC-KP4898 plasmid was 156,252 bp in size, with an average G + C content of 52.6%. Genome annotation identified 190 open reading frames (ORFs), of which 145 were transcribed in a clockwise orientation, while the remaining 45 were transcribed counterclockwise. Of these ORFs, 14 were associated with plasmid DNA replication and partition, 16 with DNA transfer, 13 with DNA-restriction and site-specific DNA methylation, 25 with DNA transposition, 19 with antimicrobial resistance, and 103 with unknown functions (**Figure 4** and Supplementary Table S3). The pIncAC-KP4898 scaffold included the *repA*, *parA*, *parB*, and *053* genes, *parM*, *kfrA*, and a putative toxin–antitoxin system, which were demonstrated to be important for maintenance and replication of IncA/C plasmid (Hancock et al., 2017). Based on RepA similarity (Carattoli et al., 2006), the pIncAC-KP4898 plasmid belongs to IncA/C1 group. The IncA/C PMLST scheme based on the *repA*, *parA*, *parB*, and *053* genes and the cgPMLST scheme that extend the 4-gene PMLST to 28 conserved genes (Hancock et al., 2017) assigned pIncAC-KP4898 to novel profiles, which corresponded to ST12 and ST12.1, respectively. The phylogeny of IncA/C 28-gene profiles showed that ST12.1 was more closely related to ST11.1, to which IncA/C1 group pRA1 plasmid (GenBank accession number FJ705807.1) has been assigned, than to the remaining 10 profiles (ST1 to ST10), which corresponded to IncA/C2 group plasmids (**Figure 5**).

Plasmid pIncAC-KP4898 contained a composite transposon of 25,716 bp (residues 139,120–156,252 and 1–8,583, G + C content: 57.7%) with four IS6 family transposase and 14 bp

TABLE 2 | Conjugative transfer of carbapenem resistance.

	MIC (mg/l)		
Antibiotic	**Strain**		
	***K. pneumoniae* ST104/A-A2**	***E. coli* J53 aziR (p1KPST104)**	***E. coli* J53 aziR**
Amoxicillin	>32	>32	4
Piperacillin–tazobactam	>128	>128	2
Ceftazidime	>64	8	0.25
Cefotaxime	>64	>64	≤0.25
Cefepime	>64	2	≤0.25
Imipenem	8	8	0.25
Meropenem	>16	≤0.25	≤0.25
Ertapenem	4	≤0.5	≤0.5
Fosfomycin	>128	≤16	≤16
Amikacin	≤2	≤2	≤2
Gentamicin	4	≤1	≤1
Ciprofloxacin	0.5	≤0.25	≤0.25
Trimethoprim–sulfamethoxazole	>320	≤20	≤20
Tigecycline	≤0.5	≤0.5	≤0.5
Colistin	≤0.5	≤0.5	≤0.5

inverted repeats (TTTGCAACAGTGCC) at residues 152,072–152,086 and 8,569–8,583 (3′-flanking region) (**Figure 4**). Within this transposon lied a class 1 integron containing a 5′-conserved structure (CS) with *int1* site-specific integrase, and five head-to-tail arranged gene cassettes consisting of the genes bla_{VIM-1}, *aacA4* family aminoglycoside N(6′)-acetyltransferase gene, aminoglycoside *O*-phosphotransferase *APH(3′)-XV* encoding gene, ANT(3″) family aminoglycoside nucleotidyl transferase gene, type B-2 chloramphenicol *O*-acetyltransferase *catB2* gene. The 3′-CS showed the *qacED1*-encoding gene, which confers resistance to quaternary ammonium compounds. The Tn3-like composite transposon included also the bla_{SHV-12} gene flanked by IS6 family transposases in inverted orientation, a macrolide 2′-phosphotransferase gene cluster consisting of macrolide 2′-phosphotransferase *mph(A)*, transporter *mfs* and macrolide 2′-phosphotransferase I repressor A *mphR* genes, quinolone resistance pentapeptide repeat protein *qnrA1* gene, and sulfonamide-resistant dihydropteroate synthase *sul1* genes. A mercury resistance gene clusters and trimethoprim-resistant dihydrofolate reductase *dfrA14* gene were carried by pIncAC-KP4898 apart from the identified transposon region. In addition, 16 genes encoding transfer functions and a conjugative apparatus were found in pIncAC-KP4898, which indicated that the plasmid was self-conjugative (**Figure 4** and Supplementary Table S3).

DISCUSSION

The emergence of multidrug-resistant and carbapenem-resistant microorganisms has become an alarming phenomenon in children and neonates (Logan, 2012; Logan et al., 2015). Intestinal carriage of carbapenemase producing Enterobacteriaceae is an important reservoir and source of dissemination of resistance to carbapenems among Gram-negative bacteria in the community and in the hospital setting (Nordmann and Poirel, 2014; Viau et al., 2016; Logan and Weinstein, 2017).

The current study investigates the molecular epidemiology and the genetic mechanism of acquisition of carbapenem resistance of multidrug-resistant *K. pneumoniae* isolated into two consecutive clusters from rectal swabs of 20 neonates in the NICU of the V. Monaldi Hospital in Naples, Italy. Our data showed the selection of a single VIM-1-producing *K. pneumoniae* epidemic genotype assigned to PFGE type A, A1, and A2 and ST104, which was isolated only from patients in the NICU but not in other wards of the hospital, thus suggesting that cross-transmission among intestinal carrier neonates may have been favored the spread of VIM-1 producing *K. pneumoniae* epidemic clone. The diffusion of VIM-1 producers *K. pneumoniae* is uncommon in Italy, where the vast majority of carbapenemase producers *K. pneumoniae* are KPC producers (Giani et al., 2017; Grundmann et al., 2017). After the first isolation of ST104 *K. pneumoniae* from milk during bovine mastitis (Paulin-Curlee et al., 2007), sporadic isolations of ST104 (Baraniak et al., 2013; Maatallah et al., 2014; Esteban-Cantos et al., 2017), were reported from human infections/colonizations. Also, *K. pneumoniae* ST1923 and ST1942, which were single-loci variants of ST104, were isolated from human blood and human feces (Yan et al., 2015), and *K. pneumoniae* isolates assigned to 21 other STs, which were double-locus variants of ST104, were isolated from difference sources including clinical specimens (**Figure 3**). Since ST104 *K. pneumoniae* human isolates were either ESBL producing (Baraniak et al., 2013; Maatallah et al., 2014; Esteban-Cantos et al., 2017) or carbapenemase producing (Esteban-Cantos et al., 2017), we can hypothesize that antimicrobial resistance might have selected ST104 *K. pneumoniae* isolates among bacterial population.

In accordance with this, data reported herein showed that *K. pneumoniae* isolates from neonates in the NICU showed a MDR phenotype, being resistant to all classes of β-lactams including third and fourth generation cephems and carbapenems, fosfomycin, gentamicin, and trimethoprim–sulfamethoxazole, but susceptible to quinolones, amikacin and colistin. This is particularly alarming in neonates, for which limited options of antimicrobial therapy are available. Also, genome sequence of VIM-1-producing *K. pneumoniae* ST104 KP4898 showed the presence of virulence-associated genes and antimicrobial resistance genes (Supplementary Table S2). Interestingly, ST104 *K. pneumoniae* isolates from neonates in the NICU showed resistance to antimicrobials frequently used in neonates, such as third and fourth generation cephems, carbapenems, gentamicin, and trimethoprim–sulfamethoxazole, while they retain susceptibility to fluoroquinolones, which are not recommended in this clinical setting. Since it has been demonstrated that previous combination antimicrobial treatment with ampicillin and gentamicin is independent risk factors for acquisition of extended-spectrum β-lactamase-producing *K. pneumoniae* and *Serratia marcescens* in neonates (Crivaro et al., 2007), we can speculate that resistance to third and

FIGURE 4 | Schematic map of pIncAC_KP4898 plasmid. Circular map of pIncAC_KP4898. The outermost ring represents the pIncAC_KP4898 plasmid with arrows indicating genes/ORFs involved in the various cellular pathways. Functions of the genes and ORFs found are color coded and shown in the left bottom corner of the figure. The middle ring and the innermost ring represent a GC plot and a GC skew plot, respectively. For both plots, magenta and olive green indicate the measures below and above the average, respectively.

fourth generation cephems and carbapenems might have been selected by their frequent use in the NICU.

Several studies demonstrate that the horizontal gene transfer through conjugative plasmids and transposons contributes to the spread of resistance to carbapenems (Bialek-Davenet et al., 2014; Nordmann and Poirel, 2014; Viau et al., 2016). Accordingly, we showed that resistance to aminopenicillins, ureidopenicillins, third and fourth generation cephems, imipenem, but not meropenem and ertapenem, was transferred from VIM-1-producing ST104 *K. pneumoniae* isolates assigned to either PFGE type A, A1, or A2 to susceptible *E. coli* along with a *bla*VIM-1 positive plasmid of IncA/C1 incompatibility group and 156,252 bp in size, which we named pIncAC_KP4898 (**Figure 4**). Based on the above data, we postulate that imipenem resistance depends mainly on the expression of VIM-1 carbapenemase carried by pIncAC_KP4898, while meropenem and ertapenem resistance might be contributed by additional resistance mechanisms in *K. pneumoniae* isolates, such as altered permeability due to changes in the expression of porins or efflux systems (Goodman et al., 2016). Further experiments

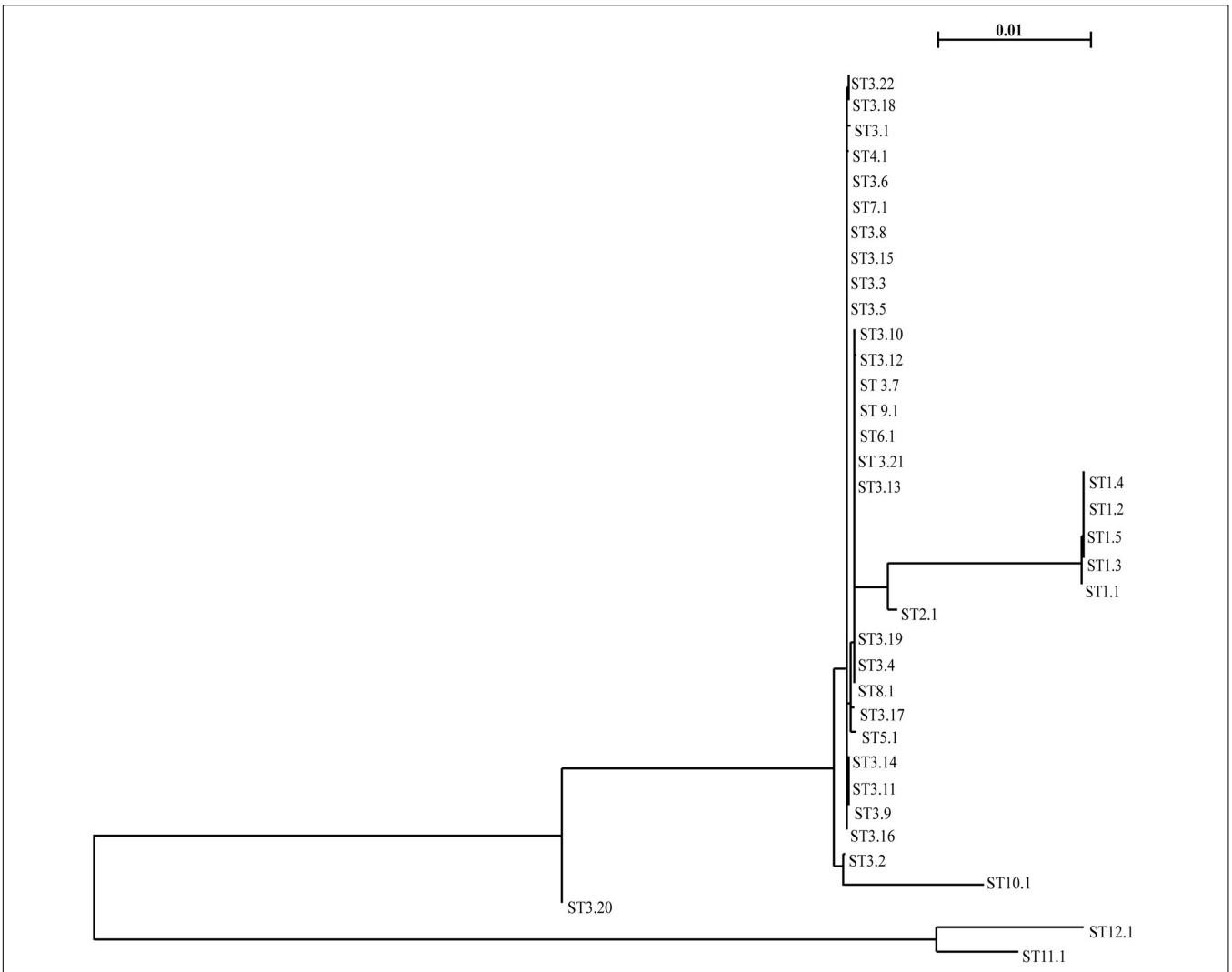


FIGURE 5 | Phylogeny of cgPMLST IncA/C plasmids. Neighbor-joining phylogenetic trees based on multiple alignments of concatenated alleles of the 28 IncA/C conserved genes. The bar at the top of the figure shows the amount of genetic change corresponding to the length of each branch.

will be necessary to validate such hypothesis. The pIncAC-KP4898 scaffold was assigned to novel ST12 and ST12.1 profiles. Interestingly, it resulted to be more closely related to IncA/C1 group pRA1 plasmid from the fish pathogen *Aeromonas hydrophila* (Fricke et al., 2009) assigned to ST11.1, than to the majority of IncA/C plasmid belonging to IncA/C2 group and ST1 to ST10 profiles (**Figure 5** and **Supplementary Figure S1**), which are associated with the carriage of bla_{NDM} or bla_{CMY} (Hancock et al., 2017). While InCA/C2 group plasmids were frequently found in Enterobacteriaceae isolated from human and non-human sources (Hancock et al., 2017), only two IncA/C1 group complete plasmid sequences are available in GenBank, pRA1 plasmid from *A. hydrophila* environmental isolate (Fricke et al., 2009) and pIncAC_KP4898 plasmid from ST104 *K. pneumoniae* clinical isolate described in this study. Of these, pIncAC_KP4898 is the first InCA/C1 plasmid carrying $bla_{VIM\text{-}like}$ sequences. Similarly to IncA/C1 group pRA1 plasmid (Fricke et al., 2009) and several IncA/C 2 group plasmids (Hancock et al., 2017), pIncAC-KP4898 carried several *tra* genes encoding transfer functions and was self-conjugative.

pIncAC_KP4898 plasmid presents a composite transposon of approximately 26 kb, which includes $bla_{SHV\text{-}12}$ extended spectrum β-lactamase gene, flanked by IS6 elements and a class I integron with $bla_{VIM\text{-}1}$ carbapenemase gene, *aacA4* family aminoglycoside N(6′)-acetyltransferase gene, aminoglycoside *O*-phosphotransferase *APH(3′)-XV* encoding gene, ANT(3″) family aminoglycoside nucleotidyl transferase gene, type B-2 chloramphenicol *O*-acetyltransferase *catB2* gene. A class I integron showing identical gene cassettes array is found in IncN plasmid pOW16C2 from *K. pneumoniae* environmental isolate (Zurfluh et al., 2015), and non-typeable plasmids pAX22 from *Achromobacter xylosoxidans* (Di Pilato et al., 2014) and plasmid of >300 kb from *E. coli* strain W1058 (GenBank accession number KF856617; Porres-Osante et al., 2014). Based on the

above all data, we can postulate that genetic structure of class I integron with bla_{VIM-1} carried by pIncAC-KP4898 might have an environmental source. In accordance with our data, VIM-1 has been shown to be the most prevalent allele variants among VIM-producing isolates, having global geographical distribution and being isolated in multiple Enterobacteriaceae species (Matsumura et al., 2017). Moreover, *Aeromonas caviae* carrying bla_{VIM-1} and bla_{VIM-35} inside class I integrons were isolated from clinical surveillance cultures in Israeli hospitals (Adler et al., 2014); *A. caviae* carrying bla_{VIM-1} and bla_{SHV-12} into a transferable plasmid were isolated from the blood cultures of 1-day-old newborn in Florence, Italy (Antonelli et al., 2016). A macrolide 2′-phosphotransferase gene cluster inside the composite transposon and a mercury resistance gene clusters and trimethoprim-resistant dihydrofolate reductase *dfrA14* gene outside the transposon region were additional resistance genes, which might have contributed to the selection of pIncAC_KP4898 into ST104 *K. pneumoniae*.

