GENOMIC ANALYSIS OF THE DIVERSITY, VIRULENCE AND ANTIMICROBIAL RESISTANCE IN CLINICAL *KLEBSIELLA PNEUMONIAE* FROM CHINA

NING DONG

PhD

The Hong Kong Polytechnic University

2019

The Hong Kong Polytechnic University
Department of Applied Biology & Chemical Technology

Genomic Analysis of the Diversity, Virulence and Antimicrobial Resistance in Clinical *Klebsiella pneumoniae* from China

Ning Dong

A thesis submitted in partial fulfilment of
the requirements for the degree of Doctor of Philosophy

November 2018

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

_____ (Signed)

Dong Ning
_____ (Name of student)

Abstract

Klebsiella pneumoniae is an opportunistic pathogen which could cause both community- and hospital-acquired infections. Hypervirulent *K. pneumoniae* (hvKp) and multidrug resistant *K. pneumoniae* represent the major branches of *K. pneumoniae* species which are commonly associated with human infections. *K. pneumoniae* relies on a battery of gene products to escape the innate immune mechanisms of the host; these include capsule, siderophores, lipopolysaccharide (LPS), fimbriae, outer membrane protein (OMPs), porins, efflux pump and transporters. HvKp strains normally exhibit enhanced capsules which could be semi-quantitatively defined by a "string test". They are mostly associated with serotype K1 or K2, with CC23 being the dominant K1 hvKp clone, whereas several genetically unrelated groups (ST25, ST86, etc.) constitute the K2 clone. *K. pneumoniae* is becoming untreatable by the last-line antibiotics since many strains exhibited resistance to multiple drugs, especially the extended-spectrum β-lactam (ESBL) and carbapenems. Classic *K. pneumoniae* (cKp) frequently acquire the antimicrobial resistance phenotype through horizontal transfer of resistance genes. The majority of KPC-producing *K. pneumoniae* worldwide belong to the notorious CC258 clone (including ST258, ST11, ST340, ST437 and ST512). Several other clonal groups (CG) were also globally distributed and associated with multidrug resistance, including CG14/15, CG17/20, CG43 and CG147.

Whole genome sequencing (WGS) is a revolutionized technology which has been widely used in both academia and clinical settings. The application of WGS in studies of *K. pneumoniae* mainly covers analysis of evolution trends and transmission of lineages of interest, as well as identification of genetic features of epidemiological or clinical importance. To gain better understanding of this notorious pathogen and to further control its evolution and transmission, we initiated this project. Through analyzing the genome sequences of clinical ST11 *K. pneumoniae* strains from China, we revealed the genetic relationship of different ST11 isolates, and identified the genetic elements associated with virulence traits and antimicrobial resistances phenotypes. We also tracked a fatal outbreak of T11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital and characterized CR-hvKp isolates that belong to different sequence types.

First, we conducted comprehensive genomic analysis of 58 clinical strains of ST11 *K. pneumoniae*, which is the dominant KPC-producing clinical clone in China. We found that these strains, collected from geographically diverse locations in the country, were genetically diverse and could be segregated into three clades, each of which exhibited distinct capsule polysaccharide loci. Various antimicrobial resistance genes and virulence genes, such as those encoding salmochelin, aerobactin and RmpA, the hallmarks of hypervirulent *K. pneumoniae,* were detected in the genome of these strains. Results in this part showed that ST11 carbapenemase-resistant, hypervirulent *K. pneumoniae* strains have widely emerged in China, and provided insight for development of strategies for prevention, diagnosis and treatment of ST11 clinical infections.

Second, we investigated a fatal ventilator-associated pneumonia outbreak of ST11 carbapenemase-resistant, hypervirulent *K. pneumoniae* (CR-hvKp) in patients in a Chinese hospital. ST11 CR-hvKp have disseminated across various regions of China, which accounted for around 3% of clinical ST11 carbapenem-resistant *K. pneumoniae* infections in China. ST11 CR-hvKp exhibits a hypervirulence phenotype which was characterized by a positive string test result, extremely high survival upon exposure to human neutrophils, and high virulence in a wax moth (*Galleria mellonella*) larva infection model. The emergence of ST11 CR-hvKp strains was due to acquisition of a 170 kbp virulence plasmid carrying the *rmpA2* and aerobactin biosynthesis genes by classic ST11 carbapenem-resistant *K. pneumoniae* strains. We suggest future research to focus on the development of intervention measures for the prevention of further dissemination of CR-hvKp isolates in hospital settings.

Lastly, we delineated the genetic structure of two different types of carbapenem-resistant, hypervirulent *K. pneumoniae*. The first belonged to ST23 CR-hvKP whose emergence was due to IS*26*-mediated insertion of $bla_{\text{KPC-2}}$ into a pLVPK-like virulence plasmid in ST23 hypervirulent *K. pneumoniae*. The second was an ST11 CR-hvKP isolate which carries 5 copies of $bla_{\text{KPC-2,}}$ which were introduced by Tnp*26*-encoded activity of the translocatable units. In the two cases, the carbapenem-resistance and hypervirulence phenotypes were both mediated by the plasmid(s) each strain harbored, indicating occurrence of an alarming evolutionary event of *K. pneumoniae,* the trend of which should be carefully monitored in the future. The emergence of diverse CR-hvKp isolates

suggest that such organisms could cause severe infections in both hospital settings and the community.

This study identified genetic elements associated with virulence traits and antimicrobial resistances phenotypes in *K. pneumoniae* and demonstrated the emergence of different types of carbapenem-resistant, hypervirulent *K. pneumoniae* via plasmid-mediated gene transfer. Findings in this study provide valuable information for future control of *K. pneumoniae* infections in China and neighboring countries.

PUBLICATIONS PRODUCED DURING THE COURSE OF THIS STUDY

1. **Dong, N.**, Zhang, R., Liu, L., Li, R., Lin, D., Chan, E. W. C., & Chen, S. (2018). Genome analysis of clinical multilocus sequence Type 11 *Klebsiella pneumoniae* from China. Microbial genomics, 4(2).
2. Gu, D.[#], **Dong, N.**[#], Zheng, Z., Lin, D., Huang, M., Wang, L., ... & Chen, S. (2018). A fatal outbreak of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital: a molecular epidemiological study. The Lancet Infectious Diseases, 18(1), 37-46. ([#]Contributed equally)
3. **Dong, N.**[#], Lin, D.#, Zhang, R., Chan, E.W., Chen, S. (2018). Carriage of bla_{KPC-2} by a virulence plasmid in hypervirulent *Klebsiella pneumoniae*. Journal of Antimicrobial Chemotherapy 73(12): 3317-3321. ([#]Contributed equally)
4. **Dong, N.**, Yang, X., Zhang, R., Chan, E. W. C., & Chen, S. (2018). Tracking microevolution events among ST11 carbapenemase-producing hypervirulent *Klebsiella pneumoniae* outbreak strains. Emerging Microbes & Infections, 7(1), 146.
5. **Dong, N.**, Liu, L., Zhang, R., Chen, K., Xie, M., Chan, E.W., Chen, S. (2019). An IncR Plasmid harbored by a hypervirulent, carbapenem resistant *Klebsiella pneumoniae* strain possesses five tandem repeats of the bla_{KPC-2}::NTE_{KPC-Id} fragment. Antimicrobial agents and chemotherapy, 63(3): e01775-18.
6. Lingbin Shu #, **Dong, N.**[#], et al. Emergence of OXA-232 carbapenemase-producing *Klebsiella pneumoniae* that carries a pLVPK-like virulence

plasmid among elderly patients in China. under revision. (#Contributed equally)

7. Zhang, R., **Dong, N.**, Huang, Y., Zhou, H., Xie, M., Chan, E. W. C., ... & Chen, S. (2018). Evolution of tigecycline-and colistin-resistant CRKP (carbapenem-resistant *Klebsiella pneumoniae*) *in vivo* and its persistence in the GI tract. Emerging microbes & infections, 7(1), 127.
8. Chen, K., **Dong, N.**, Zhao, S., Liu, L., Li, R., Xie, M., ... & Chen, S. (2018). Identification and characterization of conjugative plasmids that encode ciprofloxacin resistance in *Salmonella*. Antimicrobial agents and chemotherapy, AAC-00575.
9. Li, R., Xie, M., **Dong, N.**, Lin, D., Yang, X., Wong, M. H. Y., ... & Chen, S. (2018). Efficient generation of complete sequences of MDR-encoding plasmids by rapid assembly of MinION barcoding sequencing data. GigaScience, 7(3), gix132.
10. Zhang, Y., Zheng, Z., Chan, E. W. C., **Dong, N.**, Xia, X., & Chen, S. (2018). Molecular characterization of *qnrVC* and their novel alleles in *Vibrio* spp. isolated from food products in China. Antimicrobial agents and chemotherapy, AAC-00529.
11. Li, R., Yu, H., Xie, M., Chen, K., **Dong, N.**, Lin, D., ... & Chen, S. (2018). Genetic basis of chromosomally-encoded *mcr-1* gene. International journal of antimicrobial agents, 51(4), 578-585.
12. Chen, K., Chan, E. W. C., Xie, M., Ye, L., **Dong, N.**, & Chen, S. (2017). Widespread distribution of *mcr-1*-bearing bacteria in the ecosystem, 2015 to 2016. Eurosurveillance, 22(39).

ACKNOWLEDGEMENTS

This thesis would not have been possible without the guidance, support and encouragement of many wonderful individuals. I would like to express my deepest gratitude to each of them.

Firstly, I would like to extend my sincere gratitude to my supervisor, Prof. Sheng Chen, for providing this precious opportunity for me to work on this remarkable project. In the past three years, Prof. Chen helped me design my research project, gave me relevant advices and always encouraged me to overcome obstacles. Without his help, I could not conduct my research so smoothly. The training under his supervision will certainly benefit me throughout my life. Many thanks to my supervisor for his guidance, encouragement and gracious support.

I would also like to thank all members of the Chen laboratory for their consistent encouragement and support in my daily research work. Thanks to Dr. Edward Wai-Chi Chan for helping with manuscript proofreading and for the helpful advices on my research. Thanks to Dr. Ruichao Li for his help with next generation sequencing, to Dr. Dachuan Lin for his assistance in virulence of *Klebsiella pneumoniae*, to Lizhang Liu, Kaichao Chen and Dr. Marcus Ho Yin Wong for their help with strain collection, to Kathy Hiu Laam Po for helping with the ordering; and to Xuemei Yang, Miaomiao Xie, Lianwei Ye, Bill Kwan Wai Chan, Miaomiao Wang and Qipeng Cheng for critical discussion. Many thanks to the wonderful people in the lab.

Also, I am greatly thankful to Prof. Rong Zhang from second Affiliated Hospital of Zhejiang University for providing strains for this study and giving me suggestions on my project from the clinical view. Thanks to lab members in Prof. Zhang's lab for help with providing strains in this study. And thanks to Dr. Linfeng Huang in City University of Hong Kong for providing the opportunity to perform whole genome sequencing.

My thanks also go to my friends Fengping Chen, Chen Xu, Feng Guo, Wanlu Li, who supported me a lot in everyday life and helped me out of difficulties during the course of this study.

Above all, I sincerely thank my family members for their unconditional love, selfless support, and utmost understanding in my whole life. My special thanks must go to my parents who gave birth to me and taught me to behave. They encouraged me to pursue my passions. Whatever happens, they are always on my side. Many thanks to my sister. She provides powerful support for me which is to take care of my parents when I am not at home. Thanks to my uncles, aunts, brothers and sisters who care about my study and life very much.