CONCLUSION

The spread of carbapenem resistance in *K. pneumoniae* from neonates in the NICU was due to the acquisition of plasmid pIncAC-KP4898, carrying the bla_{VIM-1} gene and several additional resistance genes into the scaffold of an IncA/C1 group self-conjugative plasmid. The composite genetic structure of pIncAC-KP4898 might have been generated by the acquisition of different regions from different sources mediated by multiple recombination events.

AUTHOR CONTRIBUTIONS

DS and RZ conceived the study and participated in its design and coordination. VC collected the epidemiological data and performed the infection control in the hospital. MB and SC collected the microbiological data. EE, MDF, FP, and MT performed laboratory analyses. SG, PM, DS, and RZ performed data analyses. EE, SG, DS, and RZ wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported in part by grant from University of Naples "Federico II" (Fondo d'Ateneo per la Ricerca to MT and RZ).

ACKNOWLEDGMENTS

We thank Erika Scaltriti, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Parma, Italy, for technical assistance in whole genome sequencing. We thank also Dr. Steven Hancock, University of Queensland, Brisbane, Australia, for curating plasmid MLST data and making them publicly available at Plasmid MLST Databases (https://pubmlst.org/plasmid/). RZ dedicates this study to the memory of Stella Carlomagno, a mentor in bacterial genetic and life sciences.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02135/full#supplementary-material

FIGURE S1 | Minimum spanning tree showing the IncA/C core gene PMLST (cgPMLST). Numbers inside each circle are the cgST types. The size of the circle indicates the number of the isolates belonging to the same cgST type. The number on the branch indicates the different alleles between cgSTs.

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

Chapter 3

Molecular epidemiology and virulence profiles of colistin-resistant *Klebsiella pneumoniae* blood isolates from the Hospital Agency "Ospedale dei Colli," Naples, Italy.

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Front Microbiol. 2018 Jul 16;9:1463.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 July 2018
doi: 10.3389/fmicb.2018.01463

Molecular Epidemiology and Virulence Profiles of Colistin-Resistant *Klebsiella pneumoniae* Blood Isolates From the Hospital Agency "Ospedale dei Colli," Naples, Italy

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 07 March 2018
Accepted: 12 June 2018
Published: 16 July 2018

Citation:
Esposito EP, Cervoni M, Bernardo M, Crivaro V, Cuccurullo S, Imperi F and Zarrilli R (2018) Molecular Epidemiology and Virulence Profiles of Colistin-Resistant Klebsiella pneumoniae Blood Isolates From the Hospital Agency "Ospedale dei Colli," Naples, Italy. Front. Microbiol. 9:1463. doi: 10.3389/fmicb.2018.01463

Resistance to colistin is increasingly reported in *Klebsiella pneumoniae* clinical isolates. The aim of this study was to analyze the molecular epidemiology and virulence profiles of 25 colistin-resistant *K. pneumoniae* blood isolates from the Hospital Agency "Ospedale dei Colli," Naples, Italy, during 2015 and 2016. Colistin MIC values of isolates ranged from 4 to 256 mg/L. The inactivation of the *mgrB* gene, encoding a negative regulator of the PhoQ/PhoP signaling system, was the most frequent mechanism of colistin resistance found in 22 out of 25 isolates. Of these, 10 isolates assigned to ST512 and PFGE types A and A4 showed identical frameshift mutation and premature termination of *mgrB* gene; 4 isolates assigned to ST258 and PFGE types A1 showed non-sense, frameshift mutation, and premature termination; 3 and 1 isolates assigned to ST258 and PFGE A2 and ST512 and PFGE A3, respectively, had insertional inactivation of *mgrB* gene due to IS*5*-like mobile element; 2 isolates assigned to ST101 and 1 to ST392 had missense mutations in the *mgrB* gene, 1 isolate assigned to ST45 showed insertional inactivation of *mgrB* gene due to IS*903*-like mobile element. *phoQ* missense mutations were found in 2 isolates assigned to ST629 and ST101, respectively, which also showed a missense mutation in *pmrA* gene. The *mcr-1-2-3-4* genes were not detected in any isolate. Colistin-resistant *K. pneumoniae* isolates showed variable virulence profiles in *Galleria mellonella* infection assays, with the infectivity of two isolates assigned to ST45 and ST629 being significantly higher than that of all other strains ($P < 0.001$). Interestingly, colistin MIC values proved to make a significant contribution at predicting lethal doses values (LD_{50} and LD_{90}) of studied isolates in *G. mellonella*. Our data show that MgrB inactivation is a common mechanism of colistin resistance among *K. pneumoniae* in our clinical setting.

The presence of identical mutations/insertions in isolates of the same ST and PFGE profile suggests the occurrence of clonal expansion and cross-transmission. Although virulence profiles differ among isolates irrespective of their genotypes, our results suggest that high colistin MIC could predict lower infectivity capability of the isolates.

Keywords: mechanisms of colistin-resistance, *Klebsiella pneumonia*, blood isolates, *Galleria mellonella*, virulence profiles

INTRODUCTION

The occurrence of multidrug-resistant (MDR) or extensively drug-resistant (XDR) *Klebsiella pneumoniae* infections (Bialek-Davenet et al., 2014; Bradford et al., 2015; Holt et al., 2015; Pitout et al., 2015; Cerqueira et al., 2017; Logan and Weinstein, 2017; Otter et al., 2017) has favored the use of colistin-based regimens as the most frequent therapeutic options (van Duin et al., 2013). Unfortunately, resistance to colistin has been increasingly reported in *K. pneumoniae* clinical isolates worldwide (Jeannot et al., 2017; Poirel et al., 2017).

In *K. pneumoniae*, resistance to polymixins (polymyxin B and colistin) is mainly due to modification of the lipid A phosphate moieties of the lipopolysaccharide (LPS) with a sugar or ethanolamine, which reduces the electrostatic interaction between the cationic polymixins and anionic LPS (Poirel et al., 2017). Molecular mechanisms responsible for colistin resistance rely on mutations in the genes of two component transcriptional regulatory systems PhoPQ and PmrAB, which regulate the expression of *pmrC* gene that codes for the addition of phosphoethanolamine and *pmrHFIJKLM* operon genes that encode biosynthesis and lipid A transfer of 4-amino-4-deoxy-L-arabinose (Cheng et al., 2015, 2016; Jayol et al., 2015; Wright et al., 2015; Novović et al., 2017; Poirel et al., 2017; Pragasam et al., 2017). Moreover, mutations causing loss of function in the MgrB protein, a negative feedback regulator of PhoPQ two component regulatory system (Cannatelli et al., 2013, 2014, 2016; Olaitan et al., 2014; Cheng et al., 2015; Giani et al., 2015; Poirel et al., 2015; Arena et al., 2016; Jaidane et al., 2018) and mutations in the *crrB* gene, which regulates the expression of *pmrC* gene and *pmrHFIJKLM* operon through the PmrAB two component system (Wright et al., 2015; Cheng et al., 2016; Jayol et al., 2017) have been described. Also, the acquisition of plasmid-borne *mcr 1.2* gene encoding a membrane-anchored enzyme which adds phosphoethanolamine to lipid A has been recently reported in *K. pneumoniae* (Di Pilato et al., 2016).

Worryingly, mounting evidence indicates that colistin resistance caused by inactivation of the MgrB regulator is not associated with fitness cost and decreased virulence of *K. pneumoniae* (Cannatelli et al., 2015; Arena et al., 2016) and is maintained in the absence of selective antimicrobial pressure (Cannatelli et al., 2015). Also, it has been recently demonstrated that inactivation of *mgrB* can stimulate *K. pneumoniae* virulence by decreasing the expression of antimicrobial peptides and early inflammatory response of the host (Kidd et al., 2017).

The aim of this study was to investigate the molecular epidemiology and mechanism of colistin resistance a of 25 colistin-resistant *K. pneumoniae* blood isolates from the Hospital Agency (HA) "Ospedale dei Colli," Naples, Italy, during 2015 and 2016 and to assess the virulence profiles of *K. pneumoniae* isolates in a *Galleria mellonella* infection model.

MATERIALS AND METHODS

Setting and Design of the Study

The HA "Ospedale dei Colli" in Naples, Italy includes three hospitals: "V. Monaldi Hospital," which is a 580-bed tertiary-care teaching hospital providing acute medical and surgical care within cardiology, cardiothoracic surgery, and pneumology with an active heart transplantation programme also; "D. Cotugno" Hospital, which is a 209-bed hospital and is the referral center for infectious diseases; Orthopedic Trauma Center (OTC) Hospital, which is a 143-bed hospital and is the referral center for orthopedic, neurology and neurosurgery. The hospitals are provided with 6 intensive care units (ICU)s: a neonatal ICU, a cardiac surgery ICU (CS-ICU), a post-operative ICU (PO-ICU), and a cardiorespiratory ICU (CR-ICU) in "V. Monaldi" Hospital, a medical-ICU (M-ICU) in "D. Cotugno" Hospital, and a post-operative-ICU (PO-ICU) in OTC Hospital. There is one microbiology laboratory that processes samples from all three hospitals. Surveillance of carbapenem resistant Enterobacteriaceae (CRE) in the HA "Ospedale dei Colli" was performed as previously described (Esposito et al., 2017). The present study analyzed 25 colistin-resistant *K. pneumoniae* blood isolates from 25 patients who were admitted to the HA "Ospedale dei Colli," Naples from January 2015 to September 2016. The first colistin-resistant *K. pneumoniae* isolate from blood-culture was selected for each patient.

Bacterial Strains Identification

The colistin-resistant *K. pneumoniae* strains were identified using Vitek-2 and ID-GNB card for Gram-negative bacilli according to manufacturer's instructions (bioMérieux, Marcy l'Etoile, France).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibilities were performed using the Vitek 2 system and the AST-GN card (bioMérieux, Marcy l'Etoile, France). Values were interpreted according to breakpoint table for interpretation of MIC values and zone diameters (European Committee on Antimicrobial Susceptibility Testing, 2016). Colistin susceptibility assay was performed according to recommendation of joint CLSI-EUCAST guidelines: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_

documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf.

Genotype Analysis and Capsular Typing

Genotyping was performed using *XbaI* DNA macrorestriction, pulsed-field gel electrophoresis (PFGE) with dendrogram analysis as previously described (Del Franco et al., 2015). Multilocus sequence typing (MLST) was performed as reported in Diancourt et al. (2005) using primers and PCR conditions available at http://bigsdb.pasteur.fr/klebsiella/primers_used.html. Capsular typing was performed by PCR amplification and sequencing of *wzi* gene as previously described (Brisse et al., 2013). eBURST analysis of ST profiles and detection of carbapenemase genes was performed as described previously (Esposito et al., 2017).

Molecular Analysis of Colistin Resistance

The chromosomal DNA of clinical isolates was extracted using DNeasy Blood & Tissue Kit according to the manufacturer's instructions (Qiagen, Milan, Italy). Analysis of plasmid-mediated colistin resistance was performed by PCR amplification of *mcr-1*, *mcr-2*, *mcr-3,* and *mcr-4* genes as described previously (Liu et al., 2016; Xavier et al., 2016; Carattoli et al., 2017; Yin et al., 2017). Chromosomally-encoded modifications of the LPS were analyzed through amplification and sequencing of the *mgrB*, *pmrA*, *pmrB*, *crrB*, *phoP,* and *phoQ* genes as described by Cannatelli et al. (2013, 2014) and Jayol et al. (2014, 2015). The primers used are shown in **Table S1**. DNA sequencing of PCR products and Basic Local Alignment Search Tool (BLAST) analysis of nucleotide and deduced protein sequences were performed as previously described (Del Franco et al., 2015). Insertion sequences (ISs) were identified using the ISfinder tool (https://www-is.biotoul.fr/index.php). Complementation experiments for *mgrB* gene were performed as described previously (Cannatelli et al., 2013) with minor modification. *K. pneumoniae* transformants were selected on Mueller-Hinton agar plates supplemented with 10 mg/L of tetracycline. MIC testing of the complemented strains was performed in medium supplemented with 10 mg/L of tetracycline, to avoid plasmid loss.

Galleria mellonella Infection Assays

Klebsiella pneumoniae strains were grown in MH to late exponential phase. Cells were collected by centrifugation and suspended in saline. Serial 10-fold dilutions of bacterial cell suspensions in saline were injected into *G. mellonella* larvae as described (Jander et al., 2000). Ten larvae were infected with each infecting dose and 10 larvae were injected with sterile saline as negative control. Larvae were incubated at 37°C for 3 days to monitor mortality. Each strain was tested in two or three independent experiments, with at least four dilutions injected in each experiment. Dose-dependent survival curves and lethal doses 50 and 90% (LD_{50} and LD_{90}, respectively) were determined using the GraphPad Prism software as previously described (Antunes et al., 2011). For each isolate, the results obtained with the infecting doses corresponding to about 10^6 and 10^5 cells in the two or three independent assays were also pooled to generate Kaplan-Meier survival curves using GraphPad Prism.

Statistical Analysis

Statistical analysis of Kaplan-Meier survival curves was performed with the Log-rank (Mantel-Cox) Test using GraphPad Prism. Linear regression analysis of colistin MIC and LD_{50}/LD_{90} values was performed by means of SPSS v. 20.0 (Chicago, IL, USA). P-values < 0.05 were considered to be statistically significant.

Nucleotide Sequence Accession Numbers

Nucleotide sequences of mutated *mgrB* and *phoQ* genes described in this work have been deposited in GenBank under accession numbers MG210951-MG210955 and MG214776-MG214777.

Ethics Statement

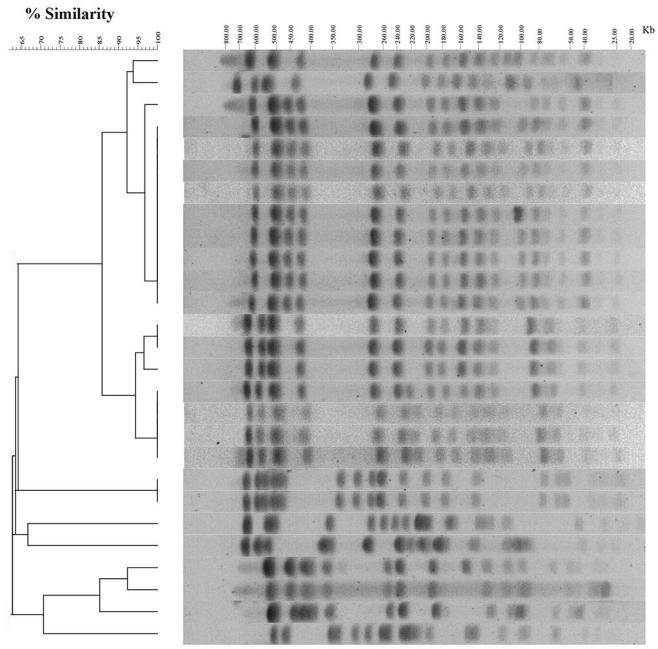
The study has been evaluated by the local Ethics committee (Comitato Etico Università degli Studi della Campania "Luigi Vanvitelli" Azienda Ospedaliera Universitaria "Luigi Vanvitelli"—HA "Ospedali dei Colli") (protocol number 52/2018). Patients included in the study were anonymized, no written informed consent was acquired because of the retrospective nature of the study.