Thank you. This thesis is dedicated to all of you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1. Introduction

1.1. *Klebsiella pneumoniae*, an opportunistic pathogen

The genus *Klebsiella*, a member of the family *Enterobacteriaceae*, is a class of Gram-negative, encapsulated, nonmotile, rod-shaped bacteria which has emerged globally as a major opportunistic pathogen (Figure 1.1) [1, 2]. Strains of this genus was first isolated in the late 19th century and named after the German microbiologist, Edwin Klebs [1, 3]. The genus comprises at least eight identified species, including *K. pneumoniae sensu stricto* (hereafter referred to as *K. pneumoniae*) and other species in the *K. pneumoniae* complex, *K. grimontii, K. michiganensis, K. oxytoca* and *K. aerogens* (Figure 1.2) [4, 5]. Recently, a novel species of the genus *Klebsiella*, *K. huaxiensis*, phylogenetically clustered with the *K. oxytoca* lineage, was reported in China [6]. The *K. pneumoniae* complex consists species including *K. pneumoniae, K. quasipneumoniae subsp. similipneumoniae, K. quasipneumoniae subsp. quasipneumoniae*, *K. variicola*, *K. quasivariicola*, and an unnamed lineage (Figure 1.2) [4, 7].

Figure 1.1 Electonmicroscopic picture of *Klebsiella pneumoniae*. (adapted from Wikepedia)

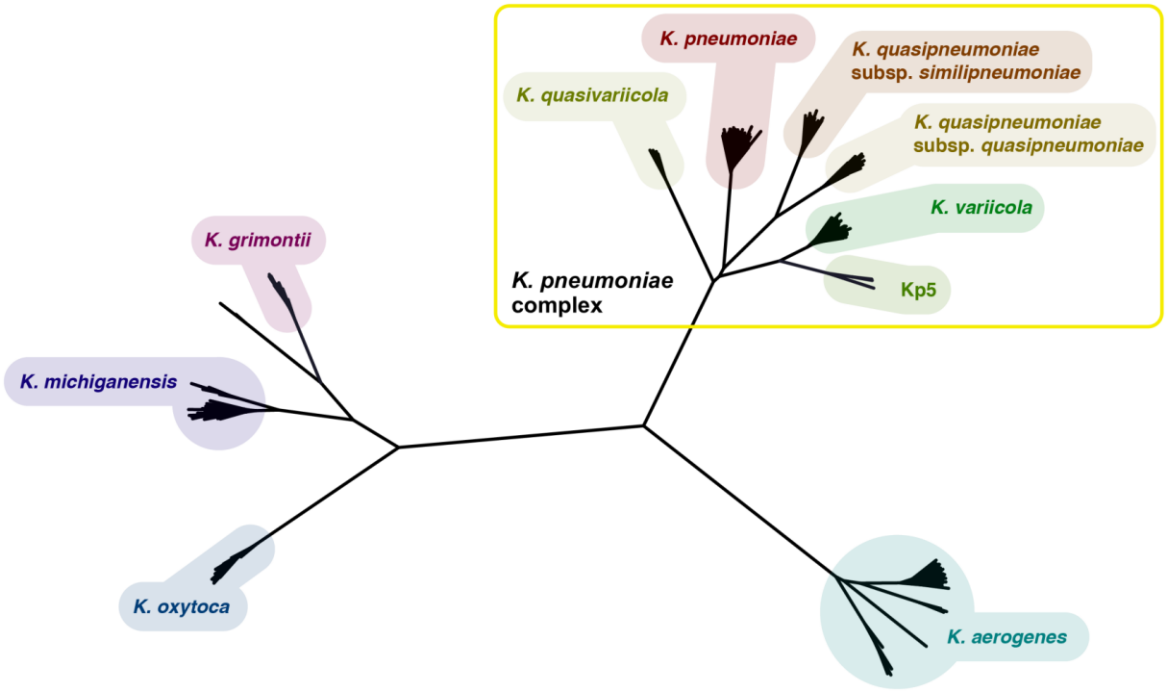


Figure 1.2 Phylogenetic tree of the genus *Klebsiella* constructed with mashtree. The *K. pneumoniae* complex was indicated with a yellow box. Adapted from [4].

Among the diverse species of *Klebsiella*, *K. pneumoniae* was most frequently associated with infections in hospital settings, with an isolation rate of approximately 80%-85% [8-10]. Also, strains of *K. pneumoniae* exhibit the highest rate of antimicrobial resistance (AMR) gene carriage within the *Klebsiella* genus, especially for those encoding resistance to third-generation cephalosporins [4, 11]. Worryingly, in the last two decades, most new AMR determinants, such as KPC, NDM, MCR-1, SHV and CTX-M was first discovered in *K. pneumoniae*, before being widely disseminated to other *Enterobacteriaceae* pathogens [12-17]. *K. pneumoniae* can normally be found in the flora of healthy individuals' nose, throat, skin and intestinal tract, but can also cause a range of infections in hospitalized patients, most commonly pneumonia, but also soft tissue and surgical wound infections, bacteremia, or urinary tract infections [8]. The organism can also cause some less common, yet serious infections such as liver abscess, invasive syndrome, septic arthritis, or generalized pustulosis [18]. In western countries, most infections caused by *K. pneumoniae* are due to the "classic" *K. pneumoniae* (cKP) strains which could cause severe infections among immunosuppressed patients in hospital

settings. cKP strains have received increased notoriety owing to their propensity to acquire AMR determinants [19, 20]. However, in the past three decades, hypervirulent *K. pneumoniae* (hvKp) strains causing community-acquired serious infections were increasingly being reported, especially in Asia [1]. The emergence and widespread dissemination of both cKP and hvKP pose serious threat to human health.

1.2.1. Classic *K. pneumoniae*

Infections caused by cKP are rapidly becoming untreatable since most clinical isolates exhibit multiple, extensive or pan-drug resistance to antibiotics, particularly through expression of β-lactamases [1, 2, 21]. Extended-spectrum β-lactamase (ESBL) and carbapenemase are two commonly observed types of β-lactamase in *K. pneumoniae* [22]. ESBL-producing *K. pneumoniae* exhibit resistance to cephalosporins and monobactams, causing 23% of the nosocomial infections (~17,000 infections) according to the work report of the US Centers for Disease Control, while carbapenemase-producing strains which cause 11% of nosocomial infections are more difficult to be eradicated since they are resistant to almost all available β-lactams [1, 23]. The multilocus sequence typing (MLST) scheme has been widely applied to distinguish the genetic relationships among *K. pneumoniae* isolates using seven house-keeping loci (*rpoB, gapA, mdh, pgi, phoE, infB, tonB*) [24]. Closely related sequence types (ST) could be further designed as clonal complexes (CC) using eBURST (http://eburst.mlst.net/) [25]. The majority of class A carbapenemase (KPC) producing *K. pneumoniae* worldwide belong to the notorious CC258 clone (including ST258, ST11, ST340, ST437 and ST512) [25]. DeLeo et al. obtained the complete sequence of two ST258 genomes (NJST258_1 and NJST258_2) and identified a recombination event related to serologic variation among ST258 *K. pneumoniae* isolates [26]. At least 22 distinct K loci imported via recombinant events were associated with strains within CC258 [27]. Several other clonal groups (CG) were also globally distributed and associated with multidrug resistance, including CG14/15, CG17/20, CG43 and CG147 (Figure 1.3).

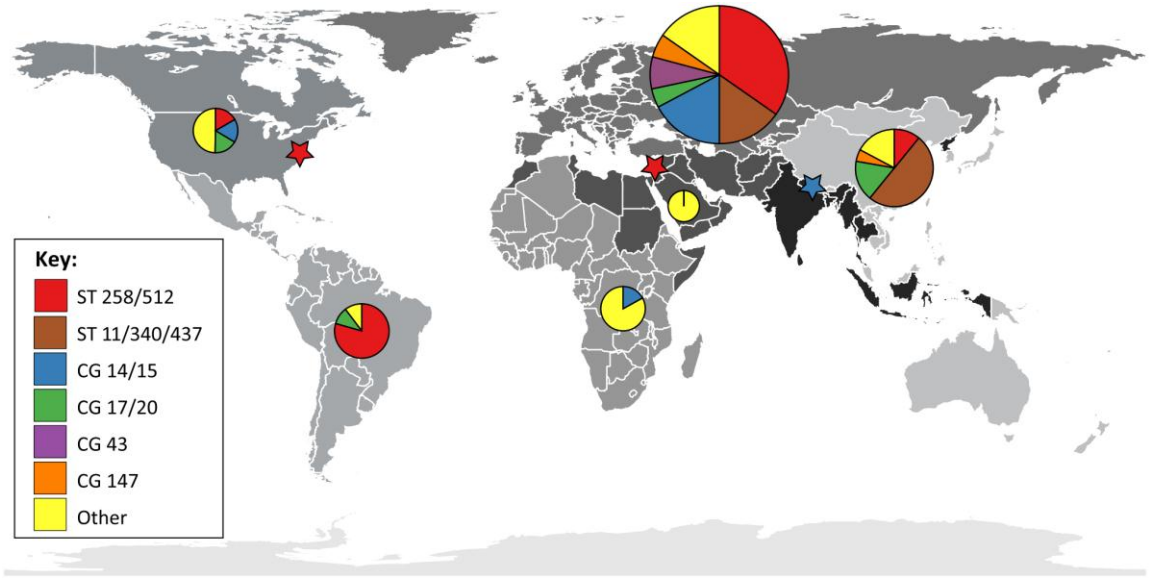


Figure 1.3 Global geographic distribution of *K. pneumoniae* outbreak by clonal group. Adapted from [12].

1.2.2. Hypervirulent *K. pneumoniae*

Initially reported in the mid-1980s from the Asian Pacific Rim, community-acquired (CA) infections caused by hvKp strains have become increasingly common [28]. International dissemination of hvKp strains are being reported recently, involving countries from North America, Europe, Australia and South Africa [1]. A recent study in China revealed a high prevalence of hvKp in the country, with a varied geographic distribution (Figure 1.4) [29]. Despite the lack of 100% specific and sensitive marker for hvKp strains, several phenotypic and clinical features have defined this *K. pneumoniae* variant [28, 30]. The first is their ability to cause severe infections in both immunocompromised and healthy hosts, typically presenting as CA-pyogenic liver abscesses (PLA) [19]. HvKp has also been observed to trigger diseases at unusual sites, including endophthalmitis, central nervous diseases (meningitis, bacteremia, *etc*.) [19]. A second trait is their propensity for metastatic spread to distinct sites, which is uncommon among enteric Gram-negative bacilli, including cKP [19, 28, 30]. Furthermore, hvKp colonies present a hypermucoviscous phenotype on agar plates which could be semi-quantitatively defined by a "string test" [28]. The "string test" is positive when a standard bacteriology loop could generate a viscous string of >5mm in length by stretching a single colony vertically on an agar plate [30]. HvKp strains which normally cause community-acquired infections

are mostly associated with serotype K1 or K2, with CC23 being the dominant K1 hvKp clone and several genetically unrelated groups (ST25, ST86, etc.) constituting the K2 clone [31]. Evidences from previous studies highlighted the importance of pLVPK-like virulence plasmids in aerobactin production and expression of the hypermucoid phenotype in hvKp isolates, since they harbor virulence-associated determinants encoding the regulators of mucoid phenotype (*rmpA* and *rmpA2*), siderophores (*iucABCDiutA* and *iroBCDN* clusters), ABC-type transporter (*fepBC*) and regulatory system for iron uptake (*fecIRA*) [31-34]. Additionally, a "high pathogenicity island" (KPHP1208) which is specially associated with CC23 harboring genes encoding yersiniabactin, colibactin, and microcin E492, is related to the hypervirulence phenotype [35, 36]. HvKp infections are frequently associated with remarkable mortality rates. Moreover, carbapenemase or ESBL carrying hvKp strains have been described in some infection cases, suggesting that hvKp strains may be the next "superbugs" in waiting [1, 28, 37-40].

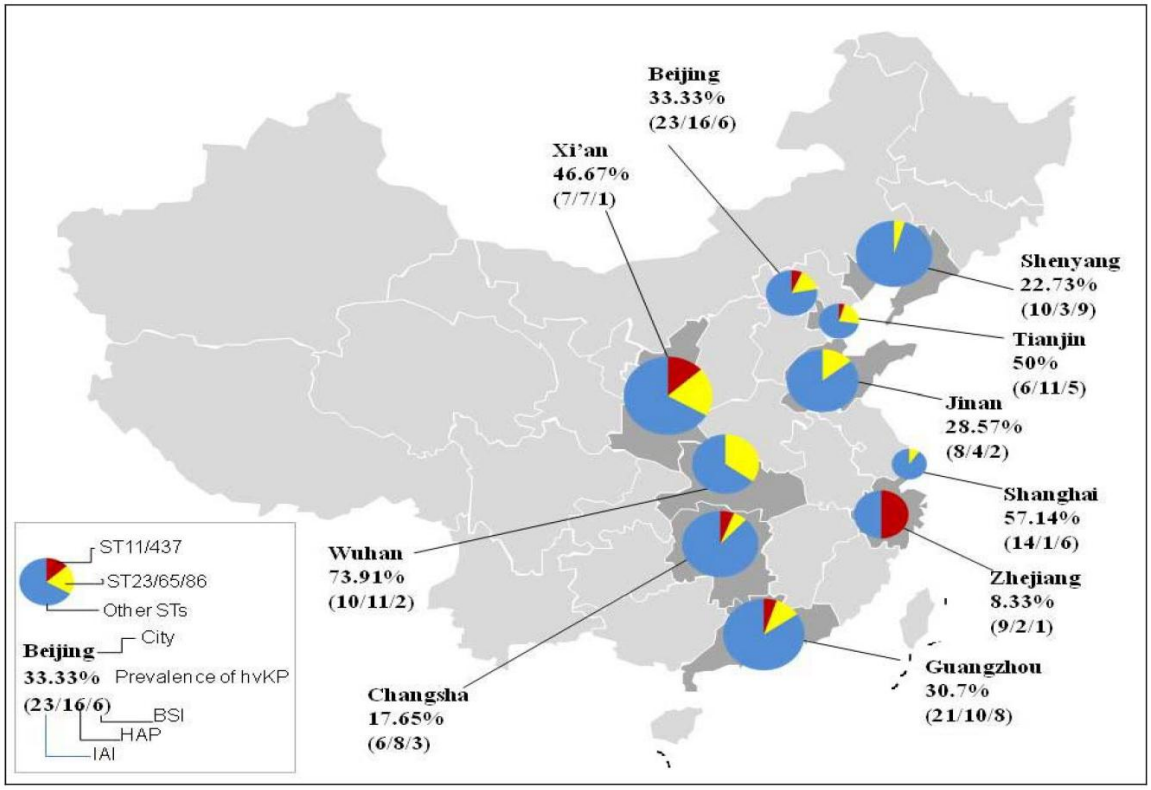


Figure 1.4. Geographic distribution of hvKP infection cases in mainland China. Adapted from [29].

1.2. Virulence factors in *K. pneumoniae*

K. pneumoniae relies on a battery of gene products to escape the innate immune mechanisms of the host [1]. The pathogenicity of *K. pneumoniae* has been attributed to several virulence factors including four categories of well characterized factors (capsule, siderophores, lipopolysaccharide (LPS) and fimbriae; Figure 1.5) and some which have not yet thoroughly characterized, including outer membrane protein (OMPs), porins, efflux pump and transporters [18, 41, 42].

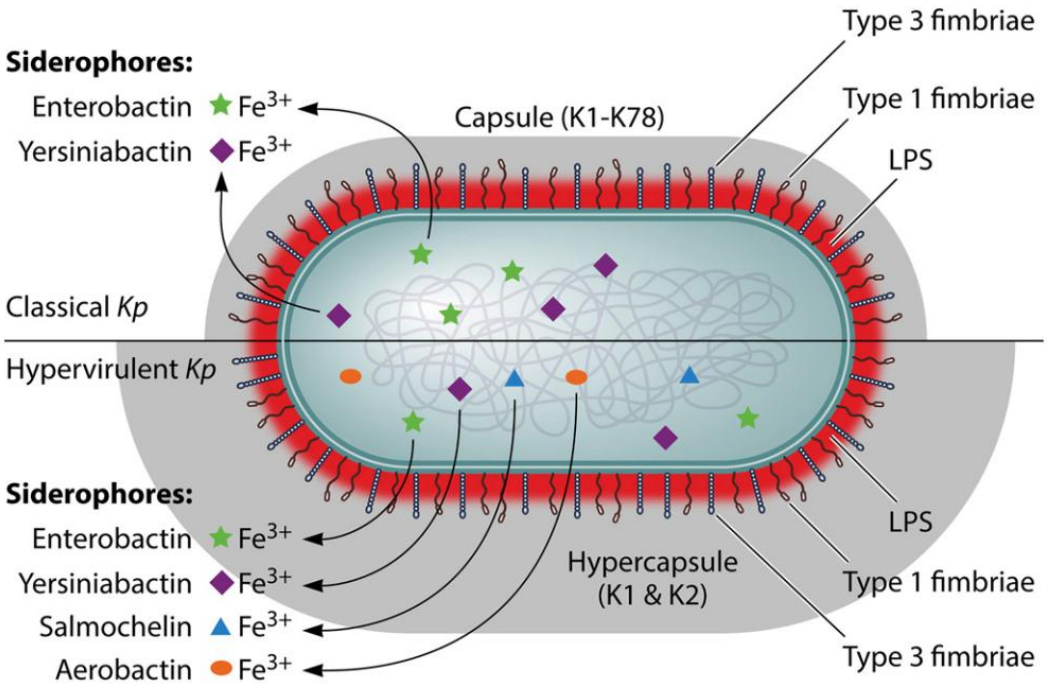


Figure 1.5 Schematic diagram of well-characterized virulence factors in *K. pneumoniae*. (Adapted from [1]).

1.2.1. Capsule

Capsule is a viscous polysaccharide matrix which constitutes the outermost layer of *K. pneumoniae* [2, 43]. It has been found to play role in evading the host immune response, including providing protection from phagocytosis, blocking complement-mediated killing, and suppressing expression of human beta-defensins [1, 2]. Capsules are termed strain-specific antigens (K antigens) based on their biochemical complexity [1, 2]. To date, at least 78 capsular serotypes (K1, K2, up to K78) have been identified for this pathogen with immunoelectrophoresis techniques [44]. The operon responsible for capsule

production is the *cps* gene cluster, which commonly harbors genes including *wzi*, *wza*, *wzb*, *wzc*, *gnd*, *galF* and *orf2* and strain-specific genes encoding different glycosyltransferases [1]. The *cps* operon displays significant sequence diversity even within one single strain type due to the frequent recombination events among *K. pneumoniae* strains [26, 27, 45]. *wzi* sequencing is widely used to determine the serotypes of most *K. pneumoniae* isolates [46]. However, in a recent study, analysis of 2503 *K. pneumoniae* genomes identified 134 distinct K loci, among which 31 were novel [45], suggesting a need for characterization of novel serotypes and establishing novel K typing methods.

The mucoviscous phenotype of hvKp has been largely linked to the enhanced production of capsule, which is under regulation by several genes, including regulator of mucoid phenotype A (*rmpA*, could be either chromosome- or plasmid-borne), *rmpA2* (plasmid-borne) and regulator of capsule synthesis genes (*rcsA* and *rcsB*) [1, 2, 19, 28]. The distribution of these regulatory alleles varies between different geographic locations and serotypes of the isolates [2]. Most hvKp strains (55% to 100%) encode at least one copy of *rmpA* or *rmpA2* gene [1]. The mucoviscosity-associated gene (*magA*), which is a wzy-like polymerase-encoding gene for K1 strains, was also reported to trigger the production of hypercapsules without the presence of *rmpA* and *rmpA2* genes [47]. Serotypes K1 and K2 were originally proposed to be a defining character for hvKp strains, yet later studies indicated the capsular types of some hvKp isolates were not K1 or K2 [28]. A study on *K. pneumoniae* liver abscess from Singapore and Taiwan using both phagocytosis assay and mouse model demonstrated that *rmpA* and *magA* were not as important as capsular serotype K1 or K2 in virulence determination [48].

1.2.2. Fimbriae

Fimbriae, also known as pili, are filamentous surface appendages which carry adhesins [49]. In Gram-negative enterobacteria, fimbrial adhesions mediate biofilm formation and attachment of the bacteria to host cell surfaces and abiotic surfaces [50]. The fimbrial adhesions documented in *K. pneumoniae* include type 1 and 3 fimbriae and KPF-28, among which the structure and *in vivo* function of KPF-28 have not been thoroughly characterized [1]. Fimbriae in *K. pneumoniae* strains are assembled by the

chaperone/usher pathway, generating structures that consist of a major pilus subunit, a specific tip adhesion and a set of adaptor molecules [2]. Both type 1 and type 3 fimbriae are not required for gastrointestinal (GI) tract or lung infections, but the former contributes to urinary tract infections (UTIs) and the latter facilitates biofilm formation [1, 51]. In UTI and probably upper respiratory tract infections (RTIs), fimbriae assist *K. pneumoniae* strains to attach to surfaces of host cells or medical devices and prevent clearance [51].

Type 1 fimbriae, frequently termed mannose-sensitive fimbriae due to the ability to bind D-mannosylated glycoproteins, are thread-like filaments expressed in 90% of *K. pneumoniae* strains [1]. They are encoded by the *fim* gene cluster (Figure 1.6.) which is homologous to that of *Escherichia coli* [52]. In this cluster, the genes *fimA* and *fimH* encode the major subunit FimA and the adhesive tip FimH, respectively; *fimK*, which is not carried by *E. coli* strains, has not been thoroughly characterized, but was hypothesized to be associated with type 1 fimbrial regulation [2]; other genes in the cluster are involved in expression of adaptor molecules like chaperone (*fimC*), minor structural subunit (*fimF* and *fimG*) and the usher protein (*fimD*) [1].

The mannose-insensitive type 3 fimbriae are helix-like protrusions encoded by the *mrkABCD* gene cluster and expressed in the large majority of *K. pneumoniae* isolates [1]. They have been demonstrated to bind extracellular matrix proteins like type V and IV collagens [53]. The main component of type 3 fimbria includes a major subunit MrkA and a minor tip adhesin MrkD, minor subunit Mrkf, chaperone MrkB, usher MrkC, *etc*. (Figure 1.6.) [54]. MrkJ, MrkH and MrkI are associated with the expression of *mrk* genes.

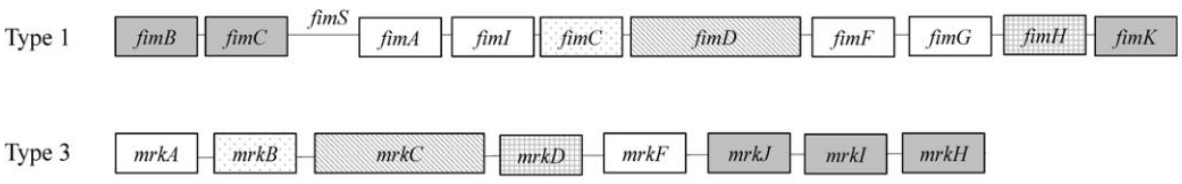


Figure 1.6 Genetic structure of the type 1 and type 3 fimbrial operons in *K. pneumoniae*. Adapted from [2].