RESULTS

Molecular Epidemiology of Colistin-Resistant *K. pneumoniae* in the HA "Ospedale dei Colli," Naples, Italy

An increase of colistin-resistant *K. pneumoniae* clinical isolates was observed in the HA "Ospedale dei Colli" during 2015 and 2016 with a prevalence of colistin-resistant *K. pneumoniae* isolates over total *K. pneumoniae* isolates of 0.15. A total of 25 isolates from blood cultures were retrospectively collected from 25 patients hospitalized in MS- ICU (11) and IDD (2) of "D. Cotugno" hospital; CR-ICU (5), CS-ICU (4), and R-ICU (2) of "V. Monaldi" hospital; PO-ICU (1) of OTC hospital (**Figure 1** and **Table S2**). Colistin resistance was detected in initial *K. pneumoniae* isolates from 18 of the 25 patients, while 7 patients showed initial colistin-susceptible *K. pneumoniae* isolate with a subsequent colistin-resistant *K. pneumoniae* isolate. *K. pneumoniae* isolates showed a MDR phenotype. In particular, all isolate were resistant to colistin and beta-lactam/beta-lactamase inhibitor combinations (clavulanic acid/amoxicillin, piperacillin/tazobactam); all isolates but one were resistant to third and fourth generation cephems; 21 isolates were resistant to imipenem and meropenem; 22 isolates were resistant to ertapenem; all isolates but one were resistant to ciprofloxacin; 23 and 18 isolates were resistant to gentamicin and amikacin, respectively; 23 isolates were resistant to tigecycline and trimethoprim-sulfamethoxazole; 19 isolates were resistant to fosfomycin. Colistin MIC values ranged from 4 to 256 mg/L values (**Table 1** and **Table S2**).

To investigate whether the increase in colistin-resistant *K. pneumoniae* isolates in the HA "Ospedale dei Colli" was due to the spread of epidemic strains, the 25 colistin-resistant *K. pneumoniae* isolates from the HA "Ospedale dei Colli" and 2 colistin-susceptible *K. pneumoniae* reference isolates were genotyped (isolates 8 and 17 in Del Franco et al., 2015). Molecular



Isolates nr.	Isolates date	Hospital	Ward	PFGE type	MLST ST	MLST CC	wzi	Colistin MIC (mg/L)
17	05/01/15	O.T.C.	PO-ICU	A3	512	258	154	128
27	05/02/14	USL Aosta	C	A5	512	258	154	0.125
8	23/08/16	Cotugno	M-ICU	A4	512	258	154	256
11	05/08/15	Cotugno	M-ICU	A	512	258	154	128
12	22/08/15	Cotugno	IDD	A	512	258	154	128
14	26/06/15	Cotugno	M-ICU	A	512	258	154	128
15	10/08/15	Cotugno	IDD	A	512	258	154	128
19	17/10/15	Cotugno	M-ICU	A	512	258	154	256
21	20/06/15	Cotugno	M-ICU	A	512	258	154	256
22	06/05/15	Cotugno	M-ICU	A	512	258	154	256
24	17/03/15	Cotugno	M-ICU	A	512	258	154	256
25	11/07/15	Cotugno	M-ICU	A	512	258	154	256
1	29/02/16	V. Monaldi	CR-ICU	A2	258	258	154	8
10	28/03/16	Cotugno	M-ICU	A2	258	258	154	4
4	01/06/16	V. Monaldi	CR-ICU	A2	258	258	154	8
13	07/12/15	V. Monaldi	CR-ICU	A1	258	258	154	64
16	24/07/15	V. Monaldi	R-ICU	A1	258	258	154	64
18	02/11/15	V. Monaldi	R-ICU	A1	258	258	154	64
23	05/08/15	V. Monaldi	CR-ICU	A1	258	258	154	64
2	16/03/16	Cotugno	M-ICU	D	629	-	485	16
5	18/01/16	V. Monaldi	CS-ICU	D	629	-	485	256
6	28/06/16	V. Monaldi	CS-ICU	F	45	-	101	64
9	07/03/16	V. Monaldi	CR-ICU	C	392	-	187	32
3	26/03/16	V. Monaldi	CS-ICU	B	101	101	137	256
7	10/09/16	V. Monaldi	CS-ICU	B1	101	101	137	32
26	23/11/13	USL Aosta	ICU	B2	1685	101	137	0.125
20	18/05/15	Cotugno	M-ICU	E	449	-	37	16

FIGURE 1 | Genotypic analysis of colistin-resistant *K. pneumoniae* isolates in the HA "Ospedale dei Colli," Naples. Dendrogram analysis of *K. pneumoniae* isolates from HA "Ospedale dei Colli," Naples. Percentage of similarity and sizes in kilobases (kb) of lambda DNA molecular mass markers are indicated. Isolate number, isolation date, hospitals, wards, PFGE type, MLST (ST and CC), *wzi* gene, and colistin MIC values are also shown. PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; ST; sequence type; CC, clonal complex.

TABLE 1 | Antimicrobial susceptibility profiles of the 25 colistin-resistant *K. pneumoniae* strains included in the study.

Antimicrobial	MIC[a] (mg/liter)		
	MIC_{50}	MIC_{90}	Range
Amoxicillin-clavulanic acid	>32	>32	16–>32
Piperacillin-tazobactam	>128	>128	32–>128
Ceftazidime	>64	>64	≤1–>64
Cefotaxime	>64	>64	≤1–>64
Cefepime	>64	>64	≤1–>64
Imipenem	>16	>16	≤0.25–>16
Meropenem	>16	>16	≤0.25–>16
Ertapenem	>8	>8	≤0.5–>8
Amikacin	>64	>64	≤2–>64
Gentamicin	4	>16	≤1–>16
Ciprofloxacin	>4	>4	≤0.25–>4
Tigecycline	4	>8	≤0.5–>8
Fosfomycin	128	>256	≤16–>256
Trimethoprim-sulfamethoxazole	>320	>320	≤20–>320
Colistin	128	256	4–256

[a]*MIC, minimal inhibitory concentration.*

typing using PFGE and dendrogram analysis identified 6 types, which we named from A to F, which differed in migration of more than 6 DNA fragments and showed a similarity of <80% at dendrogram analysis. PFGE types A and B could be further classified into 5 (A1–A5) and 2 (B1–B2) subtypes, respectively, which showed one-fragment to five-fragment variation in the macro-restriction pattern and a similarity of >80% at dendrogram analysis. Of 25 colistin-resistant *K. pneumoniae* isolates, 9 isolates showed PFGE type A; 4, 3, 1, and 1 isolates showed PFGE types A1, A2, A3, and A4, respectively; 2 isolates showed PFGE types B and B1; 2 isolates PFGE type D; three sporadic isolates showed PFGE types C, E, or F. The two colistin-susceptible isolates were assigned to PFGE types A5 and B2 (**Figure 1**). MLST analysis assigned PFGE types A, A3, A4, and A5 to ST512; PFGE types A1 and A2 to ST258; PFGE types B and B1 to ST101, PFGE type B2 to ST1685; PFGE type C to ST392; PFGE type D to ST629; PFGE types E and F to ST449 and ST45, respectively. As ST512 and ST1685 represent single locus variants of ST258 and ST101, respectively, eBURST analysis clustered ST258 and ST512 in clonal complex (CC) 258, while ST101 and ST1685 in CC101 (**Figure 1**). Capsular typing identified *wzi* allele 154 for isolates assigned to ST258 and ST512, wzi allele 137 for isolates assigned to ST101 and ST1685, wzi allele 485 for isolates assigned to ST629, wzi alleles 37, 101, and 187 for isolates assigned to ST449, ST101, and ST392, respectively (**Figure 1**). The screening for carbapenemases revealed that all colistin-resistant isolates belonging to CC258 (ST512 and ST258) and isolates assigned to ST101/B and ST392/C were KPC-3 producers, while the isolate assigned to ST101/B1 and one isolate assigned to ST629/D were VIM-1 producers. No carbapenemase genes were found in isolates assigned to ST445/E and ST45/F and in one of the isolates assigned to ST629/D (**Table 2**).

TABLE 2 | Molecular mechanisms of colistin-resistance in 25 *K. pneumoniae* blood isolates from HA "Ospedale dei Colli," Naples.

Strain	Genotype	Carbapenemase	Colistin MIC[a]	*mgrB*[b]	PhoQ[c]	CrrB[d]	PmrA[c]	PmrB[c]
1	ST258/A2	KPC-3	8	Insertional inactivation, IS5-like element at nt 75 (FW)		WT		
2	ST629/D	VIM-1	16	WT	D150G[e]	L296Q[f]	WT	R256G[e]
3	ST101/B	KPC-3	256	t95g (V32G)	D150G[e] W215G	–	A217V	WT
4	ST258/A2	KPC-3	8	Insertional inactivation, IS5-like element at nt 75 (FW)		WT		
5	ST629/D	–	256	WT	D150G[e] L257P	Q287K L296Q[f]	WT	WT
6	ST45/F	–	64	Insertional inactivation, IS903 element at nt 69 (FW)		–		
7	ST101/B1	VIM-1	32	t50g (L17R)		–		
8	ST512/A4	KPC-3	256	Δg19 (frameshift mutation)		WT		
9	ST392/C	KPC-3	32	t139a (W47R)		L296Q[f]		
10	ST258/A2	KPC-3	4	Insertional inactivation, IS5-like element at nt 75 (FW)		WT		
11	ST512/A	KPC-3	128	Δg19 (frameshift mutation)		WT		
12	ST512/A	KPC-3	128	Δg19 (frameshift mutation)		WT		
13	ST258/A1	KPC-3	64	c88t (non-sense, premature termination)		WT		
14	ST512/A	KPC-3	128	Δg19 (frameshift mutation)		WT		
15	ST512/A	KPC-3	128	Δg19 (frameshift mutation)		WT		
16	ST258/A1	KPC-3	64	c88t (non-sense, premature termination)		WT		
17	ST512/A3	KPC-3	128	Insertional inactivation, IS5-like element at nt 75 (FW)		WT		
18	ST258/A1	KPC-3	64	c88t (non-sense, premature termination)		WT		
19	ST512/A	KPC-3	256	Δg19 (frameshift mutation)		WT		
20	ST449/E	–	16	WT	D150G[e]	–	WT	WT
21	ST512/A	KPC-3	256	Δg19 (frameshift mutation)		WT		
22	ST512/A	KPC-3	256	Δg19 (frameshift mutation)		WT		
23	ST258/A1	KPC-3	64	c88t (non-sense, premature termination)		WT		
24	ST512/A	KPC-3	256	Δg19 (frameshift mutation)		WT		
25	ST512/A	KPC-3	256	Δg19 (frameshift mutation)		WT		

[a] *Colistin MICs are expressed as mg/L.*

[b] *Nucleotide (nt) numbers indicate the positions of mutations or of the insertion sites of IS (insertion sequence)s; numbering refers to the coding sequence of the mgrB open reading frame (ORF) of the colistin-susceptible K. pneumoniae KKBO-1 strain (GenBank accession no. AVFC00000000.1), considering number 1 as the first base of the GTG start codon; amino acid changes in the deduced protein sequence are indicated in parenthesis. FW indicates that the transposase gene is in the same orientation as the mgrB gene. Wild type (WT) indicates that the sequence of the mgrB ORF was identical to that of the colistin-susceptible K. pneumoniae KKBO-1 strain (Cannatelli et al., 2013). Δ, deletion.*

[c] *Positions and amino acid substitutions in PhoQ, PmrA and PmrB deduced protein sequences respect to colistin-susceptible K. pneumoniae NUTH-K2044 strain (GenBank accession no. AP006725.1) (Cheng et al., 2015) are shown.*

[d] *Positions and amino acid substitutions in the CrrB protein sequence with respect to the CrrB protein of the colistin-susceptible K. pneumoniae UHKPC27 strain (GenBank accession no. APVR00000000.1) (Wright et al., 2015) are shown; the (–) symbol indicates that the crrB gene was not amplified with the primers used in this work.*

[e] *Mutations that do not affect MIC of the colistin susceptible K. pneumoniae NUTH- K2044 strain (Cheng et al., 2015).*

[f] *Mutation previously found in the colistin susceptible K. pneumoniae XH209 strain (Hua et al., 2014).*

Based on PFGE and MLST typing data, four distinct epidemic genotypes were identified in 2 or more than 2 colistin-resistant *K. pneumoniae* isolates from patients in the HA "Ospedale dei Colli," which we named ST512/A, ST258/A1, ST512/A2, and ST629/D. Molecular epidemiology of colistin-resistant *K. pneumoniae* in different wards of the HA "Ospedale dei Colli" showed that ST512/A genotype was isolated in 7 and 2 patients from M-ICU and IDD wards of "D. Cotugno" hospital during 2015; ST258/A1 and ST258/A2 genotypes were isolated in 2, 2, and 2 patients from CR-ICU, R-ICU, and R-ICU wards of "V. Monaldi" hospital during 2015 and 2016, respectively, and 1 patients from M-ICU of "D. Cotugno" hospital during 2016; ST629/D genotype was isolated in 1 from M-ICU ward of "D. Cotugno" hospital and 1 patient from CS-ICU ward of "V.

Monaldi" hospital during 2016. Genotypes ST512/A3, ST512/A4, ST101/B, ST101/B1 ST392/C, ST/449/E, and ST45/F were isolated from single patients in different wards and were considered as sporadic (**Figure 1** and **Figure S1**).

Molecular Mechanisms of Colistin-Resistance in *K. pneumoniae* Blood Isolates From the HA "Ospedale dei Colli," Naples

In order to analyze the molecular mechanisms responsible for colistin-resistance, the presence of *mcr* genes encoding membrane-anchored enzymes which add phosphoethanolamine to lipid A was investigated in all isolates. Because the *mcr-1*, *mcr-2*, *mcr-3,* and *mcr-4* genes were not detected in any isolate, the presence of mutations in regulators of PhoQ/PhoP and PmrA/PmrB signaling systems was investigated.

Mutations in the *mgrB* gene, which encodes a negative regulator of the PhoQ/PhoP signaling system, were present in 22 out of 25 isolates. In particular, identical small deletion Δg19, which causes frameshift mutation and premature termination of MgrB (GenBank accession no. MG210954), was found in 9 and 1 isolates assigned to ST512/A and ST512/A4 genotypes, respectively; non-sense mutation c88t, which caused premature termination of MgrB, was found in 4 isolates assigned to ST258/A1 genotype (GenBank accession no. MG210955); missense mutations V32G (NCBI Reference Sequence: WP_094312677.1), L17R (GenBank accession no. MG210952), and W47R (GenBank accession no. MG210953) were found in isolates assigned to ST101/B, ST101/B1, and ST392/C genotypes, respectively (**Table 2**). The insertional inactivation of the *mgrB* gene was detected in 5 isolates. IS*5*-like mobile element at nt 75 of *mgrB* gene (GenBank accession no. MG214776) was found in 3 and 1 isolates assigned to ST258/A2 and ST512/A3 genotypes, respectively. An IS903-like element (97% identity to IS903) at nucleotide 69 of *mgrB* gene (Genbank accession no. MG214777) was found in 1 isolate assigned to ST45/F genotype. Two isolates assigned to ST629/D and 1 isolate assigned to ST449/E carried a wild type *mgrB* gene (**Table 2**).