1.2.3. Siderophores

The host plasma is normally an iron-poor environment, making it crucial for pathogens to adopt strategies to scavenge iron for survival [1]. Siderophores are high-affinity iron-chelating molecules secreted by bacteria which could steal iron released by the host [55]. Strains of *K. pneumoniae* secrete one or more types of siderophores including enterobactin, yersiniabactin, salmochelin and aerobactin, with the affinity for iron ranging from the highest to lowest, respectively [1, 8].

Enterobactin is expressed in almost all *K. pneumoniae* strains. Genes required for enterobactin biosynthesis and uptake are *entABCDEF* and *fepABCDEFG* gene clusters, respectively [56]. Lipocalin-2, a host-secreted, multifunctional protein, is capable of neutralizing enterobactin and clearance of *K. pneumoniae* strains [1].

Yersiniabactin was first characterized and described for *Yersinia* as a portion of its high-pathogenic island, and was later identified in other species [57]. The rate of carriage of yersiniabactin-encoding gene in hvKp and cKp differs significantly, being 90% and 18%, respectively [1]. Yersiniabactin is synthesised under expression of the *irp* genes, and the genes *ybt* and *fyu* are predicted to encode transporters for yersiniabactin secretion [8]. The activity of yersiniabactin is not inhibited by lipocalin-2 but by the blood concentrated protein transferrin [58].

Salmochelin, a C-glucosylated form of enterobactin, is not inhibited by lipocalin-2 and thus, not surprisingly, enhances nasal colonization [59]. The *iroBCDEN* gene cluster, located either in the plasmid or the chromosome, encodes proteins required for the synthesis, excretion, and uptake of salmochelin [60]. Like the situation of yersiniabactin, salmochelin is more prevalent in hvKp and observed in only 2%~4% of cKp [1].

Aerobactin is rarely detected in cKp (around 6%) and but present in the vast majority of hvKp (93% to 100%) [1]. The aerobactin gene cluster *iucABCD* and the transporter gene *iutA* is located in a virulence plasmid which also carries the *rmpA* or *rmpA2* gene [34]. One study reported that the siderophore required for *in vivo* and *ex vivo* hvKp growth and survival is aerobactin, but not other siderophores [61].

1.2.4. Lipopolysaccharide

Lipopolysaccharides (LPSs), also called endotoxins or lipoglycans, are essential elements of the outer membrane in Gram-negative bacteria [62]. They are large molecules typically composed of a lipid A, a core oligosaccharide and an O antigen [63]. Lipid A inserts into the membrane and the other components stick out from the cell surface [64]. The three components are encoded by the *wb*, *waa*, and *lpx* gene clusters, respectively [1]. Lipid A acts potentially as a potent inflammation activator and protects the bacteria against the bactericidal action of cationic antimicrobial peptides [1]. Nine O-antigen types have been identified in *K. pneumoniae*, with O1 being the most common serotype among clinical isolates [1, 2, 65]. O-antigen confers resistance to the complement system of the host through preventing binding of C1q and C3b from the outer membrane [1, 66].

1.2.5. Other Virulence Factors

Apart from the aforementioned virulence factors which have been well-characterized, several other factors which have also been identified in *K. pneumoniae* remained to be further characterized. These include: (1) OMPs: Murein lipoprotein (Lpp) and peptidoglycan-associated lipoprotein (Pal) which are highly conserved structural OMPs among enteric Gram-negative bacteria and released during sepsis; they both are important for the maintenance of cell integrity in *K. pneumoniae* and play a role in conferring protection against phagocytosis and serum killing [67]. (2) porins: Outer membrane porins OmpK35 and OmpK36 not only serve as channels which allow entry of antibiotics into *K. pneumoniae*, but also play a role in virulence determination, as downregulation of expression level of such porins may affect metabolic fitness [68]. (3) The efflux pump, AcrAB, is also a factor implicated in both antibiotic resistance and virulence. It plays a role in resisting innate immune defense mechanisms of the lung [69]. (4) Kfu, an ABC iron transport system strongly associated with hvKp and clinical infection, is involved in iron acquisition and is related to the virulence of *K. pneumoniae* [1]. (5) The operon involved in allantoin metabolism was reported to be related to the virulence of hvKp [70].

Despite the clinical relevance of *K. pneumoniae*, understanding of the role of bacterial determinants in promoting colonization, dissemination, or the ability to cause disease at specific sites such as lung, sepsis, and urinary tract is still far from being complete [51].

1.3. Antimicrobial resistance in *K. pneumoniae*

Extensive use of antibiotics in clinical settings resulted in a worldwide crisis of antimicrobial resistance in modern medicine [71]. *K. pneumoniae* was classified as one of the ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), which represent the major nosocomial pathogens that exhibited a high resistance rate [72, 73]. Antibiotic resistance in bacteria arises via several broad categories of mechanisms including removal of drugs from the cell by alteration of cell permeability, mechanical protection via biofilm formation, degradation or alteration of the antimicrobial agent, and target modification to prevent the drug from binding to a cellular target [4, 72].

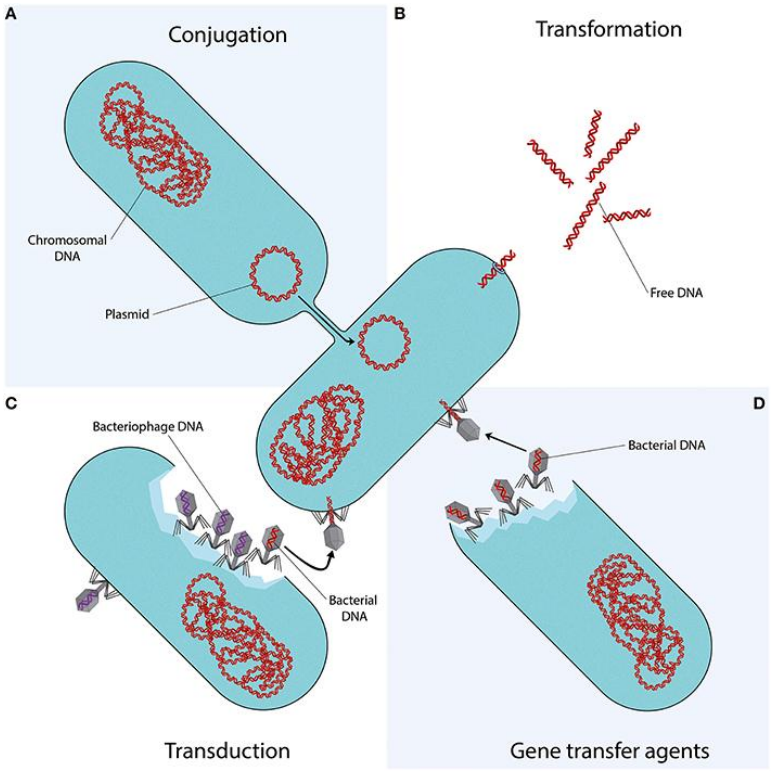


Figure 1.7 Mechanisms of horizontal gene transfer [74].

Broadly, antimicrobial resistance in bacteria could be classified into two types, namely intrinsic and acquired resistance [75, 76]. Intrinsic resistance refers to the inherent functional or structural characteristics of a bacterial species to resist the action of a particular antibiotic [76]. This definition encompasses all chromosomally-encoded elements which are not acquired as a result of antibiotic exposure or horizontal gene transfer [77]. The reduced membrane permeability due to porin loss or increased efflux of antibiotics, absence of bacterial target for a specific antibiotic and action of antibiotic-inactivating enzymes by chromosomally-encoded elements are the major causes of intrinsic resistance [75]. The advance of high-throughput screening technologies has led to the discovery of different genes conferring intrinsic resistance to various classes of antibiotics, including aminoglycosides, fluoroquinolones and β-lactams [76]. *Klebsiella* spp. are intrinsically resistant to penicillin by encoding different types of β-lactamases, such as SHV in *K. pneumoniae* (KpI), OKP in *K. quasipneumoniae* (KpII) and LEN in *K. variicola* (KpIII) [78, 79]. KpI, KpII and KpIII are commonly resistant to quinolone and fosfomycin at low-level by expressing the efflux pump genes *oqxAB* and the *fosA* gene, respectively [12].

K. pneumoniae strains also have the capacity to acquire drug resistance phenotype through accumulation of mutations and/or horizontal gene transfer (HGT) [80]. HGT was mainly mediated by the well-recognized mechanisms including transduction, transformation and conjugation, among which conjugation was considered the most significant mechanism (Figure 1.7) [81, 82]. Acquired AMR genes transferred among bacteria via mobile genetic elements include those located in conjugative and mobilizable plasmids, integrative conjugative elements (ICE), integrons, insertion sequences and transposons [75]. More than 100 diverse acquired AMR genes have been identified in *K. pneumoniae*, which encodes different products that confer resistance towards distinct classes of antibiotics including β-lactams, aminoglycosides, quinolones, tigecycline and polymyxins (Figure 1.9) [12, 73]. The terms multidrug resistance (MDR), extensive drug resistance (XDR) and pandrug resistance (PDR) were used worldwide to define the non-susceptibility level of a specific isolate, which are resistant to at least one agent in three or more antimicrobial categories, to at least one agent in all but two or fewer categories

and to all agents in all categories, respectively [83]. Production and dissemination of carbapenemase, including Ambler class A (KPCs), B (NDMs, IMPs, VIMs), and D (OXA-48) β-lactamases, is of clinical concern [12, 84]. All these carbapenemase genes have been reported in *K. pneumoniae* and were known to be associated with a broad range of plasmids [12]. The *K. pneumoniae* carbapenemase, KPC, the most prevalent carbapenemase in *K. pneumoniae*, was first identified in the mid-1990s in North Carolina, USA [73, 85]. KPC is frequently mobilized by Tn*4401*, a ~10 kb long, Tn*3*-based transposon bracketed by two 39-bp imperfect inverted repeats (IR) [25, 86]. Five isoforms of Tn*4401* have been reported with the typical structure harboring the bla_{KPC} gene, a transposase gene (*tnpA*), a resolvase gene (*tnpR*), and two insertion sequences (IS*Kpn6* and IS*Kpn7*) (Figure 1.8) [86, 87]. A novel genetic environment which is a chimera form of several transposon-associated elements with the structure of *tnpA-tnpR-ISKpn8-*bla_{KPC-2}*-*IS*Kpn6* was reported in China and many other countries [25, 87-89]. According to the nationwide surveillance data in China, the most dominant type of carbapenemase-producing *K. pneumoniae* was ST11 [89].

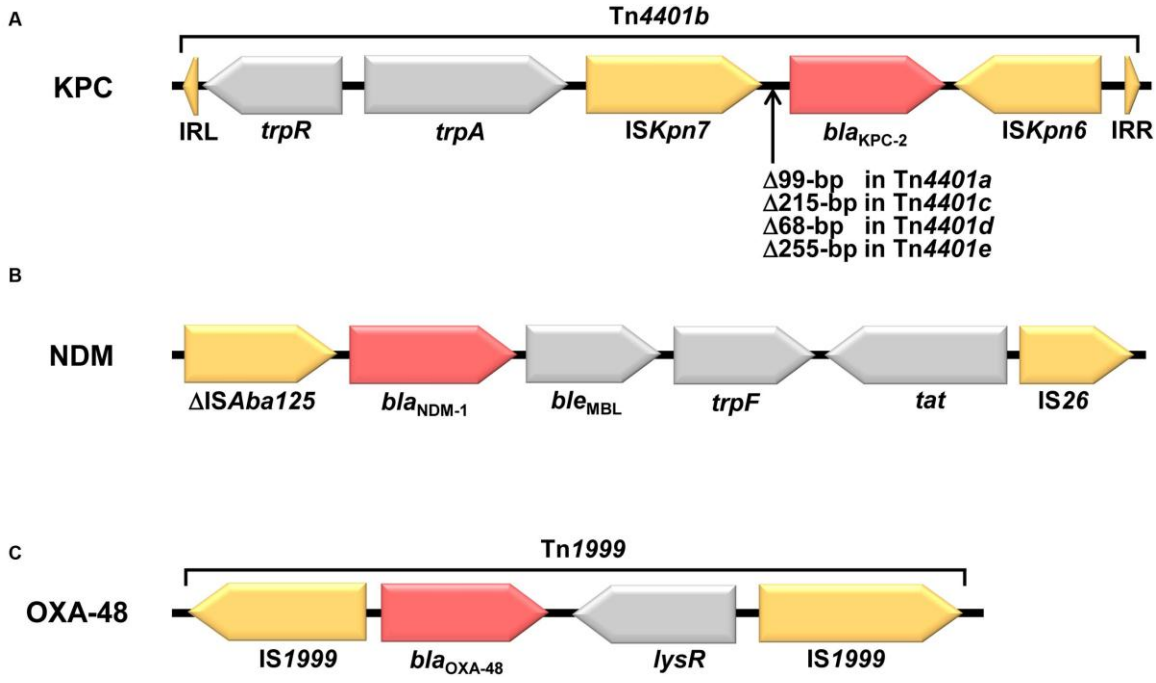


Figure 1.8 Structure of representative genetic environments of major carbapenem-resistance genes [87].

Beta-lactamases		*bla* Genes conferring resistance (*intrinsic)
Class A		CARB-3, PSE-1, SCO-1, SHV-1*, TEM-1
- ESBL		CTX-M, SHV-5, TEM-10, VEB
- Carbapenemase		KPC, GES-5
Class B (Metallo-beta-lactamase)		CphA, IMP, NDM, SIM, VIM
Class C (Cephalosporinase)		AmpC, CMY, DHA, FOX, MIR
Class D		OXA-1, OXA-2, OXA-7, OXA-9, OXA-10, OXA-12
- ESBL		OXA-11, OXA-15
- Carbapenemase		OXA-48, OXA-51, OXA-181, OXA-237
Other AMR	Genes conferring resistance (*intrinsic)	Mutations
Aminoglycosides	*aac, aadA, aadB, aph, armA, rmt, strAB*	–
Carbapenems	(see carbapenemase *bla* genes, class A & D above)	Mutations in *ompK35*, *ompK36*
Colistin	*mcr-1, mcr1.2*	Inactivation of *pmrB, mgrB*; mutations in *crrB*
Fluoroquinolones	*qepA, qnrA, qnrB, qnrD, qnrS*	SNPs in *gyrA, parC;* Upregulation of *acrAB* or *oqxAB* efflux
Macrolides	*ereA, ereB, ermB, mef, mph, msrE*	–
Phenicols	*catA, catB, cml, floR*	–
Rifampin	*arr*	
Sulfonamides	*folP, sul1, sul2, sul3*	–
Tetracycline	*tet* genes	–
Tigecycline	-	Upregulation of *acrAB* or *oqxAB* efflux; mutation in *rpsJ*
Trimethoprim	*dfr* genes	–

Figure 1.9 Major genes conferring antimicrobial resistance in *K. pneumoniae* [12].

Tigecycline and polymyxins are the last resort antibiotics used to treat extensively drug-resistant, carbapenemase-producing *K. pneumoniae* [90]. However, resistance to these drugs has also been reported in *K. pneumoniae*. Tigecycline resistance in *K. pneumoniae* is commonly mediated by mutations in chromosomal genes including *ramA, ramR, acrR* and *rpsJ*, which lead to the increased expression of the AcrAB efflux pump [91-94]. Resistance to polymyxin B and colistin arises through mutations that trigger the action of two-component regulatory systems (PmrAB, PhoPQ and the recently reported CrrAB) which further upregulate the *pmrHFIJKLM* operon [95-97]. Inactivation or down-regulation of the *mgrB* gene via IS insertion or nonsense mutations is a common

mechanism conferring polymyxin resistance [12, 98, 99]. Moreover, the acquisition of plasmid-borne *mcr-1* gene and its variants that mediate colistin resistance was reported recently [13, 100].

1.4. Whole genome sequencing and its application in *K. pneumoniae* research

Whole genome sequencing (WGS) is the process of determining the entire DNA sequence of an organism through using next generation sequencing (NGS) methods [101]. It is a revolutionized technology which has been widely used in both academia and clinical settings. NGS was named after first generation sequencing which is represented by Sanger and Maxam-Gilbert sequencing methods [102]. Short-read sequencing and long-read sequencing involving different methods including sequencing by ligation, sequencing by synthesis, and single-molecule real-time long reads sequencing are the major paradigms in NGS technology [103]. NGS could be divided into two generations based on the occurrence time, performances and their applications, namely the second- and third-generation sequencing technologies [75]. The major platforms of second-generation high-throughput sequencing include 454 (Roche), SOLiD (Thermo Fisher), Ion Torrent (Thermo Fisher) and Illumina, and that for the third-generation sequencing are represented by the real-time sequencing platforms Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) [75, 103, 104]. Detailed information of NGS methods can be found in previous reviews [103-106]. With the advent of WGS technologies, different databases and bioinformatics tools were developed to process the high-throughput data produced. BIGSdb-Kp is an online database and an integration of tools for the rapid identification of the *K. pneumoniae* genomes [107]. Kleborate is a tool recently developed for molecular typing with the *K. pneumoniae* genome assemblies, including the screening of species, MLST, typing of ICE*Kp*, virulence and resistance related loci, K (capsule) and O antigen (LPS) serotype predictions [45, 108-111]. Kaptive is used for tracking of capsule loci from the assembled *K. pneumoniae* genomes [45, 110]. Besides, Center for Genomic Epidemiology (http://www.genomicepidemiology.org/), SRST2, ISfinder, ISmapper, and other tools for typing of general bacteria genome, could be applied for study of *K. pneumoniae* genomes [101, 112-117].

Since the genome sequencing and assembly of the first organism, *Haemophilus influenzae*, in 1995, WGS has been used by microbiologists to investigate the bacterial genome architectures, population structures and transmission routes [4, 118]. Compared with traditional molecular typing methods including Pulse Field Gel Electrophoresis (PFGE), MLST and repetitive element palindromic PCR (rep-PCR), WGS provides much superior resolution into single nucleotide polymorphism (SNP) level in pathogen surveillance [119]. The complete genome sequence of *K. pneumoniae* was first published in the early 21st century, revealing a single circular chromosome of around 5.2 Mb to 5.6 Mb which bears approximately 4800 to 6500 genes [120-122]. To date (24 September 2018), 268 *K. pneumoniae* genomes have been completely sequenced and around 6,000 *K. pneumoniae* draft genomes have been sequenced by next-generation sequencing (http://www.ncbi.nlm.nih.gov/genome/genomes/815). A study based on the genome analysis of 328 distinct isolates demonstrated that *K. pneumoniae* has a small core genome with less than 2000 genes encoding essential cell functions, and a large accessory genome of approximately 30,000 protein-coding genes [8]. Functional genomics analysis assigned the accessory genes into several functional groups, including those responsible for carbohydrate metabolism (19%), other metabolic pathways (18%), membrane transport (13%), exopolysaccharide capsule (11%), iron resistance and metabolism (2%), and resistance to antibiotics, heavy metals, and stress (1%), while the function of one-third of the protein-coding genes remains unknown [8, 12]. This indicates the potential of *K. pneumoniae* isolates to sample genes from a large reservoir of accessory genome, which further provides solutions for its adaptive survival in diverse ecological niches.

The application of WGS in *K. pneumoniae* mainly covers the study of evolution and transmission of lineages of interest and the identification of genetic features of epidemiological or clinical importance [4]. Most studies focused on the hypervirulent or multidrug resistant clones which caused severe or untreatable infections [123-126]. For example, Zhou et al. investigated the transmission of a high-risk CTX-M-15-producing *K. pneumoniae* clone (ST15) between patients with no obvious epidemiological association. Through genomic phylogenetic analysis including published ST15 genomes, the outbreak isolate was found to exhibit close homology with isolates previously found in the USA. Lack of consistent patient screening and environmental contamination were identified as

being responsible for clonal dissemination. The investigation addresses the advantages of WGS in the early detection of high risk clones with a high propensity of nosocomial transmission and prolonged circulation in the regional patient population [127]. Struve et al. investigated the phylogenetic background of hvKP isolates using WGS, with an emphasis on CC23, and demonstrated that this clonal lineage was distinct from non-hypervirulent strains [31]. Lam et al further analyzed the genome sequences of 97 hvKp strains belonging to CG23, and detected several deep-branching sublineages of the clonal group related to ICE*Kp* acquisition [128]. Most strains analyzed belonged to sublineage CG23-I carrying the element ICE*Kp*10, which encoded both yersiniabactin and colibactin [128]. Lery et al. determined the complete genome of the *K. pneumoniae* K2 strain and identified several genomic islands comprising putative elements of pathogenicity. The role of gene pld1 (encoding PLD1) in pathogenesis was demonstrated for the first time and suggests that lipid metabolism is a novel virulence mechanism of *K. pneumoniae* [21]. Studies to date reveal that the *K. pneumoniae* species is diverse and intriguing, and warrants further study.

1.5. Summary and objectives of this study

K. pneumoniae is rapidly becoming untreatable by last-line antibiotics [8]. Through combining the epidemiological data and the genome sequences of clinical *K. pneumonia* strains from China, we aimed at identifying the genetic elements associated with virulence traits and antimicrobial resistance phenotypes. Our genomic study indicated the potential emergence of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in China. We then tracked a fatal outbreak of ST11 CR-hvKP isolates in a Chinese hospital and further identified and characterized CR-hvKp isolates that belonged to different sequence types (ST23 and ST11). Work in this study covers three major parts:

Part 1 (Chapter 2) A comprehensive genomic analysis of 58 clinical strains of ST11 *K. pneumoniae,* the dominant KPC-producing clinical clone in China, was performed in this study. We found that that these strains, collected from geographically diverse locations in the country, were genetically diverse and could be segregated into three clades, each of which exhibited distinct capsule polysaccharide loci. Various antimicrobial resistance genes and virulence genes, such as those encoding for salmochelin, aerobactin and RmpA,

the hallmarks of hypervirulent *K. pneumoniae,* were detected in the genome of these strains, indicating the potential emergence of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae*. This study provides insights into the development of strategies for prevention, diagnosis and treatment of ST11 clinical infections.

Part 2 (Chapter 3) An outbreak of ST11 carbapenemase-resistant, hypervirulent *K. pneumoniae* causing fatal ventilator-associated pneumonia in patients a Chinese hospital was invigilated. Such strains have disseminated across various regions of China, accounting for as much as 3% of clinical ST11 carbapenem-resistant *K. pneumoniae* infections in the country. ST11 carbapenem-resistant hypervirulent *K. pneumoniae* has a hypervirulence phenotype characterized by a positive string test result, extremely high survival on exposure to human neutrophils, and high virulence in a wax moth (*Galleria mellonella*) larva infection model. The emergence of ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains was due to acquisition of a roughly 170 kbp virulence plasmid carrying the *rmpA2* and aerobactin biosynthesis genes by classic ST11 carbapenem-resistant *K. pneumoniae* strains. Future research should focus on the development of intervention measures to prevent further dissemination of such organisms in hospital settings.