Missense mutations were also found in *pmrA*, *pmrB*, *phoQ,* and *crrB* genes when compared to the genes present in colistin-susceptible *K. pneumoniae* NUTH-K2044 strain (GenBank accession no. AP006725.1) (Cheng et al., 2015). For PhoQ deduced protein sequences, a common mutation D150G was found in isolates assigned to ST629/D, ST101B-B1, and ST449/E genotypes. This mutation has been previously described and was not related with colistin resistance (Cheng et al., 2015). The substitutions W215G (Genbank accession no. MG210951) and L257P (NCBI Reference Sequence: WP_087760419.1) were also found in isolates assigned to ST101/B and ST629/D genotypes; however, no information is available about their implication in colistin resistance. In *pmrA*, a mutation leading to the substitution A217V was found in the isolate assigned to ST101/B genotype. This mutation has been already reported in *K. pneumoniae* (NCBI Reference Sequence: WP_032419166.1), but there is no information regarding colistin-susceptibility of the isolates. Finally, a mutation causing the substitution R256G in PmrB, which has been previously demonstrated to not confer colistin resistance (Cheng et al., 2015), was found in 1 isolate assigned to ST629/D genotype.

The sequence of the signal-transducing histidine kinase of the two-component regulatory system CrrAB was also investigated. In accordance with previous data (Wright et al., 2015), the *crrB* gene was found in 21 out of 25 isolates. Of these, 18 isolates assigned to ST512/A-A3-A4 and ST258/A1-A2 genotypes had CrrB identical to that of the colistin-susceptible *K. pneumoniae* UHKPC27 strain (GenBank accession no. APVR00000000.1) (Wright et al., 2015), while 2 and 1 isolates assigned to ST629/D and ST392/C genotypes, respectively, carried a missense mutation leading to L296Q substitution in the CrrB protein sequence. This CrrB substitution has been described already in colistin-susceptible *K. pneumoniae* XH209 strain (Hua et al., 2014), suggesting that is not responsible for colistin resistance of our isolates.

The above data indicate that MgrB inactivation was the most common mechanism of colistin resistance among *K. pneumoniae* isolates. In support of this, complementation experiments demonstrated that plasmid pACYC-*mgrB*, carrying WT *mgrB* gene fused to its own promoter, but not the empty plasmid pACYC184, was able to restore colistin susceptibility in selected *K. pneumoniae* isolates showing either insertional inactivation (isolates 1 and 17) or different mutations (isolates 3, 7, 16, and 22) in the *mgrB* gene (**Table 3**).

Virulence Profiles of Colistin-Resistant *K. pneumoniae* Isolates

In order to verify the effect of colistin resistance on pathogenicity, we assessed the virulence profile of 16 colistin resistant *K. pneumoniae* isolates, representative of all different genotypes identified in our collection and of different levels of colistin resistance for each genotype, and of the 2 colistin sensitive strains in the insect *G. mellonella*, which has been extensively used as

TABLE 3 | Colistin MICs of selected *K. pneumoniae* isolates carrying either the pACYC184 or the pACYC-*mgrB* plasmid.

Strain	*mgrB* mutation	Colistin MIC (mg/L)[a]	
		+ pACYC184	+ pACYC-*mgrB*
1	Insertional inactivation, IS5-like element at nt 75 (FW)	8	0.5
3	t95g (V32G)	256	1
7	t50g (L17R)	32	0.5
16	c88t (premature termination)	64	0.5
17	Insertional inactivation, IS5-like element at nt 75 (FW)	128	0.25
22	Δg19 (frameshift mutation)	128	0.5

[a] *MIC assays were performed using Mueller-Hinton Broth II supplemented with 10 mg/L of tetracycline.*

an infection model to investigate the pathogenic potential of *K. pneumoniae* strains (Insua et al., 2013; McLaughlin et al., 2014; Arena et al., 2016).

We generated dose-dependent survival curves for all isolates, which allowed to determine their LD_{50} and LD_{90} values in *G. mellonella* larvae (**Table 4**). High variability in the infectivity of the different isolates in *G. mellonella* was observed, with LD_{90} values ranging from few thousands of cells (isolate 6) to more than 40 millions of cells (isolate 21) (**Table 4**). This huge variability is however mainly related to the presence of a couple of hyper-virulent strains (isolates 6 and 2), characterized by LD_{90} values more than 100- or 10-fold lower than those of all other isolates respectively, and an isolate (21) which showed very low infectivity, with an LD_{90} value >5-fold higher than those of all other isolates (**Table 4**). The median LD_{50} and LD_{90} values for the remaining isolates were about 1.5×10^5 and 5×10^6 cells respectively, in line with previous reports on the pathogenicity of different *K. pneumoniae* strains in *G. mellonella* larvae (Insua et al., 2013; Wand et al., 2017). The higher infectivity of the isolates 2 and 6 was also confirmed by Kaplan-Maier survival curves generated with two different infecting doses (about 10^6 and 10^5 cells; **Figure 2** and **Figure S2**). Indeed, larvae infected with these isolates died much faster as compared to those infected with other *K. pneumoniae* isolates, with survival curves at both infecting doses significantly different from those obtained with all other strains ($P < 0.001$) (**Figure 2** and **Figure S2**). A relationship was found between LD_{50} and LD_{90} values and colistin MIC values among isolates, those with elevated colistin MIC values having higher LD_{50} and LD_{90} values (**Table 4**). Although correlation coefficients were not significant, simple regression analysis showed that colistin MIC values make a significant contribution at predicting both LD_{50} and LD_{90} values, though the latter appears to be more affected. In detail, colistin MIC accounts for 27.9% (F value 6.183, Sig. 0.024) and 42.7% (F value 11.937, Sig. 0.003) of LD_{50} and LD_{90} variations, respectively.

TABLE 4 | Lethal doses 50% (LD_{50}) and 90% (LD_{90}) in *G. mellonella* larvae for the indicated *K. pneumoniae* strains[a].

Strain[b]	Genotype[c]	Colistin MIC[d]	LD_{50}	LD_{90}	R^2
6	ST45/F	64	5.98×10^2	2.21×10^3	0.988
2	ST629/D	16	8.56×10^3	3.51×10^4	0.964
3	ST101/B	256	1.98×10^4	4.84×10^5	0.807
8	ST512/A4	256	6.51×10^4	4.83×10^6	0.768
4	ST258/A2	8	7.18×10^4	1.71×10^6	0.659
10	ST258/A2	4	7.46×10^4	2.58×10^6	0.740
11	ST512/A	128	9.45×10^4	2.21×10^6	0.825
20	ST449/E	16	9.93×10^4	3.43×10^6	0.832
17	ST512/A3	128	1.02×10^5	3.57×10^6	0.822
19	ST512/A	256	1.35×10^5	2.82×10^6	0.749
26	ST1685/B2	0.125	2.29×10^5	4.99×10^6	0.702
7	ST101/B1	32	4.46×10^5	6.03×10^6	0.790
9	ST392/C	32	7.24×10^5	8.27×10^6	0.824
5	ST629/D	256	7.86×10^5	6.42×10^6	0.997
27	ST512/A5	0.125	8.46×10^5	5.32×10^6	0.970
1	ST258/A2	8	8.80×10^5	6.58×10^6	0.933
13	ST258/A1	64	1.01×10^6	7.58×10^6	0.940
21	ST512/A	256	2.91×10^6	4.24×10^7	0.893

[a] *LD_{50}, LD_{90}, and R^2-values were determined using the GraphPad Prism software and the dose-dependent survival curves shown in the* ***Figure S3****. LD_{50} and LD_{90} are expressed as number of viable cells.*

[b] *Strains have been ordered according to increasing LD_{50} values.*

[c] *ST and PFGE profiles are shown.*

[d] *Colistin MICs are expressed as mg/L.*

DISCUSSION

In the present study, we analyzed the molecular epidemiology, mechanism of resistance, and virulence profiles of colistin-resistant *K. pneumoniae* blood isolates from HA "Ospedale dei Colli," Naples. Our data demonstrate the cross transmission and clonal expansion of 4 epidemic genotypes in different patients and wards: ST512/A genotype was isolated in M-ICU and IDD wards of "D. Cotugno" hospital, ST258/A1 and ST258/A2 genotypes were isolated in CR-ICU, R-ICU and R-ICU wards of "V. Monaldi" hospital and M-ICU of "D. Cotugno," ST629/D genotype in M-ICU ward of "D. Cotugno" hospital and CS-ICU ward of "V. Monaldi" hospital. This is in agreement with previous data reporting clonal expansion of *K. pneumoniae* isolates assigned to CC258 in Italy and world-wide (Bialek-Davenet et al., 2014; Gaiarsa et al., 2015; Giani et al., 2015; Conte et al., 2016; Cerqueira et al., 2017). Of the 7 *K. pneumoniae* sporadic isolates, 2 were assigned to the emerging epidemic clonal lineage CC101 (Del Franco et al., 2015; Conte et al., 2016; Novović et al., 2017; Jaidane et al., 2018). In agreement with previous data (Cannatelli et al., 2014; Jayol et al., 2014; Bradford et al., 2015; Giani et al., 2015; Wright et al., 2015), 20 out of 25 colistin-resistant *K. pneumoniae* isolates were carbapenem-resistant and KPC-3 producing, while 2 were VIM-1 producing isolates.

Colistin-resistant *K. pneumoniae* was the initial isolate from 18 patients (72%), while isolates were initially colistin-susceptible and subsequently colistin-resistant in 7 patients (28%). The inactivation of MgrB, the negative regulator of the PhoQ/PhoP signaling system, was found in 22 (88%) colistin-resistant *K. pneumoniae* isolates from HA "Ospedale dei Colli" and was caused by 7 different mechanisms of *mgrB* alterations. This is consistent with a recent study showing that the emergence of colistin-resistant *K. pneumoniae* in two England Hospitals was due to the transmission among patients of two distinct genotypes harboring three distinct mechanisms of colistin resistance (Otter et al., 2017). In agreement with previous data (Cannatelli et al., 2014; Olaitan et al., 2014; Giani et al., 2015; Poirel et al., 2015, 2017; Wright et al., 2015; Jaidane et al., 2018), a truncated MgrB was generated by one single nucleotide deletion, which causes frameshift mutation and premature termination in 10 isolates assigned to ST512/A and ST512/A4 genotypes, or by non-sense mutation c88t, which causes premature termination in 4 isolates assigned to ST512/A1. Mutated MgrB was generated by 3 distinct mutations in 3 isolates assigned to ST101/B, ST101/B1 and ST392/C genotypes. The insertional inactivation of *mgrB* gene

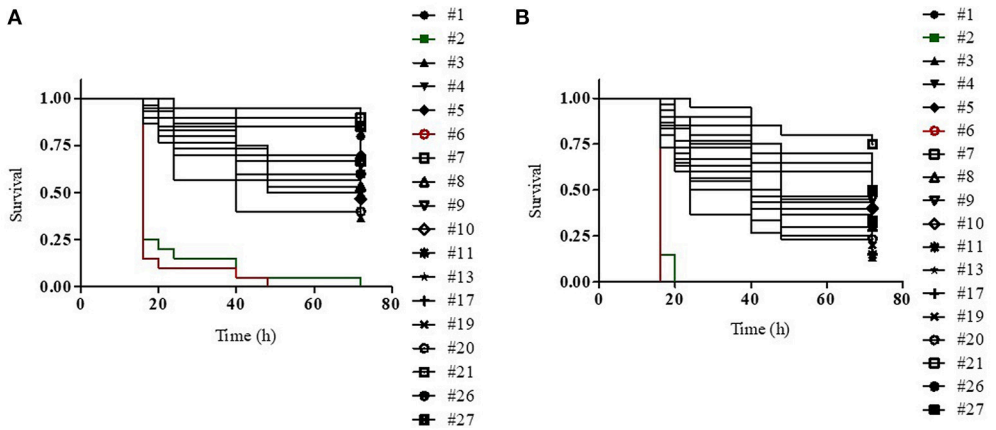


FIGURE 2 | Kaplan–Meier survival curves of *G. mellonella* larvae infected with **(A)** 1 (±0.26) × 10^5 or **(B)** 1 (±0.26) × 10^6 viable cells of the indicated *K. pneumoniae* strains. For each strain, 20 or 30 larvae were infected in two or three independent assays, respectively. In both experiments, the infectivity of isolates 2 and 6 (green and red lines, respectively) is significantly higher than that of all other strains ($P < 0.001$).

due to IS*5*-like or IS-903-like mobile elements was observed in 2, 1, and 1 isolates assigned to ST512/A2, ST512/A3, and ST45/F, respectively, similarly to data reported in Cannatelli et al. (2013), Olaitan et al. (2014), and Poirel et al. (2015). In particular, the insertion of IS*5*-like mobile element at nt 75 of *mgrB* gene was in the same position to that found in *K. pneumoniae* KKBO-4 strain (Cannatelli et al., 2013), suggesting the existence of a specific hot spot for IS*5* insertion in the *mgrB* gene. The presence of identical *mgrB* alterations in isolates from the same ward/hospital assigned to the same ST and PFGE profile demonstrates the occurrence of clonal expansion and cross-transmission of the colistin-resistant isolates within hospitals. Allelic variants with respect to the genes present in a colistin-susceptible *K. pneumoniae* reference strain were also found for *pmrA*, *pmrB*, *phoQ,* and *crrB* genes in some *K. pneumoniae* isolates described in this study; these variants have been already described in the literature and proposed to be not related with colistin resistance.

The above all data suggest that colistin-resistance in *K. pneumoniae* isolates was caused by alterations identified in the *mgrB* gene. In support of this, complementation with a wild type *mgrB* allele successfully restored colistin susceptibility in isolates having mutations or insertional inactivation of *mgrB* gene (**Table 3**). In particular, colistin susceptibility was restored also in isolate 3, which carries mutations in both *mgrB* and *phoQ* genes, further confirming that resistance was mainly caused by inactivation of MgrB. Notably, three isolates showed a wild type *mgrB* gene and no mutations in the other colistin-resistance related genes here analyzed, suggesting the presence of still-unidentified mechanism(s) alternative to mutations of *mgrB* gene.

We also demonstrate that colistin-resistant *K. pneumoniae* blood isolates from HA "Ospedale dei Colli" showed high variability in the infectivity in *G. mellonella* larvae, with up to 10-fold differences in lethal dose values among isolates assigned to the same ST and PFGE genotype and two hyper-virulent isolates assigned to ST45 and ST629 having LD_{90} values more than 100- or 10-fold lower than those of all other isolates, respectively. This is consistent with previous studies showing that *K. pneumoniae* has a high genome variability and a large accessory genome, which includes virulence functions associated with invasive disease in humans and antimicrobial resistance genes associated with hospital-acquired infections (Bialek-Davenet et al., 2014; Holt et al., 2015; Cerqueira et al., 2017). *K. pneumoniae* isolates assigned to CC258 and to other STs associated with hospital-acquired infections have acquired antimicrobial resistance genes and are usually devoid of virulence genes (Bialek-Davenet et al., 2014; Cerqueira et al., 2017), although the acquisition of yersiniabactin has been observed in many isolates of the epidemic KPC-producing CC258 (Holt et al., 2015). In further agreement with our data, carbapenem-resistant *K. pneumoniae* ST258 isolates associated with nosocomial infections exhibit variability in the infectivity of *G. mellonella* and in other virulence-associated traits (Diago-Navarro et al., 2014). Our data also showed no significant reduction in the infectivity in colistin-resistant isolates assigned to ST101 or ST258/ST512 genotypes with inactivation of the *mgrB* gene as compared to their respective colistin susceptible isolates (26 and 27; **Figure 2**). This is an agreement with previous studies showing that colistin resistance caused by the inactivation of the MgrB regulator is not associated with fitness cost and decreased virulence of ST258 KPC carbapenemase-producing *K. pneumoniae* (Cannatelli et al., 2015; Arena et al., 2016). Our data are also in partial agreement with a recent study showing that inactivation of MgrB actually enhanced virulence of *K. pneumoniae* Kp52145 strain, serotype O1:K2, belonging to the virulent CC65 (Kidd et al., 2017), although it should be considered that, even when they belong to the same ST and PFGE type, our clinical isolates are not isogenic, and thus a direct causal relationship between specific mutations and infectivity cannot be inferred. In this regard, it is worth noting that another study indeed reported that colistin-resistant *K. pneumoniae* strains carrying distinct mutations in *pmrB* or *mgrB* genes were highly variable in

their pathogenicity in the *G. mellonella* infection model (Wand et al., 2017). Overall, all the above data strongly suggest that the pathogenicity of *K. pneumoniae* isolates is independent of specific colistin-resistance mechanisms and might be influenced by variability in the genetic background of the different strains.

Finally, it is interesting to note that some isolates assigned to the same genotype, carrying the same mutation in *mgrB* and showing identical alleles for all the other colistin-resistance related genes here investigated, showed variability in colistin MIC values (**Table 2**), indicating that other, still-unidentified genetic mechanisms likely contribute to the acquisition of high levels of colistin resistance in *mgrB* defective backgrounds. Notably, although we did not observe any significant correlation between colistin-resistance mechanisms and virulence, in line with previous reports (Wand et al., 2017), our data suggest that the level of colistin MIC of *K. pneumoniae* isolates is predictive of their lethality (LD_{50} and LD_{90} values) in *G. mellonella*. Indeed, in our experimental setting high colistin MIC values are predictive of lower virulence of the isolates, suggesting that genetic adaption to high levels of colistin resistance could somehow impair *K. pneumoniae* infectivity. Further studies using larger collections of isolates, isogenic *in vitro* evolved colistin-resistant mutants and/or different infection models are however required to verify this hypothesis. Moreover, as whole genome sequencing has not been performed in this study, at this stage we cannot rule out that the acquisition of specific virulence-related gene(s) could partly account for the different pathogenic behavior of our isolates.

AUTHOR CONTRIBUTIONS

FI and RZ conceived the study and participated in its design and coordination. MB, SC, and VC collected the microbiological and epidemiological data. EE, MC, and MB performed laboratory analyses. FI, VC, and RZ performed data analyses. EE, FI, and RZ wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported in part by grant from University of Naples Federico II (Fondo d'Ateneo per la Ricerca to RZ) and Sapienza University of Rome (Ateneo 2017 to FI). MC was supported by a grant from Regione Lazio, Italy.

ACKNOWLEDGMENTS

We thank the team of curators from the Institute Pasteur MLST system (Paris, France) for importing *K. pneumoniae* novel *wzi* alleles and isolates at http://bigsdb.web.pasteur.fr. We thank Dr. Antonio Cannatelli, University of Siena, Italy and Dr. Gian Maria Rossolini, University of Florence, Italy, for providing *mcr-1* positive *E. coli* strain and pACYC-*mgrB* plasmid.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01463/full#supplementary-material

Figure S1 | Circulation of colistin-resistant *K. pneumoniae* genotypes in the HA "Ospedale dei Colli" from January 2015 to September 2016. The number of isolates and wards are shown.

Figure S2 | Kaplan–Meier survival curves of *G. mellonella* larvae infected with 1 ($\pm$0.26) $\times$ 10^6 (continuous line) or 1 ($\pm$0.26) $\times$ 10^5 (dashed line) viable cells of the indicated *K. pneumoniae* strains. For each strain, 20 or 30 larvae were infected in two or three independent assays, respectively. In both experiments, the infectivity of strains #2 and 6 is significantly higher than that of all other strains ($P < 0.001$).

Figure S3 | Dose-dependent survival curves, generated by the GraphPad Prism software, of *G. mellonella* larvae infected with different doses of the *K. pneumoniae* strains #1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 17, 19, 20, 21, 26, and 27. These curves have been used to retrieve the LD50, LD90, and R^2 values reported in **Table 4**.

Table S1 | Oligonucleotides used in the study.

Table S2 | Phenotypic and genotypic features of *K. pneumoniae* isolates included in the study.

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

Chapter 4

Diversity, virulence, and antimicrobial resistance in isolates from the newly emerging *Klebsiella pneumoniae* ST101 lineage.

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Front Microbiol. 2019 Apr 2; 10:542.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 02 April 2019
doi: 10.3389/fmicb.2019.00542

Diversity, Virulence, and Antimicrobial Resistance in Isolates From the Newly Emerging *Klebsiella pneumoniae* ST101 Lineage

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The global dissemination of *Klebsiella pneumoniae* and *Klebsiella pneumoniae* carbapenemase (KPC) has been largely attributed to a few high-risk sequence types (STs) (ST258, ST11, ST512) associated with human disease. ST101 is an emerging clone that has been identified in different parts of the world with the potential to become a global, persistent public health threat. Recent research suggests the ST101 lineage is associated with an 11% increase in mortality rate in comparison to non-ST101 infections. In this study, we generated a high-quality, near-finished genome assembly of a multidrug-resistant (MDR) isolate from Italy (isolate 4743) that is a single locus variant of ST101 (ST1685). We demonstrate that the 4743 genome contains virulence features such as an integrative conjugative element carrying the yersiniabactin siderophore (ICEKp3), the mannose-resistant *Klebsiella*-like (type III) fimbriae cluster (mrkABCDFHIJ), the ferric uptake system (kfuABC), the yersiniabactin receptor gene *fyuA*, a capsular K type K17, and an O antigen type of O1. *K. pneumoniae* 4743 carries the *bla*KPC-2 carbapenemase gene along with genes conferring resistance to aminoglycosides, beta-lactams, fluoroquinolones, fosfomycin, macrolides, lincosamides, and streptogramin B. A comparative genomics analysis of 44 ST101 genomes as well as newly sequenced isolate 4743 identified variable antimicrobial resistance (AMR) resistance profiles and incompatibility plasmid types, but similar virulence factor profiles. Using Bayesian methodologies, we estimate the common ancestor for the ST101 lineage emerged in 1990 (95% HPD: 1965 to 2007) and isolates within the lineage acquired bla_{KPC} after the divergence from its parental clonal group and dissemination. The identification of virulence factors and antibiotic resistance genes acquired by this newly emerging clone provides insight into the reported increased mortality rates and highlights its potential success as a persistent nosocomial pathogen. With a combination of both colistin resistance, carbapenem resistance, and several known virulence factors, the ST101 genetic repertoire may be a "perfect storm" allowing for a newly emerging, high-risk, extensively antibiotic resistant clone. This high-risk clone appears adept at acquiring resistance and may perpetuate the dissemination of extensive antimicrobial resistance. Greater focus on the acquisition of virulence factors and antibiotic resistance genes is crucial for understanding the spread of antibiotic resistance.

Keywords: *Klebsiella*, phylogenetics, antimicrobial resistance, ST101, genomics

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *20 November 2018*
Accepted: *01 March 2019*
Published: *02 April 2019*

Citation:
Roe CC, Vazquez AJ, Esposito EP, Zarrilli R and Sahl JW (2019) Diversity, Virulence, and Antimicrobial Resistance in Isolates From the Newly Emerging Klebsiella pneumoniae ST101 Lineage. Front. Microbiol. 10:542. doi: 10.3389/fmicb.2019.00542

INTRODUCTION

The Gram-negative bacterium, *Klebsiella pneumoniae,* is abundantly distributed in the environment and has traditionally been considered an opportunistic pathogen associated with hospital-acquired and community-acquired infections (Holt et al., 2015). Also, the rapid and global dissemination of multidrug-resistant (MDR) and extensively drug resistant (XDR or pan-resistant) *Klebsiella pneumoniae* was recently recognized by the CDC as an urgent public health threat requiring immediate and aggressive action (CDC, Antibiotic resistance threats in the U.S., 2013). Currently, carbapenem and broad-spectrum antibiotics are considered last resort treatment options, but the increasing incidence of extended-spectrum beta-lactamase (ESBL) and carbapenem resistant (CRE) isolates coupled with their global distribution highlights the potential for rapid dissemination of mobile MDR genes to other highly virulent, nosocomial pathogens (Ventola, 2015). *K. pneumoniae* contains significant genome variability and large accessory genomes, which includes virulence functions associated with invasive disease in humans and antimicrobial genes associated with hospital-acquired infections (Bialek-Davenet et al., 2014a; Holt et al., 2015; Cerqueira et al., 2017). Recent genomic epidemiology studies of *K. pneumoniae* isolates identified two types of high-risk clonal groups among bacterial populations: hyper-virulent clonal complexes (CCs) responsible for community-acquired invasive infections and multidrug-resistant CCs responsible for health-care associated infections (Struve et al., 2015; Cerqueira et al., 2017). The existence of hyper-virulent clones is a key feature of *K. pneumoniae* and typically show increased pathogenesis not commonly associated with antimicrobial resistance (Struve et al., 2015).

Klebsiella pneumoniae isolates responsible for health-care associated infections are usually CRE due to production of either class B metallo-β-lactamases (MBLs) (IMP,VIM,NDM) or class A (KPC) or class D (OXA-48) serine carbapenemases (Pitout et al., 2015; Grundmann et al., 2017; Logan and Weinstein, 2017). Multidrug-resistant (MDR) *K. pneumoniae* isolates have primarily been assigned to CC258 (Gaiarsa et al., 2015) and to additional emerging genotypes ST11, ST15 and ST101 (Bialek-Davenet et al., 2014a; Bowers et al., 2016; Conte et al., 2016; Cerqueira et al., 2017; Moradigaravand et al., 2017). *K. pneumoniae* isolates associated with hospital-acquired infections have acquired antimicrobial resistance genes and are usually devoid of virulence genes (Bialek-Davenet et al., 2014a; Cerqueira et al., 2017). However, the acquisition of yersiniabactin (an iron sequestering system crucial for disease establishment) has been observed in many isolates of the epidemic KPC-producing CC258 (Holt et al., 2015). High-risk clones have a highly flexible accessory genome and are adept at both acquiring resistance as well as switching resistance profiles (Woodford et al., 2011). Their global success as a nosocomial pathogen can be correlated with excessive genome plasticity (Conlan et al., 2016). Understanding high-risk clones' ability to adapt and survive in a hospital environment is vital to assuage further spread of antibiotic resistance. While hyper-virulent and drug-resistant *K. pneumoniae* populations remain mostly non-overlapping, combinations of these groups (isolates within CC23) have been described (Bialek-Davenet et al., 2014a). The risk of combination of these two high-risk genetic profiles highlights the importance of understanding the spread of genomic regions from the *K. pneumoniae* accessory genome.

Among the newly emerging genotypes, *K. pneumoniae* isolates belonging to ST101 are associated with hospital-acquired infections and epidemics worldwide (Giani et al., 2013; Skálová et al., 2016; Gonçalves et al., 2017). *K. pneumoniae* isolates assigned to ST101 are CRE because of the production of KPC-2 (Oteo et al., 2016) or OXA-48 (Skálová et al., 2016; Avgoulea et al., 2018). Also, the emergence of colistin resistance has been observed in KPC-2 producing (Del Franco et al., 2015) and OXA-48 producing (Giani et al., 2013; Del Franco et al., 2015; Papagiannitsis et al., 2016) *K. pneumoniae* ST101 isolates. The focus of this study was to characterize the phylogenetic and genomic diversity within the emerging nosocomial high-risk sequence type ST101. MDR *K. pneumoniae* isolates typically belong to specific high-risk sequence types (ST11, ST258, ST512) (Bialek-Davenet et al., 2014a), with extensive comparative genomics research investigating the genomic backbone of these clones, however, few studies have investigated the global genomic diversity of the newly emerging and clinically relevant ST101 clone (Gonçalves et al., 2017; Moradigaravand et al., 2017; Avgoulea et al., 2018). In this study, we investigated the lineage relatedness, resistance determinants, plasmid profiles, core and accessory genome content, and the evolutionary rate of the ST101 sequence type through whole-genome sequencing. We propose a timeline for emergence as well as provide a better understanding of the evolution and composition of an increasingly successful MDR global lineage that may aid in the identification of newly emerging novel *K. pneumoniae* lineages and help understand the proliferation of extensively antibiotic resistance pathogens.

MATERIALS AND METHODS

Isolate Information of an MDR Strain From Italy

Klebsiella pneumoniae 4743 strain was isolated from rectal swab of a patient in the intensive care unit ward of public hospital of USL Valle D'Aosta, Aosta, Italy on November 24th 2013. To compare this isolate in a phylogenetic context, a global collection of 1,723 publicly available *K. pneumoniae* isolates were downloaded and analyzed (**Supplementary Table S1**).

Antimicrobial Resistance Profiling

Antimicrobial susceptibilities were performed for 4743 using the Vitek 2 system and the AST-GN card (bioMérieux, Marcy l'Étoile, France). Values were interpreted according to breakpoint table for interpretation of MIC values and zone diameters (European Committee on Antimicrobial Susceptibility Testing, 2016). Colistin susceptibility assay was performed according to recommendations of joint CLSI-EUCAST guidelines: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf.

DNA Extraction, Sequencing, and Assembly

For Illumina MiSeq sequencing of strain 4743, genomic DNA was extracted with the GenElute DNA extraction kit (Sigma-Aldrich, Milan, Italy). A sequence library was generated for paired-end sequencing using previously described methods (Stone et al., 2016) and sequenced to an average depth of 50*x*. For PacBio sequencing, genomic DNA of sample 4743 was extracted using a DNeasy Blood and Tissue Kit according to the manufacturer's instructions (Qiagen, Milan, Italy). Approximately 10 μg of DNA was fragmented to 10–20 kbp using the G-tube apparatus (Covaris) following the manufacturer's recommendations. A PacBio Sequencing library was constructed using the SMRTbell^{TM} Template Prep Kit 1.0 and by following the PacBio 20 kb library protocol; the library molecules, after adapter ligation and damage repair, were size selected for 15 kb and larger using Blue Pippin instrument (Sage Sciences) by following the manufacturer's instructions. The final library was processed for sequencing by using PacBio MagBead kit v2 with the P6/C4 chemistry and following PacBio protocols. Sequencing was performed on PacBio RSII instrument in one SMRT cell (v3) for 6 h.

The 4743 genome was assembled using Unicycler v0.4.7 (Wick et al., 2017), resulting in seven contigs. The genome was polished by running six rounds of Pilon (Walker et al., 2014) and was then processed with Circlator (Hunt et al., 2015). Assembly statistics are shown in **Supplementary Table S2**. The assembly and raw reads were deposited in NCBI under BioProject PRJNA477005. Additionally, genome annotation was performed using PROKKA v1.13 (Seemann, 2014).

In silico MLST Typing

Multi-locus sequence typing (MLST) analysis was performed on all genome assemblies using an *in silico* MLST script[1] which implemented the Institut Pasteur's MLST scheme as previously described (Diancourt et al., 2005).

In silico K Typing and O Typing

Polysaccharide capsule (K typing) and lipopolysaccharide O antigen typing was performed using the Kaptive tool (Wyres et al., 2016). Briefly, the source code for the command-line version of Kaptive was downloaded from the GitHub repository[2]. Both K and O typing of 45 ST101 isolates was accomplished running default settings within the Kaptive.py script.