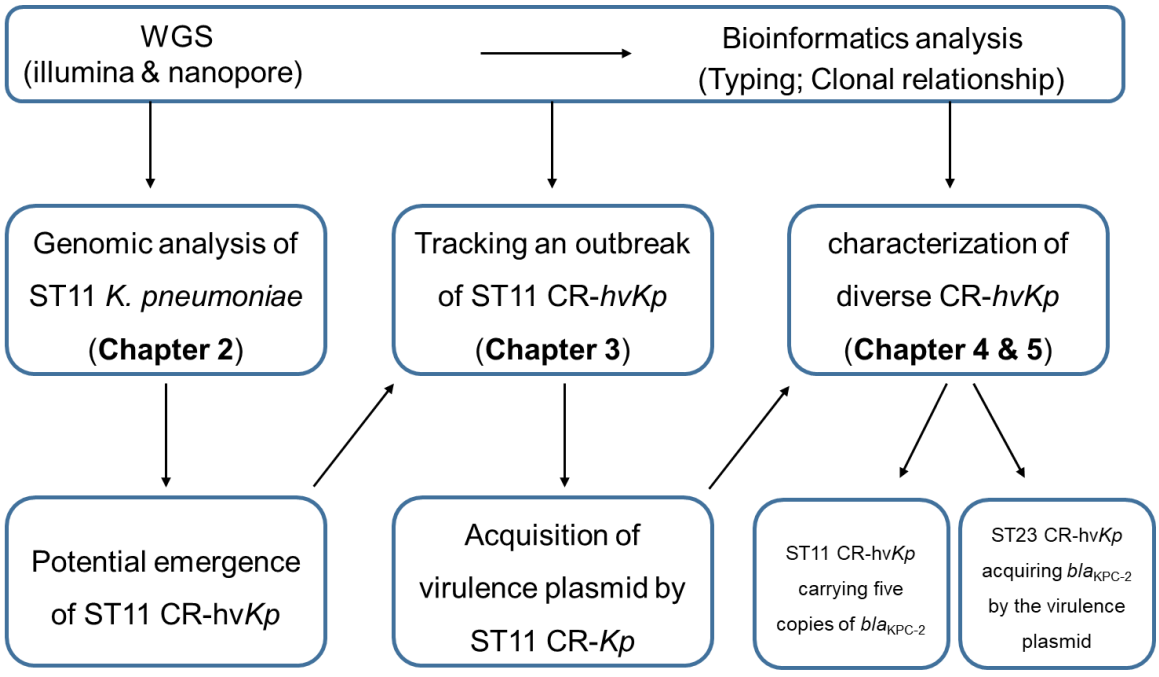


Figure 1.10 Flowchart of work conducted in this study.

Part 3 (Chapter 4 and Chapter 5) The third part of this study focused on the genomic study of two different types of carbapenem-resistant, hypervirulent *K. pneumoniae*. The first belonged to ST23 CR-hvKP whose emergence was due to IS*26*-mediated insertion of bla_{KPC-2} into a pLVPK-like virulence plasmid in ST23 hypervirulent *K. pneumoniae*. The second case reported the carriage of 5 copies of bla_{KPC-2} by an ST11 CR-hvKP mediated by Tnp*26*-catalyzed conservative reaction through activity of translocatable units. In the two cases, the carbapenem-resistance and hypervirulence phenotypes were both mediated by the plasmid(s) each strain harbored, indicating an alarming evolutionary event of *K. pneumoniae* which should be carefully monitored. The emergence of diverse CR-hvKp isolates suggests that such organisms could cause severe infections in both hospital settings and the community.

Findings in this study provide valuable information that facilitate future development of strategies to control of *K. pneumoniae* infections in China and neighboring countries.

CHAPTER 2. Genome analysis of clinical multilocus sequence Type 11 *Klebsiella pneumoniae* from China

The content in this chapter has been published in following paper:

Dong, N., Zhang, R., Liu, L., Li, R., Lin, D., Chan, E. W. C., & Chen, S. (2018). Genome analysis of clinical multilocus sequence type 11 *Klebsiella pneumoniae* from China. Microbial genomics, 4(2).

2.1 Abstract

The increasing prevalence of KPC-producing *K. pneumoniae* strains in clinical settings has been largely attributed to dissemination of organisms of specific multilocus sequence types, such as ST258 and ST11. Compared with the ST258 clone, which is prevalent in North America and Europe, ST11 is common in China but information regarding its genetic features remains scarce. In this study, we performed detailed genetic characterization of ST11 *K. pneumoniae* strains by analyzing whole genome sequences of 58 clinical strains collected from diverse geographic locations in China. The ST11 genomes were found to be highly heterogeneous and clustered into at least three major lineages based on the patterns of single-nucleotide polymorphisms. Exhibiting five different capsular types, these ST11 strains were found to harbor multiple resistance and virulence determinants such as the notorious $bla_{\text{KPC-2}}$ gene that encodes carbapenemase, and the yersiniabactin-associated virulence genes *irp*, *ybt* and *fyu*. Moreover, genes encoding the virulence factor aerobactin and the regulator of the mucoid phenotype (*rmpA*) were detectable in six genomes, whereas genes encoding salmochelin were found in three genomes, indicating the potential emergence of ST11 carbapenem resistant, hypervirulent *K. pneumoniae*. In conclusion, our data showed that carriage of a wide range of resistance and virulence genes constitutes the underlying basis of high-level prevalence of ST11 in clinical settings. Such findings provide insight into development of novel strategies for prevention, diagnosis and treatment of *K. pneumoniae* infections.

2.2 Introduction

K. pneumoniae has gained notoriety as a major opportunistic pathogen which causes a range of hospital-acquired infections [2]. The emergence of multidrug resistant *K. pneumoniae* strains which cause untreatable infections has drawn extensive public concerns [1]. Notably, *K. pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae*, one of the most clinically significant carbapenem-resistant Enterobacteriaceae (CRE) strains, has not only disseminated globally, but are also associated with high morbidity and mortality rates [129]. To date, sixteen different KPC variants (KPC-2~KPC-17) classified on the basis of single amino-acid mutations have been identified, among which KPC-2 and KPC-3 are the best-studied enzymes [1, 130]. Transmission of the bla_{KPC} gene involves multiple mechanisms ranging from clonal spread to horizontal transfer mediated by plasmids and other transposable genetic elements, notably Tn*4401* [25].

The clinical prevalence of KPC-producing *K. pneumoniae* has been largely attributed to dissemination of strains of the clonal group (CG) 258, with ST258 and ST11 being the dominant multilocus sequence types (ST) [25, 26, 129, 131, 132]. ST258 has disseminated worldwide since its emergence during the early to mid-2000s, especially in North America, Latin America and several countries in Europe [25, 84, 132]. In Asia and South America, however, the dominant KPC-producing clone is ST11 [132, 133], which is a single locus (*tonB*) variant of ST258, with the *tonB4* gene in ST11 differing from *tonB79* in ST258 by four single-nucleotide polymorphisms (SNPs) [133]. According to Gaiarsa S. *et al*, a putative recombination event (happened sometime before 1985) during which a donor related to *K. pneumoniae* ST1628 contributed ~1.3 Mbp to an ancestor of ST11 (CG258) gave rise to CG258 [134]. A recent genome-based study indicated that ST258 *K. pneumoniae* differed significantly from ST11 in terms of genetic composition. ST258 was found to be a hybrid clone adopting 80% of its genome from ST11-like strains and 20% of the genome from ST442-like strains [1, 25, 135]. Strains of the ST258 type was found to comprise at least two distinct lineages, namely clade 1 and clade 2, which differ mainly in a genomic region, where the gene encoding capsule polysaccharide (CPS), *cps*, is located [26]. In contrast, the genetic composition of ST11, which is the dominant carbapenem-resistant *K. pneumoniae* clone in China, remains poorly understood [124, 133]. The complete genomic sequences of two clinical ST11

multidrug resistant strains, HS11286 and JM45, are publicly available and widely used as references for various genetic studies [25, 136-138]. A previous study demonstrated that the ST11 genomes exhibited a relatively high degree of diversity [27]. Jiang *et al.* tracked the outbreak of ST11 *K. pneumoniae* in a single Chinese hospital by performing whole genome sequencing of twelve strains; in this previous work, phylogenetic analysis resulted in partitioning of these strains into three separate clades [138]. However, a comprehensive clonal lineage map of ST11 strains prevailing in China is currently not available. Also, data regarding the pathogenicity and antimicrobial resistance profiles of the ST11 strains is scarce. To address these issues, we performed whole genome sequencing to obtain the genome complete of one KPC-producing ST11 clinical isolate, followed by phylogenetic analysis and genome mining of a total of 58 ST11 clinical isolates collected from diverse geographic locations of China. Findings in this work led to identification of unique genetic traits of the ST11 clone and helped promote better understanding of the genetic basis of virulence of this important clone.

2.3 Materials and Methods

2.3.1 *K. pneumoniae* isolates and sequencing

Thirty-one carbapenem-resistant *K. pneumoniae* isolates were collected in a retrospective study from blood, sputum and stool specimens of patients in eight hospitals located at different provinces in China. The isolates were non-outbreak-related and were selected based upon the presence of KPC. Antimicrobial susceptibilities were determined by the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guideline [139]. Genomic DNA was extracted from overnight cultures by using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Genomic libraries were prepared with a ~350bp insert size using the NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs) and sequenced by the Illumina NextSeq 500 sequencing platform. One of these strains, GD4, was simultaneously randomly selected for sequencing with the PacBio RSII single-molecule real-time (SMRT) sequencing platform (Wuhan Institute of Biotechnology, Wuhan, China). All ST-11 *K. pneumoniae* genomes that were publicly available in the NCBI Pathogen Detection database (https://www.ncbi.nlm.nih.gov/pathogens/)

as of January 2017 (a total of 27 genomes), were also included for analysis in this study. Genetic information of the test isolates is listed in Table 2.1 and Table 2.2.

2.3.2 Genome assembly and annotation

Raw reads generated in this study and the Illumina reads of 11 strains obtained from the NCBI database were trimmed or filtered to remove low-quality sequences and adaptors. Finally, at least 500 Mb raw sequencing reads were obtained for each isolate to ensure a coverage of at least 100 ×. Both Illumina and PacBio reads were *de novo* assembled with the SPAdes Genome Assembler v3.9.1 [140, 141]. Illumina reads of strain GD4 was aligned to the corresponding PacBio contigs to improve the accuracy of the genome sequence data and obtain the complete genome sequence of strain GD4. The 30 draft and 1 complete genome sequences generated in this study, and the 27 genome sequences retrieved from the NCBI database were all annotated with the RAST tool [142] and Prokka [143]. The genome sequences obtained in this study are deposited in GenBank under Bioproject numbers PRJNA422332 and PRJNA422171.

2.3.3 Genome profiling

Genome profiling was conducted using the assembled genome sequences. Integrative and conjugative elements (ICEs) were predicted as described previously [137]. Acquired antibiotic resistance genes were identified with ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/) [114]. Plasmid replicons were analyzed using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) [91]. Insertion sequences (ISs) and identified using Isfinder and ISsaga (https://www-is.biotoul.fr/index.php) [117]. Phage-associated regions were identified by PHAST (http://phast.wishartlab.com/index.html) [144]. Multilocus sequence types, virulence-associated genes encoding yersiniabactin, aerobactin, salmochelin and the regulators of mucoid phenotype were determined with Kleborate [111, 145]. Heatmap of the antimicrobial resistance genes and virulence determinants were generated using an in-house script.