Core Genome SNP Phylogenies

All external *K. pneumoniae* genomes (n = 3,352) were downloaded from GenBank in February 2018. Genomes were filtered from the dataset if they contained: (1) greater than 10 ambiguous nucleotides; (2) an anomalous number of contigs (>462); (3) an anomalous genome assembly size (<5,195,738, >5,992,749) and; (4) an anomalous pairwise MASH distance (>0.017) (Ondov et al., 2016); this filtering resulted in a final dataset of 1,504 genomes. All genome assemblies were aligned against the completed reference ST101 genome GCA_001902435 using NUCmer (Delcher et al., 2002) and SNPs were identified as part of the NASP pipeline (Sahl et al., 2016). A global maximum likelihood phylogeny was generated using the TVM+ASC+G4 nucleotide substitution model in IQTREE v1.6.1 (Nguyen et al., 2015) with 1,000 bootstrap replicates. Trees were visualized in FigTree[3]. The Retention Index (Farris, 1989) was calculated with Phangorn (Schliep, 2011).

For the ST101 clade analysis, assembled genomes were aligned to sample Kp_Goe_33208 (GCA_001902435) using the previously described methods above. A ST101-only maximum likelihood phylogeny was inferred using the K3P+ASC substitution model with 1,000 bootstrap replicates in IQTREE. Phylogenetic trees were visualized in FigTree. The Retention Index was calculated with Phangorn. Additionally, the presence of recombination within this dataset was determined using the PHI statistic from the package PhiPack (Bruen et al., 2006).

LS-BSR Analysis

The Large-Scale Blast Score Ratio (LS-BSR) pipeline (Sahl et al., 2014) was used to identify differential coding region conservation within the ST101 clade. This pipeline predicted coding regions (CDSs) using the program Prodigal v2.60 (Hyatt et al., 2010) and clustered the putative CDS regions with 90% identity using VSEARCH v1.11.1 (Rognes et al., 2016). The resulting regions were aligned against themselves using the program BLAT v35×1 (Kent, 2002) to generate a reference bit score. Genomic regions were then aligned back to every sample in the ST101 dataset using BLAT to generate a query bit score. The query bit score was divided by the reference bit score for each region to obtain the BLAST Score Ratio (BSR) (Rasko et al., 2005). The core CDSs were identified as genomic regions that had a BSR value of > 0.80 across all genomes. Unique genomic regions were identified based on a BSR value < 0.40 in all but a single genome.

Genes Associated With Virulence, Heavy Metal Resistance, and Drug Resistance

Genes associated with virulence (120), colibactins (91), yersiniabactins (165), heavy metal resistance (203), efflux pumps and regulators (489), and non-scheme genes (278) were downloaded from BIGSdb-Kp database (Bialek-Davenet et al., 2014b). LS-BSR was run on the ST101-only lineage with the genes parameter flagged using the virulence, heavy metal resistance, and efflux pump databases as input. LS-BSR was run using nucleotides and the BLAT alignment option. A BSR value > 0.95 was considered present.

QRDR Mutations

Quinolone resistance determining regions (QRDRs) within the ST101 genomes were inspected for mutations conferring increased fitness within gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE)* genes. Reference genes for *parE (*AFQ64062.1), *parC* (AFQ64068.1), *gyrA* (AFQ64815.1),

[1]https://gist.github.com/jasonsahl/2eedc0ea93f90097890879e56b0c3fa3

[2]http://github.com/kelwyres/Kaptive-Web

[3]http://tree.bio.ed.ac.uk/software/figtree/

and *gyrB* (AFQ63426.1) were downloaded from NCBI. Blastx from blast+ v2.2.29 (Camacho et al., 2009) was used to identify the composition of each gene in the ST101 genomes. Genes were extracted from each genome and alignments were performed using ClustalW (Thompson et al., 1994) in MEGA7 (Kumar et al., 2016).

Plasmid Analysis

The *Enterobacteriaceae* plasmid database from PlasmidFinder (Carattoli et al., 2014) was used to identify plasmid types within the ST101 clade. The PlasmidFinder database consists of representative genes of varying incompatibility groups. Here we screened 122 typing genes from PlasmidFinder against the ST101 genomes using LS-BSR/BLAT. BSRs were generated and visualized in Rstudio using the shinyheatmap program (Khomtchouk et al., 2017). Typing genes with a BSR value > 0.95 were considered to be present.

Antibiotic Resistance Gene Screening

The distribution of genes associated with virulence and antimicrobial resistance was identified for all ST101 genomes. Acquired genes associated with antimicrobial resistance were identified using the ResFinder database (Kleinheinz et al., 2014) with LS-BSR. The ResFinder database included genes that may confer resistance to the following antibiotics: aminoglycosides, beta-lactams, colistin, fluoroquinolone, fosfomycin, fusidic acid, glycopeptide, macrolide, lincosamide, and streptogramin B (MLS), nitroimidazole, oxazolidinone, phenicol, rifampicin, sulphonamide, tetracycline, and trimethoprim. Mutations within the *mgr*B regulator gene that are known to confer resistance to colistin were also investigated (Esposito et al., 2018). Briefly, the program BLAT was used to identify the *mgrB* gene within the ST101 samples and the sequence was extracted from each alignment. The *mgrB* gene sequences were aligned using MUSCLE (Edgar, 2004) in MEGA7 (Kumar et al., 2016) and subsequently translated into amino acids. The amino acid sequences were visually inspected for premature stop codons. Additionally, genes associated with virulence were obtained from the BIGSdb-KP database and screened for within the ST101 genomes using LS-BSR. A heatmap representing presence/absence of antimicrobial resistance genes was produced using shinyheatmap in *R*.

Population Structure

In order to assess the genomic relationships within the ST101 lineage, we applied Plink v1.07 (Purcell et al., 2007) and fastStructure (Raj et al., 2014), a Bayesian model-based clustering algorithm; the Plink output was used as input for fastStructure. Briefly, SNP positions with more than two allele states were removed from the dataset. Plink was implemented using default parameters. As the *K* parameter represents populations in fastStructure, we implemented $K = 2$ through $K = 9$ with default parameters. An optimal *K* value was chosen based on model complexity that maximizes marginal likelihood as well as optimum model components used to explain the structure.

Beast Timing Analysis

BEAST analysis included 36 of the 45 ST101 genomes (**Supplementary Table S3**). Strains were only included in this analysis if the collection date was listed under the biosample data on NCBI. A core SNP matrix was generated by aligning raw reads to sample 4743 using the NASP pipeline. The presence of recombination within a dataset can confound molecular clock analyses and was therefore identified and removed using ClonalframeML running default parameters (Didelot and Wilson, 2015). A regression analysis implementing root-to-tip genetic distance as a function of the sample collection year was conducted using the software package TempEst version 1.5.1 (Rambaut et al., 2016). A measure of clocklike behavior was assessed using the determination coefficient R^2 and the rooted ST101-only phylogeny. Additionally, a 10,000 date-randomization permutation of sampling collection dates was performed in an effort to compare our regression coefficient to that observed by random chance (Murray et al., 2016).

RESULTS

4743 Strain Details

Klebsiella pneumoniae 4743 was isolated from a rectal swab of a patient admitted to Intensive Care Unit of USL Valle D'Aosta, Aosta, Italy on November 24th 2013. *K. pneumoniae* 4743 isolate was representative of an epidemic of KPC-2 producing *K. pneumoniae* CC101 occurring from November 2013 to August 2014 (Del Franco et al., 2015). *K. pneumoniae* 4743 isolate was able to transfer resistance to carbapenems and ESBL activity along with conjugative plasmids carrying $bla_{\mathrm{KPC-2}}$ and $bla_{\mathrm{CTX-M-group1}}$ genes, respectively (Del Franco et al., 2015).

4743 Genome Assembly

The genome assembly using PacBio reads combined with Illumina MiSeq reads produced a total of 7 contigs, 6 of which are plasmids and 1 of which is chromosomal; 4 of the plasmids were determined to be circular. The total size of sample 4743 is 5,857,478 bases. PHASTER identified four intact phage within the large chromosome contig, and two more complete phage in two of the plasmid contigs (4743 plasmid unnamed 3 and 4743 plasmid unnamed 6).

Antimicrobial Resistance Profiling

Antimicrobial susceptibilities were performed for sample 4743 using the Vitek 2 system and the AST-GN card. *Klebsiella pneumoniae* 4743 isolate was resistant to imipenem, meropenem, ertapenem, beta-lactam/beta-lactamase inhibitor combinations (clavulanic acid/amoxicillin, piperacillin/tazobactam), third and fourth generation cephems, ciprofloxacin, but susceptible to trimethoprim-sulfamethoxazole and colistin (**Supplementary Table S4**).

External Genomes

External genome assemblies from *K. pneumoniae* were downloaded from the assembly database in GenBank and

filtered based on several quality criteria, resulting in 1,504 genome assemblies. *in silico* MLST typing was performed on all assemblies using the previously described Pasteur system (Diancourt et al., 2005). Of all *K. pneumoniae* isolates analyzed, 41 were assigned to ST101, three were assigned to ST2017, and one sample (4743) was assigned to ST1685. Both ST2017 and ST1685 are single locus variants (locus *rpoB*) of ST101 and were included in subsequent analyses.

Phylogenetics and Comparative Genomics

WGS of one isolate (4743) was performed and compared to whole genome sequences of 44 *K. pneumoniae* isolates assigned to the ST101 lineage available in GenBank and 1,504 non-ST101 *K. pneumoniae* reference genomes (**Supplementary Table S1**). The core genome phylogeny based on 1.9 Mb of conserved sequence and 179,342 SNPs demonstrated the position of the ST101 lineage (**Figure 1**) in relation to most global lineages. The retention index (RI) of the global isolate SNP alignment was 0.958, indicating little homoplasy throughout the *K. pneumoniae* core genome. The presence of recombination introduces incongruences in the phylogenetic placement of lineages with deeply branching nodes and long branches (Schierup and Hein, 2000). The PHI statistic was used to test for evidence of recombination. The *p*-value for the PHI statistic was 0.00e+00, providing evidence, but not quantification, of recombination.

A phylogeny of the ST101 lineage only, as defined by a monophyletic clade in the global phylogeny, was based on 3,992 concatenated SNPs and demonstrated little homoplasy with an RI value of 0.95, indicating that the genomes in this lineage are closely related and their evolution was minimally driven by recombination. However, the PHI statistic revealed statistically significant recombination within this dataset (*p*-value = 0.0). ClonalframeML identified 372 recombination events that spanned 138,212 bases, both monomorphic and polymorphic, across the chromosome of the ST101 lineage. Masking of these regions removed 1,882 SNPs from the ST101 core genome. The high-quality, non-recombination core genome size of the ST101 clade was 4.85 Mb.

Population Structure in the ST101 Lineage

In order to visualize the shared genomic regions within the ST101 lineage, fastStructure was applied using the ST101-only dataset that included 3,992 SNPs. We evaluated our dataset for shared ancestry using a range of K values (number of expected populations) from 2 to 10. Model complexity that maximized the marginal likelihood was 2. Model components used to explain the structure in the data was 3. When K = 2, fastStructure revealed two subpopulations within the ST101 lineage with limited genome sharing between the two populations; only two samples (GCA 001720745 Pakistan 2013 and GCA 001720815 Pakistan 2013) displayed genome sharing (**Figure 2**). For K = 3, fastStructure revealed limited genome sharing between two of the three of the populations; eight samples demonstrated admixture. In both models, samples GCA 002187295 (Nigeria 2014) and GCA 002247645 (Thailand 2015) make up a separate ST101 population.

In silico Antimicrobial Resistance Profiling

As a complement to the laboratory-determined antimicrobial susceptibility profile for strain 4743 and in order to compare the ST101 lineage antimicrobial susceptibility patterns, *in silico* antimicrobial susceptibility testing was performed using the ResFinder database. Genes responsible for conferring aminoglycoside resistance, beta-lactam resistance, and fluoroquinolone resistance were conserved across all ST101 clade genomes (**Figure 3** and **Supplementary Table S5**). Fluoroquinolone resistance genes were identified within every ST101 genome (*oqx*A, *oqx*B). Genes responsible for fosfomycin resistance were identified in 97.8% of ST101 genomes (*fosA*). Phenicol resistance genes (*cat*B4, *cat*A2, *cml*A1, *floR*) were conserved in 73% of the ST101 genomes. In total, 26% ST101 isolates carried genes responsible for macrolide, lincosamide, and streptogramin B resistance (*mphE*, *msrE*, *ereA*, *ereB*, *mphA*). Rifampicin resistant genes (*arr*-2 and *arr*-3) were present in 24% of genomes, and 71.1% of isolates carried genes responsible for sulphonamide resistance (*sul*1, *sul*2, *sul*3). Additionally, 60% of the isolates harbored genes conferring Tetracycline resistance (*tetD*, *tetX*, and *tetA*) and 86.7% carried at least one gene responsible for trimethoprim resistance (*dfrA*1, *dfrA*5, *dfrA*14, *dfrA*16, or *dfrA*27). A total of 15 different beta-lactamase genes, including ESBLs, were identified within the ST101 lineage. The most prevalent ESBL gene identified was $bla_{\mathrm{CTX-M-15}}$ (84%); $bla_{\mathrm{CTX-M-14}}$ gene was found in two ST101 genomes. The blaSHV-1 gene was identified in 87% of the ST101 samples, conferring resistance to broad spectrum beta-lactams. Sample 4743 carried the $bla_{\mathrm{CTX-M-15}}$ gene. Two genomes, sample 4743 and ST101 (Oteo et al., 2016), carried the $bla_{\mathrm{KPC-2}}$ gene (pKP048_p019, NC_014312.1). Additionally, 33.3% of the 45 genomes carried the $bla_{\mathrm{OXA-48}}$ gene. Alarmingly, 7 of 45 (15.5%) ST101 genomes contain alterations in *mgrB* gene (4 genomes harbor either a frameshift mutation or IS insertional inactivation while 3 genomes show a deletion of the *mgrB* locus) conferring colistin resistance (Esposito et al., 2018).

Plasmid Composition

Plasmid typing was analyzed in ST101 genomes using 122 previously characterized *K. pneumoniae* plasmids from the database PlasmidFinder. The results demonstrate the plasmid diversity across the ST101 lineage (**Figure 4**), although this methodology does not reveal whether the typing genes are present chromosomally or on plasmids. Plasmid replicon analysis revealed various types of plasmid incompatibility groups among the ST101 isolates, none of which were detected in all 45 ST101 samples. However, incompatibility plasmid groups FIB, FII, and R were detected in the majority of ST101 samples [IncFIB (K) in 84%, IncFII (K) in 75%, IncR in 61%, IncFIA

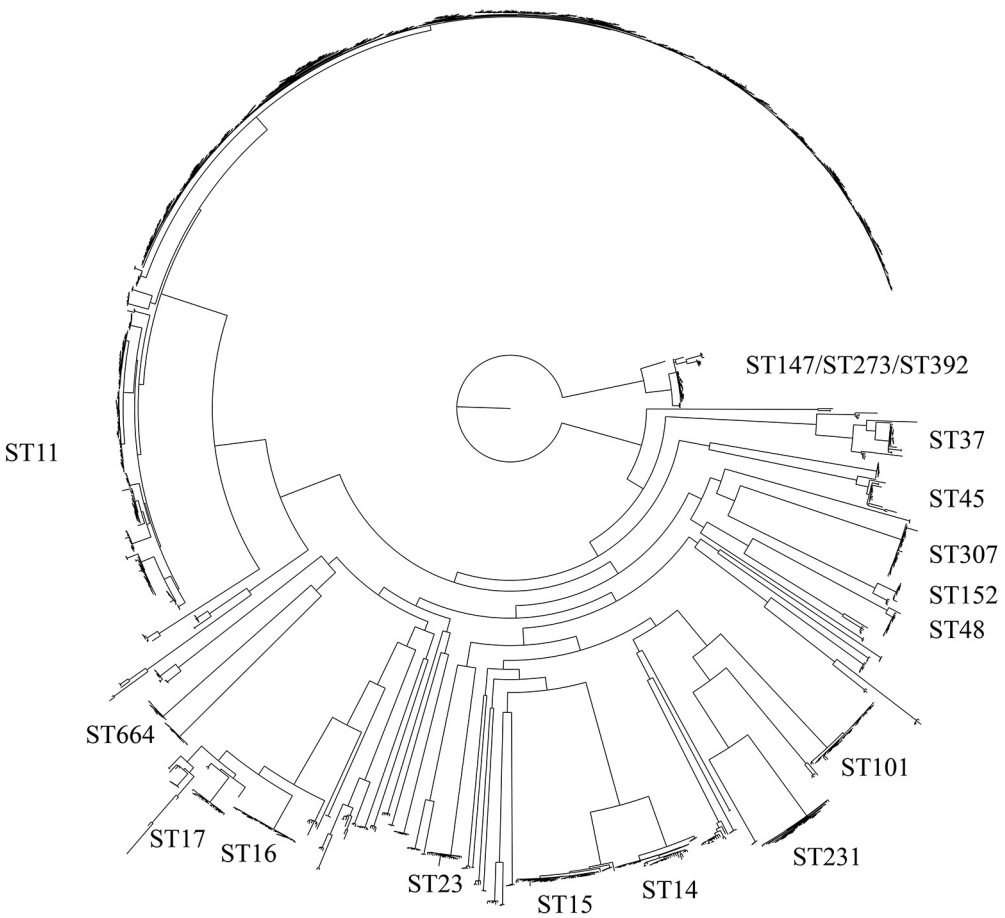

FIGURE 1 | Maximum-likelihood phylogeny of the *Klebsiella pneumoniae* species. This tree includes 1,724 genomes that collectively cover 38.59% of the GCA 001902435 reference genome and is based on 118,011 total SNPs. Within this species, several large clades exist and are labeled according to their MLST type.

(HI1) in 61%, and IncL/M (pOXA-48) in 29%]. The following incompatibility groups were found in 3 out of 4 plasmids identified in sample 4743 as well as one non-circular contig: IncFIB(K) (4743 plasmid unnamed 2), IncFII(K) (4743 plasmid unnamed 2), IncR (4743 plasmid unnamed 3), IncFIA (HI1) (4743 plasmid unnamed 3), IncFII (4743 plasmid unnamed 4), and ColRNAI (4743 plasmid unnamed 5). Plasmid pKP-KPC2 corresponding to 4743 plasmid unnamed 3 (111,854 nts) carried IncFII(K) and bla_{KPC-2} gene, plasmid pKP-CTXM-15 corresponding to 4743 plasmid unnamed 4 (67,867 nts) carried IncFII and $bla_{CTXM-15}$ gene. Interestingly, the blastn alignment of plasmid pKP-KPC2 from isolate 4743 and plasmid pKP048 from ST101 *K. pneumoniae* isolate described by Oteo et al., 2016 showed that the bla_{KPC-2} gene is included in Tn1721 transposon structure of 14,470 nts (residues 11,810–26,279 in plasmid pKP048).

Unique Genomic Regions

The complete gene content for 1,504 *K. pneumoniae* genomes was compared using LS-BSR. By using default values in LS-BSR, we did not identify any coding regions that were unique to the ST101 clade and absent from all others. The core genome for the ST101 lineage consisted of 4,465 coding region sequences (CDSs). The ST101 lineage accessory genome had a total of 2,247 CDSs. There were a total of 564 CDSs found only in a single genome within ST101 and the average number of unique CDSs per genome was 12.8. While these gene are unique within the ST101 lineage, all 564 CDSs were identified throughout the global *K. pneumoniae* genomes.

Virulence and AMR Associated Genes

Known genes associated with virulence (**Supplementary Table S6**) from the BIGSdb-Kp database were screened against the ST101 lineage using LS-BSR and BLAT. These genes included heavy metal resistance genes, efflux pumps, colibactin and yersiniabactin genes. Additionally, we screened for the yersiniabactin-encoding mobile element ICE*Kp* as well as capsule and lipopolysaccharide serotyping. Samples within the ST101 lineage all carry the iron-scavenging siderophore

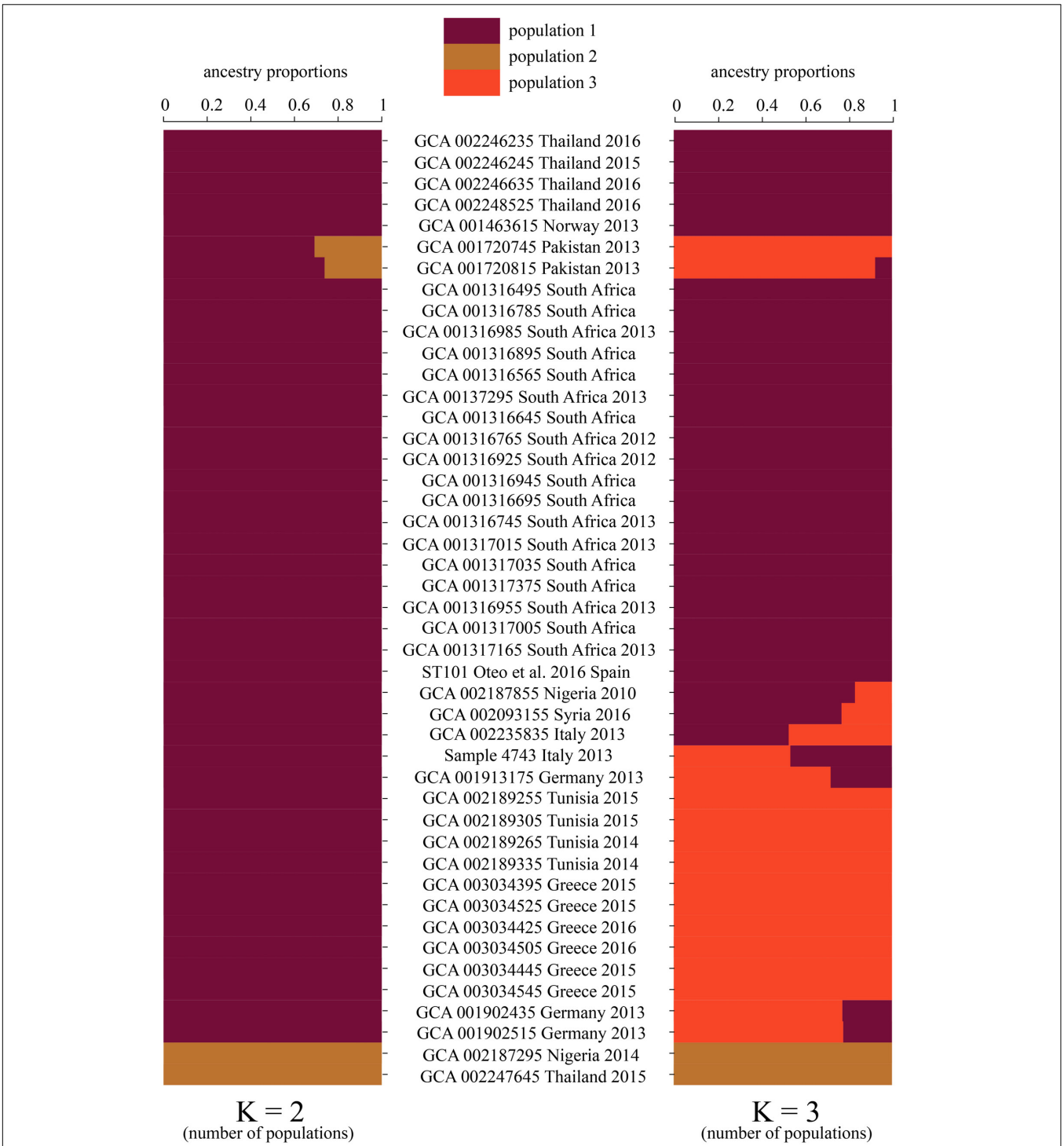

FIGURE 2 | Ancestry proportions were inferred for the ST101 lineage using fastStructure v1.0 with the optimal choices of *K* (populations). Two different *K*-values (number of populations) were selected as most appropriate, $K = 2$ and $K = 3$. $K = 2$ graph represents two populations with little shared ancestry between them. Population one is represented by dark red. Population two is distinguished as brown. When running fastStructure with the number of expected populations set at three ($K = 3$), we observed limited shared ancestry.

yersiniabactin on the mobile genetic element ICE*Kp3* except for two samples, GCA 002187295 and GCA 002247645, which fall outside the main ST101 clade (**Figure 1**). The ST101 lineage was screened for a total of 489 efflux pump genes from the BIGSdb-KP database. We identified 115 conserved efflux pump genes within all ST101 samples including the

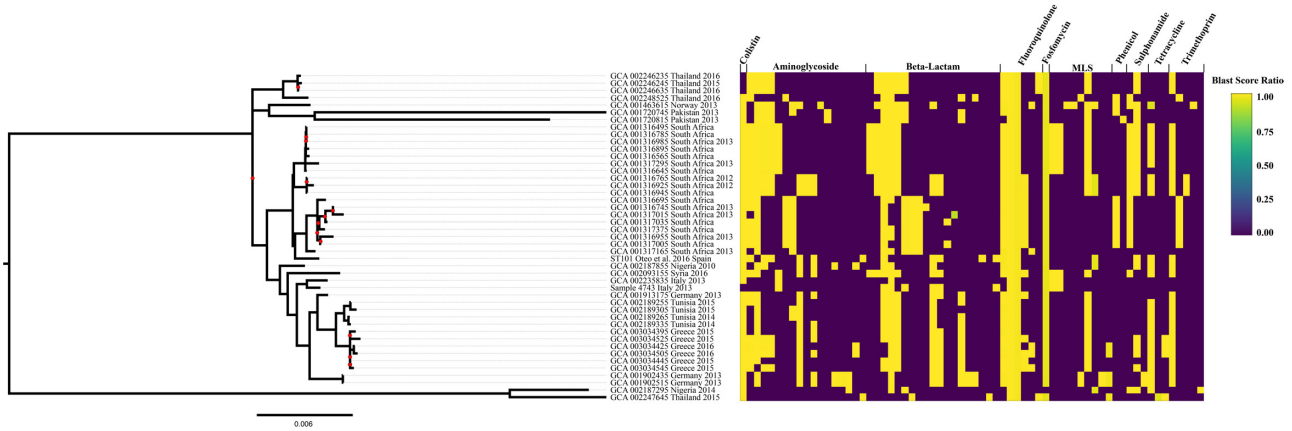

FIGURE 3 | Maximum-likelihood phylogeny of the *Klebsiella pneumoniae* ST101 lineage. The tree is based on 4,077 SNPs using the K3P+ASC+G4 nucleotide substitution model and includes 45 genomes that collectively cover 85.95% of the reference genome. The PHI test found statistically significant evidence for recombination in this dataset (P = 0.00e+00). All samples were screened for antibiotic resistance using ResFinder. The percent identity for each resistance gene was plotted using shinyheatmap alongside the phylogenetic tree. Gene lists are in **Supplementary Table S6**. MLS includes genes that confer resistance to macrolides-lincosamides-streptogramin B.

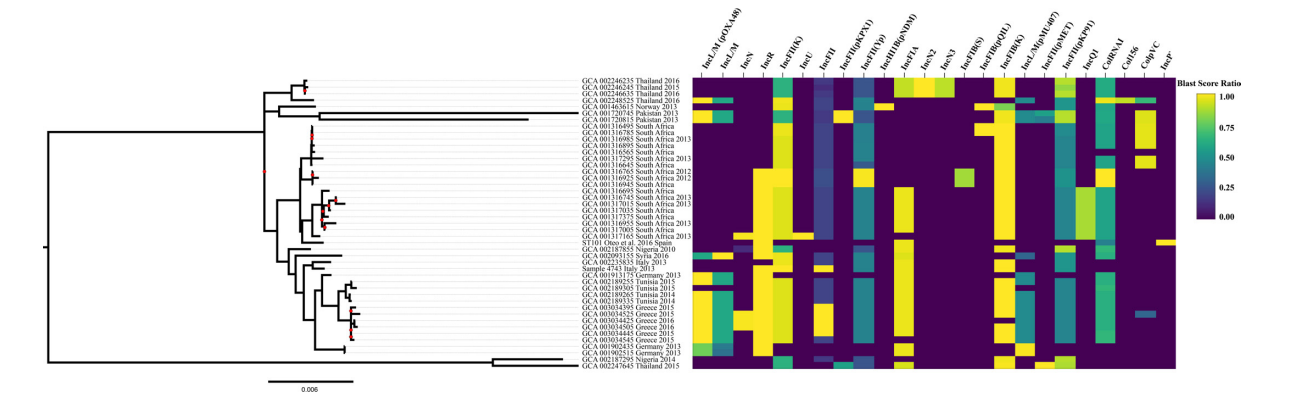

FIGURE 4 | Plasmid replicon gene screening. The ST101 lineage assemblies were screened for plasmid types using PlasmidFinder. The percent identity for each plasmid typing gene was plotted using shinyheatmap alongside the maximum-likelihood phylogenetic tree.

virulence and antibiotic cross-resistance associated AcrAB and OqxAB efflux pumps as well as the *rarA* regulator gene (**Supplementary Table S5**). A total of 203 heavy metal resistance genes were screened against the ST101 lineage. The complete pcoABCDRSE operon conferring copper resistance was identified in 36 of the 44 ST101 samples while 41 of the genomes carry genes responsible for silver resistance (silCERS).

Both K typing and O typing have meaningful clinical and epidemiological significance. Capsular polysaccharide characterization (K typing) is widely used to define clinical *K. pneumoniae* isolates (Hansen et al., 2002; Vimont et al., 2008; Woodford et al., 2011). The polysaccharide capsule is often considered a virulence determinant as K-type variations have been linked to specific presence/absence of genes within the locus. Specific lipopolysaccharide antigens (O typing) also contribute to *K. pneumoniae* pathogenicity. In an effort to better understand the pathogenicity of the ST101 clone, we performed both K and O typing using the program Kaptive Web. Limited capsular diversity was observed with 43 samples having the KL17 loci and 2 samples with the KL106 loci. O loci typing revealed the majority of the ST101 lineage as O1V1. Interestingly, the O1 antigen has been previously described as a major contributor to the virulence of pyogenic liver abscess causing *K. pneumoniae*. Three ST101 samples (GCA 001316955, GCA 002189265, GCA 001316985) were typed as O1/O2v1. O1/O2 signifies that either gene *wbbY* or *wbbZ* could not be found within the sample (Wick et al., 2018).