Table 2.1 Genomic information and minimal inhibitory concentration (MIC) of strains from this study.

Strain ID	origin Province	Year of isolation	assembly level	assemly method	number of scaffold (>500bp)	sequence length	N50 (bp)	N90 (bp)	MIC (μg/mL)									
									Cefotaxime	Ceftazidime	Cefepime	Ertapenem	Imipenem	Meropenem	Amikacin	Ciprofloxacin	Amoxicillin	Polymyxin E
AH10	Anhui	2015	scaffold	SPAdes v.3.9.1	121	5693242	151791	34549	> 256	256	> 256	> 128	64	> 128	> 256	32	> 256	1
AH11	Anhui	2015	scaffold	SPAdes v.3.9.1	96	5547999	195948	61211	> 256	256	> 256	> 128	> 128	> 128	> 256	16	> 256	1
AH12	Anhui	2015	scaffold	SPAdes v.3.9.1	99	5549595	176066	55162	> 256	256	> 256	> 128	> 128	> 128	> 256	16	> 256	1
BJ-A1040	Beijing	2015	scaffold	SPAdes v.3.9.1	161	5475333	110021	26346	> 256	> 256	64	32	32	16	2	≤0.5	> 256	≤0.25
BJK6	Beijing	2015	scaffold	SPAdes v.3.9.1	139	5489720	151791	31828	> 256	> 256	64	32	4	4	1	≤0.5	> 256	≤0.25
BJK7	Beijing	2015	scaffold	SPAdes v.3.9.1	137	5488635	156763	26346	> 256	> 256	64	> 128	64	64	> 256	256	> 256	≤0.25
BJK8	Beijing	2015	scaffold	SPAdes v.3.9.1	131	5485139	157690	31828	> 256	> 256	64	32	16	8	8	16	256	≤0.25
FJ10	Fujian	2015	scaffold	SPAdes v.3.9.1	100	5620804	151791	46437	> 256	> 256	128	> 128	16	64	> 256	32	> 256	≤0.25
FJ11	Fujian	2015	scaffold	SPAdes v.3.9.1	109	5742636	156044	37781	> 256	> 256	> 256	128	32	64	1	16	> 256	≤0.25
GD4	Guangdong	2015	Complete genome	SPAdes v.3.9.1	2	5537631	5366808	170823	> 256	> 256	256	32	16	16	2	1	64	≤0.25
GD-KPC30	Guangdong	2015	scaffold	SPAdes v.3.9.1	132	5741272	169916	28544	> 256	> 256	64	> 128	64	64	> 256	> 256	> 256	≤0.25
HN-26	Henan	2015	scaffold	SPAdes v.3.9.1	140	5785890	151373	30927	> 256	256	128	> 128	32	64	≤0.5	32	> 256	≤0.25
HN34-1	Henan	2015	scaffold	SPAdes v.3.9.1	167	5519507	97294	19808	> 256	> 256	> 256	128	32	128	> 256	32	> 256	2
HN34-2	Henan	2015	scaffold	SPAdes v.3.9.1	113	5792460	151791	34549	> 256	> 256	256	> 128	16	128	1	32	> 256	≤0.25
JXR113	Zhejiang	2015	scaffold	SPAdes v.3.9.1	122	5712976	176066	31828	> 256	64	128	> 128	32	128	> 256	16	> 256	≤0.25
JXR114	Zhejiang	2015	scaffold	SPAdes v.3.9.1	111	5562831	141255	34549	> 256	64	256	> 128	64	128	> 256	16	> 256	≤0.25
FJ8	Fujian	2015	scaffold	SPAdes v.3.9.1	90	5619946	176066	52283	> 256	> 256	> 256	> 128	128	> 128	> 256	2	> 256	≤0.25
SH-1	Shanghai	2015	scaffold	SPAdes v.3.9.1	129	5719880	129798	34549	> 256	256	256	> 128	64	128	> 256	≤0.5	> 256	1

SH-16	Shanghai	2015	scaffold	SPAdes v.3.9.1	136	5739574	129690	31828	> 256	128	128	128	32	64	> 256	64	> 256	1
SH-2	Shanghai	2015	scaffold	SPAdes v.3.9.1	117	5826799	132102	37781	> 256	64	256	> 128	64	> 128	> 256	64	≤ 0.5	1
SH-9	Shanghai	2015	scaffold	SPAdes v.3.9.1	129	5739793	129690	32363	> 256	128	128	> 128	16	128	> 256	64	> 256	≤0.25
XJ126	Shanxi	2015	scaffold	SPAdes v.3.9.1	87	5603218	134034	52283	> 256	> 256	128	128	16	64	> 256	64	> 256	1
AH-29	Anhui	2015	scaffold	SPAdes v.3.9.1	91	5579775	178089	55162	> 256	128	256	128	> 128	> 128	> 256	≤0.5	> 256	≤0.25
AH-9	Anhui	2015	scaffold	SPAdes v.3.9.1	92	5582007	198350	51358	> 256	256	> 256	> 128	> 128	> 128	> 256	8	> 256	1
BJK20	Beijing	2015	scaffold	SPAdes v.3.9.1	130	5477670	156320	26346	> 256	> 256	128	8	8	8	1	4	> 256	≤0.25
BJK22	Beijing	2015	scaffold	SPAdes v.3.9.1	124	5481029	159606	31828	> 256	> 256	64	32	16	8	> 256	16	> 256	≤0.25
BJK23	Beijing	2015	scaffold	SPAdes v.3.9.1	127	5477543	151791	31703	> 256	> 256	256	32	16	32	2	≤0.5	> 256	≤0.25
GD108	Guangdong	2015	scaffold	SPAdes v.3.9.1	96	5415704	190461	55162	>256	>256	256	>128	16	128	>256	64	>256	≤0.25
HA2-17	Henan	2015	scaffold	SPAdes v.3.9.1	99	5557502	203714	45955	> 256	256	128	> 128	32	64	> 256	32	> 256	≤0.25
HA-74	Henan	2015	scaffold	SPAdes v.3.9.1	95	5567939	196385	48207	> 256	64	32	8	32	64	> 256	64	> 256	4
HA-99	Henan	2015	scaffold	SPAdes v.3.9.1	105	5593438	190942	45955	> 256	> 256	> 256	32	64	64	> 256	64	256	≤0.25

Table 2.2. Information of genomes downloaded from the NCBI database.

Strain ID	Accession	origin	isolation source	assembly_method	assembly level	length (bp)	number of contigs	reference	Note
KP41	GCA_001856605.1	Xinhua Hospital, Shanghai Jiao Tong University School of Medicine	central venous catheter	SPAdes v. 3.9.0; Mauve v. 2.4.0	Scaffold	5565024	110	[146]	*
KP6	GCA_001866785.1	Xinhua Hospital, Shanghai Jiao Tong University School of Medicine	urine	SPAdes v. 3.9.0	Scaffold	5640726	129	[146]	*
SWU01	GCA_001902475.1	Southwest Medical University	blood specimen	SOAPdenovo v. v2.04	Complete Genome	5699058	2	[147]	*
TR187	GCA_001613565.1	Chinese Center for Disease Control and Prevention	abdominal drainage	SOAPdenovo v. 2.04	Scaffold	5546703	87	-	*
TR191	GCA_001613715.1	Chinese Center for Disease Control and Prevention	sputum	SOAPdenovo v. 2.04	Scaffold	5534841	104	-	*
TR198	GCA_001613595.1	Chinese Center for Disease Control and Prevention	sputum	SOAPdenovo v. 2.04	Scaffold	5544407	74	-	*
TR200	GCA_001613655.1	Chinese Center for Disease Control and Prevention	urine	SOAPdenovo v. 2.04	Scaffold	5546912	102	-	*
TR262	GCA_001613665.1	Chinese Center for Disease Control and Prevention	sputum	SOAPdenovo v. 2.04	Scaffold	5574386	103	-	*
TRqt-37	GCA_001613585.1	Chinese Center for Disease Control and Prevention	nose	SOAPdenovo v. 2.04	Scaffold	5541511	85	-	*
TRqt-41	GCA_001613575.1	Chinese Center for Disease Control and Prevention	nose	SOAPdenovo v. 2.04	Scaffold	5550293	92	-	*
TRqt-49	GCA_001613645.1	Chinese Center for Disease Control and Prevention	nose	SOAPdenovo v. 2.04	Scaffold	5545016	76	-	*
WCHKP649	GCA_001887985.1	West China Hospital, Sichuan University	Secreta	SPAdes v. 3.9.0	Contig	5582397	150	[148]	*
XL-1	GCA_001939845.1	Fifth Affiliated Hospital of Wenzhou Medical University	blood	SPAdes v. 3.5	Scaffold	5520425	84	[149]	*
kp10	GCA_001856585.1	Xinhua Hospital, Shanghai Jiao Tong University School of Medicine	blood	SPAdes v. 3.9.0; plasmidspades v. 3.9.0	Scaffold	5727930	107	[146]	*
HS11286	GCA_000240185.2	Huanshan hospital, Shanghai,China	sputum	Newbler	Complete Genome	5682322	7	[136]	*
JM45	GCA_000445405.1	the Second Affiliated Hospital Zhejiang University School of Medicine, Hangzhou, China	blood	Newbler	Complete Genome	5603174	3	[150]	*
K6	SRR2064877	QuZhou People's Hospital, Zhejiang, China,	Blood	SPAdes v.3.9.1	Scaffold	5591567	79	[138]	**
K11	SRR2064844	QuZhou People's Hospital, Zhejiang, China,	Catheter	SPAdes v.3.9.1	Scaffold	5684140	94	[138]	**
K15	SRR2064879	QuZhou People's Hospital, Zhejiang, China,	Blood	SPAdes v.3.9.1	Scaffold	5537094	88	[138]	**

K22-1	SRR2064885	QuZhou People's Hospital, Zhejiang, China,	Blood	SPAdes v.3.9.1	Scaffold	5525229	119	[138]	**
K22-2	SRR2064887	QuZhou People's Hospital, Zhejiang, China,	Blood	SPAdes v.3.9.1	Scaffold	5524438	112	[138]	**
K28-1	SRR2064888	QuZhou People's Hospital, Zhejiang, China,	Blood	SPAdes v.3.9.1	Scaffold	5522729	111	[138]	**
K45	SRR2070616	QuZhou People's Hospital, Zhejiang, China,	Catheter	SPAdes v.3.9.1	Scaffold	5538061	87	[138]	**
K47	SRR2070618	QuZhou People's Hospital, Zhejiang, China,	Blood	SPAdes v.3.9.1	Scaffold	5539584	88	[138]	**
K48	SRR2070619	QuZhou People's Hospital, Zhejiang, China,	Catheter	SPAdes v.3.9.1	Scaffold	5540063	91	[138]	**
K49	SRR2070620	QuZhou People's Hospital, Zhejiang, China,	Pleural effusion	SPAdes v.3.9.1	Scaffold	5540513	89	[138]	**
K50	SRR2070621	QuZhou People's Hospital, Zhejiang, China,	cerebrospinal fluid	SPAdes v.3.9.1	Scaffold	5538471	87	[138]	**

*, assembled scaffolds were downloaded from the database; **, Only original reads available in the database, genome was assembled in this study

2.3.4 Phylogenetic analysis

The harvest suite, which was designated for analyzing intraspecific microbial genomes and encompasses three modules (Parsnp, Gingr and Harvest Tools) was applied to filter recombination (-x), run core genome alignment and variant calling, and generate the phylogenetic tree with the 58 assembled genomes using default settings [151]. Interactive tree of life (iTOL) v3 (http://itol.embl.de/) was applied to modify and visualize the generated phylogenetic tree [111]. Genome assemblies for two representative ST258 *K. pneumoniae* isolates, NJST258_1 (GenBank accession No. CP006923) and NJST258_2 (GenBank accession No. NZ_CP006918), and one CG258-unrelated isolate, *K. pneumoniae* MGH78578 (ST38, GenBank accession No. AB720665) were retrieved from GenBank and used to generate a phylogenetic tree with three ST11 isolates (each from a different evolutionary clade) using the harvest suite. This tree as well as the variants was visualized with gingr [151]. The chromosome sequence of *K. pneumoniae* strain GD4 was used as references for both phylogenetic analyses.