QRDR Mutations

All of the ST101 genomes but two (GCA_002187295 and GCA_002247645) showed amino acid substitutions in codon 83 (Ser83Tyr) and codon 87 (Asp87Gly, Asp87Asn, Asp87Ala) of the *gyrA* gene (**Supplementary Table S7**). Additionally, a Ser80ILe substitution in the

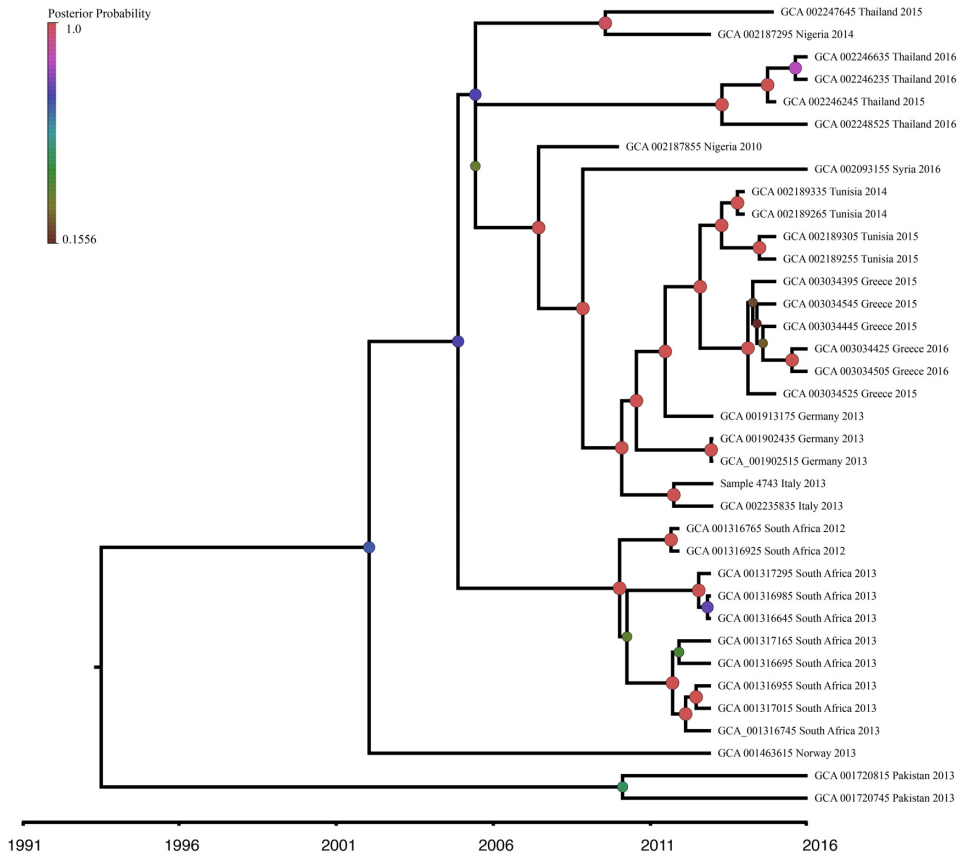

FIGURE 5 | Bayesian phylogenetic analysis of *K. pneumoniae* ST101 isolates. Beast v1.8.4 was used to produce a calibrated maximum clade credibility (MCC) phylogeny. The analysis was performed on WGS data from 36 *K. pneumoniae* ST101 genomes. Posterior probabilities are indicated by node size and color. The timeline represents years before the present.

parC gene was detected in all ST101 genomes with the exception of two samples (GCA_002187295 and GCA_002247645). Amino acid substitutions within QRDR of the *parE* or the *gyrB* genes were not observed in any of the ST101 genomes.

Beast Analysis

The ST101-only Bayesian dataset consisted of 35 genomes with reported collection dates from NCBI and the newly sequenced sample 4743. This dataset contained 3,977 SNPs, with 1,889 SNP positions falling within genomic regions determined by ClonalframeML as recombining and subsequently removed from this analysis. The root-to-tip regression analysis identified weak clocklike behavior (R^2 = 0.025) within the ST101 lineage with 2.5% of the diversity explained by time. However, we believe we are capturing little of the true temporal signal due to our narrow sampling dates (2010–2016) and small sample size (n = 36). Given the positive regression slope and the detection of a clocklike signal, we determined molecular clock analysis was appropriate and reliable for SNP accumulation rate estimation (Duchêne et al., 2016). MEGA7 identified the best fitting nucleotide substitution model as the GTR model and was applied in the BEAST analysis. In an effort to determine if the observed R^2 value was better than random chance, a 10,000-date permutation testing on the recombination removed ST101-only dataset. This testing produced a P value of 0.339, indicating that our observed R^2 value was not statistically different than random chance.

In order to investigate the evolution of *K. pneumoniae* ST101, BEAST analysis was performed using the recombination-free, high quality SNP dataset previously described. The constant population demographic model with a relaxed molecular clock was selected as the most appropriate model and clock combination to describe ST101 evolution. Molecular clock calibration estimated the evolutionary rate for the ST101 lineage as 2.8527×10^{-6} substitutions per site per year (95% highest posterior density [HPD], 1.0762×10^{-6} to 4.7396×10^{-6}). The mean time to most recent common ancestor (TMRCA) was estimated at 26.6 years ago (95% HPD, 8.35 to 51.24 years ago) from the time of the last sample date, which was 2016 (**Figure 5**). This dataset has narrow sampling dates with a limited number of samples and it is likely we are capturing very little of the temporal signal. Given a wider sampling time and additional samples, we would likely narrow our MRCA estimate.

DISCUSSION

The focus of this study was to perform a comprehensive genomics analysis of the ST101 lineage to better understand the genomic content of this newly emerging, extensively resistant lineage. Previous studies have identified this sequence type as high risk for furthering the spread of carbapenem resistance (Del Franco et al., 2015; Oteo et al., 2016). Here we demonstrate that ST101 is a distinct lineage in which one sample in this study, 4743, acquired the KPC-2 enzyme. Additionally, a recent study identified 13 ST101 samples carrying the KPC-2 gene (WGS data not yet available) (Del Franco et al., 2015; Oteo et al., 2016). The inconsistent carriage of KPC-2 within the ST101 clone suggest the KPC enzyme was acquired through horizontal transfer of plasmids after the divergence of the ST101 lineage rather than vertical gene transfer among the entire lineage. In further support of this, our data showed that ST101 *K. pneumoniae* isolate 4743 and ST101 isolate described by Oteo et al., 2016 both carry plasmids containing the bla_{KPC-2} gene into Tn1721 transposon.

We found multiple genomic features that may provide this clone with an advantage for adaptation within a hospital environment as well as a human host. Multiple putative virulence factors were identified throughout the ST101 lineage including the siderophore genes *irp1* and *irp2*, the yersiniabactin receptor gene *fyuA,* the ICE*Kp*-3 element that carries the yersiniabactin siderophore cluster (ybtAEPQSTUX), the mannose-resistant *Klebsiella*-like (type III) fimbriae cluster (mrkABCDFHIJ), and the ferric uptake system (kfuABC). The *irp* and *irp2* genes encode iron-repressible high molecular weight proteins that are involved with yersiniabactin production (Schubert et al., 1998); these were identified in all but two samples within the ST101 lineage. The *fyuA* (ferric yersiniabactin uptake) gene is also involved with the iron-acquiring yersiniabactin system by coding for yersiniabactin receptors (Schubert et al., 1998). In *E. coli*, the *fyuA* gene is required for biofilm formation in urinary tracts and is important for disease establishment in iron-poor environments such as the urinary tract (Hancock et al., 2008). The ST101 lineage also carry the yersiniabactin siderophore cluster on an ICE, which has been identified as a frequent virulence factor in *K. pneumoniae* (Schubert et al., 2004; Holt et al., 2015; Lam et al., 2018). The ICEKp element increases the ST101 clone's ability to cause disease by coding for an iron scavenging system; this element also allows this clone to further spread this important virulence factor because it is located on an integrative conjugative element (Lam et al., 2018). The mannose-resistant *Klebsiella*-like (type III) fimbriae cluster (mrkABCDFHIJ) is considered a virulence factor and has been previously described in several species of *Enterobacteriaceae* as contributors to mucous adherence, tissue colonization, and biofilm formation (Monroy et al., 2005; El Fertas-Aissani et al., 2013). Finally, the ferric uptake system (kfuABC) is also associated with increased virulence (Ma et al., 2005). In pyogenic liver abscess causing *K. pneumoniae*, the kfu system is described as usually more prevalent in invasive tissue strains, however, the presence of multiple iron-acquisition systems suggests the ST101 lineage is capable of acquiring and using iron from diverse sources similar to pyogenic liver causing strains (Ma et al., 2005; Hsieh et al., 2008). Previous research suggests iron acquisition gene diversity allows for more capable environmental iron acquisition (Lee et al., 2012). This increased efficiency of iron uptake has been hypothesized to result in increased capsule production, which is an important phenotype of hypervirulence (Russo et al., 2011; Chaturvedi et al., 2012; Shon et al., 2013; Moradigaravand et al., 2017).

Hyper-virulent and drug-resistant *K. pneumoniae* populations remain mostly non-overlapping, although combinations of these groups have been previously reported (Bialek-Davenet et al., 2014a; Gu et al., 2018). Here we demonstrate the presence of several known virulence factors within the ST101 lineage as well as highlight the extensive drug resistance gene repertoire this clone harbors (colistin, beta-lactams, aminoglycosides, fluoroquinolones, and fosfomycin). The ST101 lineage is an example of a dual-risk clone combining the genetic profiles of hypervirulent *K. pneumoniae* as well as extensive drug resistant strains. Alarmingly, the ST101 resistome is similar to the antibiotic resistant profile of the global ST258 lineage. The majority of both ST101 and ST258 clones possess genes that are responsible for resistance to aminoglycosides [aac(6′), aph(6′), aadA genes], third generation-cephalosporins and aztreonam (*bla*CTXM), fosfomycin (*fosA*), and fluoroquinolones [aac(6′)-Ib-cr, *oqxA*, *oqxB*, *qnrB*, *qnrS* genes]. Both lineages confer resistance to colistin due to inactivation of the *mgrB* gene and to carbapenems due to the acquisition of the *bla*KPC-2 gene. Both lineages carry the mannose-resistant *Klebsiella*-like (type III) fimbriae cluster (mrkABCDFHIJ), and the ICE*Kp* containing the yersiniabactin siderophore cluster (ybtAEPQSTUX). While drug-resistance has been shown to be associated with a large loss of fitness, researchers have demonstrated the presence of three amino acid substitutions within QRDR of *parC* and *gyrA* genes that result in no loss of vitality in multiple bacterial species, including *K. pneumoniae* (Marcusson et al., 2009; Toth et al., 2014). In addition to extensive drug resistance, genomes from the ST101 lineage harbor these three amino acid substitutions within the *gyrA* and *parC* genes. *K. pneumoniae* minor sequence types that are fluoroquinolone resistant were reported to lack these mutations entirely, carry a "non-serine" mutation, or only one of the three mutations (Fuzi et al., 2017). However, major clones, specifically ST258, were all shown to carry the *gyrA* Ser83Ile and *parC* Ser80Ile double mutations (Bowers et al., 2015). Given the global success of ST258 and similar virulence, antibiotic resistance, and QRDR mutation profiles compared to the ST101 lineage, this newly emerging clone has the potential to become a global epidemic dual-risk clone and major public health threat.

DATA AVAILABILITY

The datasets generated for this study can be found in NCBI, PRJNA477005.

ETHICS STATEMENT

The study was approved by the ethics committee of the Aosta Regional Hospital (protocol number 836/2015). All

microbiological samples were taken as part of standard care procedures. Patients included in the study were anonymized, no written informed consent was acquired because of the retrospective nature of the study.

AUTHOR CONTRIBUTIONS

CR analyzed the data and wrote the manuscript. AV and EE performed laboratory analyses. RZ designed the study and helped to write the manuscript. JS designed the study, analyzed the data, and helped to write the manuscript.

FUNDING

This work was funded under the State of Arizona Technology and Research Initiative Fund (TRIF), administered by the Arizona Board of Regents, through Northern Arizona University.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00542/full#supplementary-material

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

Chapter 5

Concluding remarks

K. pneumoniae is currently recognized as an urgent threat for the public health worldwide because of its capability to cause hospital and community acquired infections. The selection and the spread of MDR, XDR and HV strains is due to the great genomic plasticity of *K. pneumoniae* that allows it to acquire and exchange a large number of accessory genes, such as AMR genes and virulence factors, that allow it to grow and persist in several environmental conditions in addition to humans and animals. This phenomenon is responsible for the emergence of *K. pneumoniae* high-risk isolates, which conjugate AMR and HV phenotypes and leave clinicians with very few therapeutic options.

This PhD thesis reported the emergence of carbapenem and colistin resistant *K. pneumoniae* isolates assigned to distinct genotypes in different hospital setting of Naples and focused on an emerging ST101 *K. pneumoniae* high-risk clone, analyzing the genomic features, which explain its spread in clinical setting.

CRE *K. pneumoniae* ST104 was detected in neonates recovered in the NICU of the V. Monaldi Hospital in Naples, Italy. The spread was due to the acquisition of plasmid pIncAC-KP4898, carrying the *bla*VIM-1 gene and several additional resistance genes into the scaffold of an IncA/C1 group self-conjugative plasmid. The pIncAC-KP4898 presents a composite genetic structure, which might have been generated by the acquisition of different genomic regions from different sources mediated by multiple recombination events (Esposito et al, 2017).

Twenty-five colistin-resistant *K. pneumoniae* blood isolates were collected from the Hospital Agency "Ospedale dei Colli," Naples, Italy, during 2015 and 2016. MgrB inactivation was the mechanism of resistance found in our strains. The presence of identical mutations/insertions in isolates of the same ST and PFGE profile suggests the occurrence of clonal expansion and cross-transmission. In addition, we performed virulence assays in *Galleria mellonella* model to demonstrate the infectious capacity of colistin-resistant *K. pneumoniae* belonging to different genotypes. The results showed different virulence profiles among isolates with the infectivity of the two isolates assigned to ST45 and ST629 being significantly higher than that of all other isolates. Moreover, high colistin MIC correlated with low infectivity (Esposito et al, 2018). These data confirm what is happening in the global epidemiological scenario where antibiotic resistant and HV phenotypes are overlapping in clinical isolates worldwide.

Finally, we demonstrated that the ST101 lineage was an example of dual-risk clone combining the genetic profiles of HV *K. pneumoniae* as well as extensive drug resistant strains. Alarmingly, the ST101 resistome was similar to the antibiotic resistant profile of the global ST258

lineage. The identification of virulence factors and antibiotic resistance genes acquired by this newly emerging clone provides insight into the reported increased mortality rates and highlights its potential success as a persistent nosocomial pathogen. With the combination of both colistin resistance, carbapenem resistance, and several known virulence factors, the ST101 genetic repertoire may be a “perfect storm” allowing for a newly emerging, high-risk, extensively antibiotic resistant clone (Roe et al., 2019).

List of publications

List of papers included in the thesis

Esposito EP, Gaiarsa S, Del Franco M, Crivaro V, Bernardo M, Cuccurullo S, Pennino F, Triassi M, Marone P, Sassera D, Zarrilli R. A Novel IncA/C1 Group Conjugative Plasmid, Encoding VIM-1 Metallo-Beta-Lactamase, Mediates the Acquisition of Carbapenem Resistance in ST104 *Klebsiella pneumoniae* Isolates from Neonates in the Intensive Care Unit of V. Monaldi Hospital in Naples. Front Microbiol. 2017 Nov 3;8:2135. doi: 10.3389/fmicb.2017.02135.

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Additional papers not included in the thesis

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Acknowledgments

I would like to thank Prof. Raffaele Zarrilli for guidance, encouraging, support and constructive criticism to my PhD work.

I would like to extend my thanks to my colleagues Dr. Mariateresa Del Franco, Dr. Eliana De Gregorio and Dr. Maria Bagattini who provided me their precious help and fruitful discussions and to the other colleagues of 19th floor of “Biological Tower”.

I wish to thank Matteo Cervoni and Antonella Migliaccio for the time we spent working together and for all the fun we had.

A special mention should be made to Prof. Ohad Gal-Mor and his group, who I was lucky to collaborate with. They all supported me during the experience in Israel and I felt like at home.

I would like to express my immense gratitude to my friends Marika Errico from Spain, Blerina Marku, Caterina De Mauro, Carmen Iannone, Francesco Laudato and Francesco Chiuso for sharing the most beautiful and the hardest moments of my university career.

A big thank to my family for their continuous and unconditional support, for love, for encouraging and suffering me from the beginning to the end of my PhD, and for teaching me to try to give always the best of me.

Lastly, I want to thank God because when the difficulties seem insurmountable he stayed with me and has paved the way.