2.3.5 Core- and pan- genome analysis

Conserved core genes among the ST11 isolates were analyzed with Roary [152] with a blastp percentage identity of 95 %. The Prokka [143] annotated files were used as inputs for Roary. The percentage of isolates a gene must be in to be core was set to be 99%.

2.3.6 Comparative genomics analysis

Genome sequences of *K. pneumoniae* ST11 strains GD4, JM45 and HS11286, originated from China, were compared with seven other closed genomes, including ATCC BAA-2146, NJST258_1, NJST258_2, NTUH-K2044, MGH78578, CG43, KCTC2242, using the BLAST Ring Image Generator **(**BRIG) [153]. Capsular typing was performed using Kaptive and the assembled sequences [45]. Comparison of the *cps* locus was conducted by using EasyFig [154]. Plasmid homology search was performed for the plasmid pKPGD4 harbored by strain GD4 using BLASTN on NCBI's Nucleotide collection (nr/nt) database. Sequence comparison between plasmid pKPGD4 with related plasmids was also conducted with BRIG [153].

2.4 Results and Discussion

2.4.1 Genome sequence of carbapenem-resistant ST11 *K. pneumoniae* clinical isolates

ST11 was found to be the dominant type of carbapenem-resistant *K. pneumoniae* strains in China in our recent Chinese nationwide surveillance, accounting for 60% of such strains [89]. The species *K. pneumoniae* has been reported to be highly genetically diverse, with a large accessory genome that comprises 30,000 protein-coding genes [8]. In this study, the complete genome sequence of a multidrug-resistant *K. pneumoniae* strain GD4 isolated from a sputum specimen in 2015 at Huashan Hospital, Guangdong, China (MIC profile in Table 2.1) was obtained. The genome of this strain was found to comprise a chromosome of 5,366,808 bp in size, and a 170,823 bp plasmid designated pKPGD4. The overall G+C content of the chromosome, in which 5215 coding sequences (CDSs) and 80 tRNA molecules were detectable, is 57.5%. The genetic diversity of *K. pneumoniae* genomes was previously demonstrated to be primarily due to elements that migrate frequently by horizontal gene transfer, including plasmids, phages, integrated conjugative elements (ICEs) and insertion sequences (ISs) [25]. Resembling the ST258 strains [26], the chromosome of strain GD4 was found to harbor numerous mobile genetic elements including eight putative prophages (designated prophages 11.1—11.8), two ICEs (designated ICE*Kpn*HS11286-1 and ICE*Kpn*HS11286-2 based on the name of the parent strain HS11286) and 40 insertion sequences (ISs) (Figure 2.1). The chromosomal features of three completely sequenced ST11 *K. pneumoniae* strains, namely GD4, HS11286 and JM45, were compared and summarized in Table 2.3. Information of all 58 strains tested in this study was listed in Table 2.1 and Table 2.2. Apart from the completely assembled sequences, the genomes of other ST11 isolates in this study were found to comprise 74~161 scaffolds. Like other *K. pneumoniae* genomes [26], the genome sizes of the ST11 strains ranged from approximately 5.4 Mbp to around 5.7 Mbp. Pangenome analysis identified a total of 8285 genes in the genomes of the 58 ST11 *K. pneumoniae* isolates, among which 4297 (about 82.4% of the total genes in isolate GD4) were core to all isolates. The ST11 *K. pneumoniae* clone harbor large set of accessory genes to render the clone adapt to different environments.

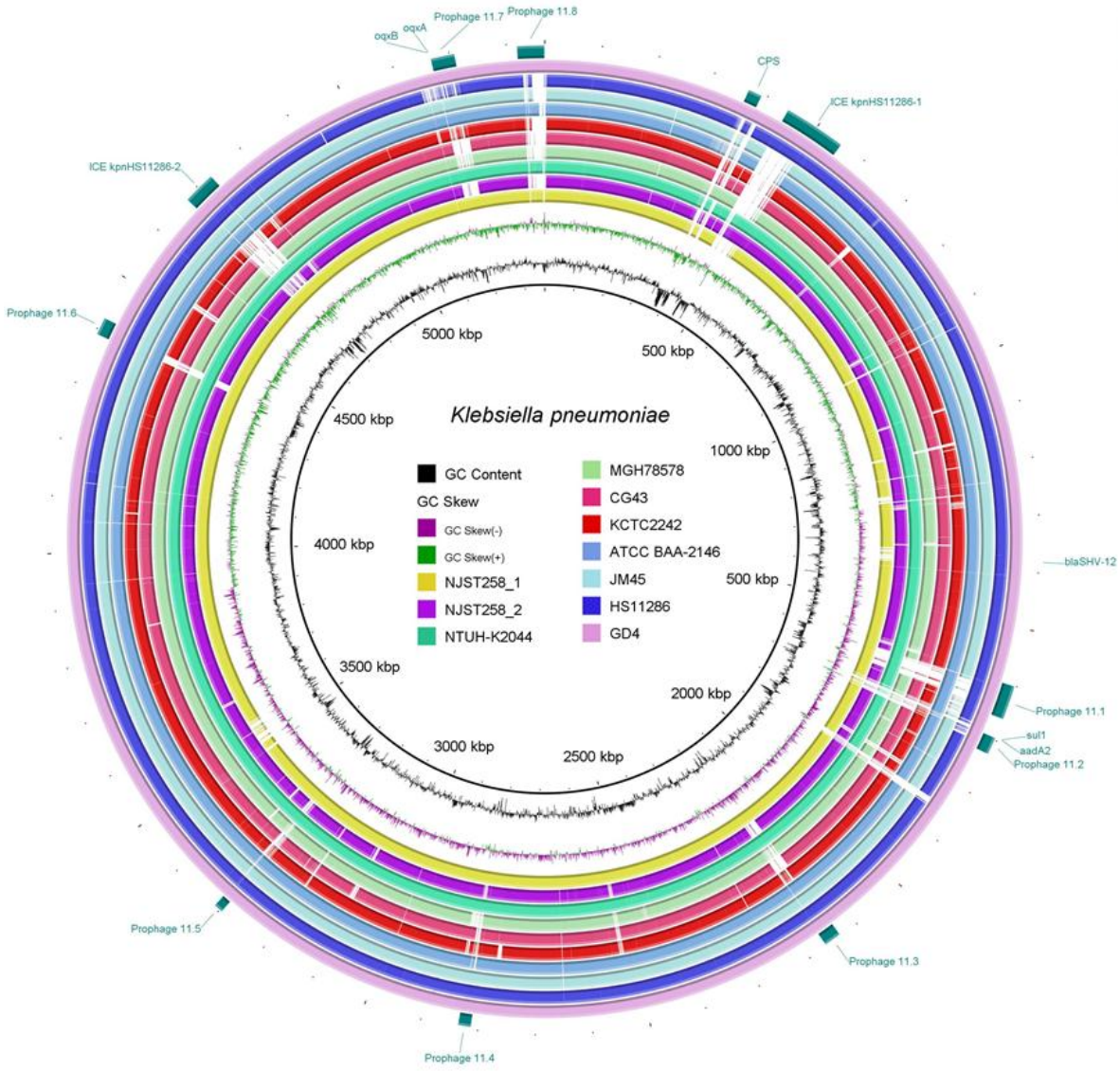


Figure 2.1 Alignment of the *K. pneumoniae* genomes.

A total of 10 *K. pneumoniae* genomes were compared using the chromosome sequence of strain GD4 (outermost circle) as a reference. Prophages (prophages 11.1—11.8), integrated conjugative elements (ICE*Kpn*HS11286-1 and ICE*Kpn*HS11286-2) and the capsule polysaccharide region are indicated by rectangles. Antimicrobial resistance genes are indicated.

Table 2.3 Comparison of key features of the ST11 chromosomes.

	GD4	HS11286 [a]	JM45 [b]
Size (bp)	5,366,808	5,332,752	5,273,813
G+C content (%)	57.5	57.5	57.5
Number of CDS	5215	5316	4872
rRNA	25	25	25
16S	8	8	8
23S	8	8	8
5S	9	9	9
tRNA number	80	87	83
tmRNA number	1	1	1
Plasmid number	1	6	2
Prophages	8	7	8
ICEs number	2	2	1
ICE*Kpn*HS11286-1	+	+	-
ICE*Kpn*HS11286-2	+	+	+
IS number	40	30	24
ISNCY	7	6	7
IS6	2	-	-
IS5	15	8	-
IS481	1	-	1
IS3	10	8	15
IS1380	1	6	1
IS1182	1	-	-
IS1	3	1	-
IS66	-	1	-
bla genes	bla_{KPC-2}, $bla_{CTX-M-65}$, bla_{TEM-1}	bla_{KPC-2}, $bla_{CTX-M-14}$, bla_{TEM-1}	bla_{KPC-2}, $bla_{CTX-M-24}$, bla_{VEB-3}

a. Data for this column are from reference [137]. b. Data for this column are from reference [135] except for IS numbers which derived from this study. ICE, integrative and conjugative elements; IS, insertion sequence.

2.4.2 Phylogenetic analysis

After filtering recombination, a 4,205,312 bp conserved core genome was identified among the genomes of the 58 ST11 strains. A total of 6749 SNPs was identified in the core genomes and were used to construct an approximately maximum-likelihood phylogenetic tree. Similar to the ST258 *K. pneumoniae* strains which comprised at least two distinct lineages [26], the 58 ST11 isolates were found to be clustered into three major groups, namely clade 1, clade 2 and clade 3 with respectively 4, 10 and 44 strains in each clade, indicating there is widespread of clade 3 within Chinese hospitals (Figure 2.2). Isolates within clade 1 and clade 2 were found to differ from that of clade 3 by an average of 2848 (a range of 2595-3353, around one SNP per 1884 nucleotides) and 1198 (a range of 1181-1244, about one SNP per 4480 nucleotides) SNPs, respectively (Table 2.4). This finding was similar to the previous study, in which the 12 ST11 outbreak strains from a single hospital could also be grouped into three clades [138], indicating that the ST11 lineage should not be regarded as a single clone. It should be noted that strains in clade 2 and 3 of the previous study were mostly clustered in clade 3 of this study, whereas, strains in clade 1 of the previous study was clustered into clade 2 in this work (Figure 2.2). Importantly, none of strains belonging to Clade 1 in this study was detectable in the previous study. The discrepancy between the clustering data of the two studies was probably due to the large sample size and inclusion of more genetically diverse strains in our work. Phylogeographic clustering could not be properly resolved in the current study, owing to the shortage of samples as well as the detection of different lineages in a single province (Beijing, Zhejiang, Shanxi, Shanghai, etc).

Table 2.4 Distribution of SNPs in genomes of ST11 clinical isolates from distinct clades*.

	number of SNP in the ~5.3Mbp whole genome (average/range)	number of SNP in the ~24Kbp *cps* cluster (average/range)
Clade 1	2848 / 2595-3353	113 / 83-161
Clade 2	1198 / 1181-1244	155 / 155-155
Clade 3	57 / 28-166	0 / 0-0

*, Numbers of SNP in this table were calculated with harvest suite using the sequence of strain GD4 as a reference.

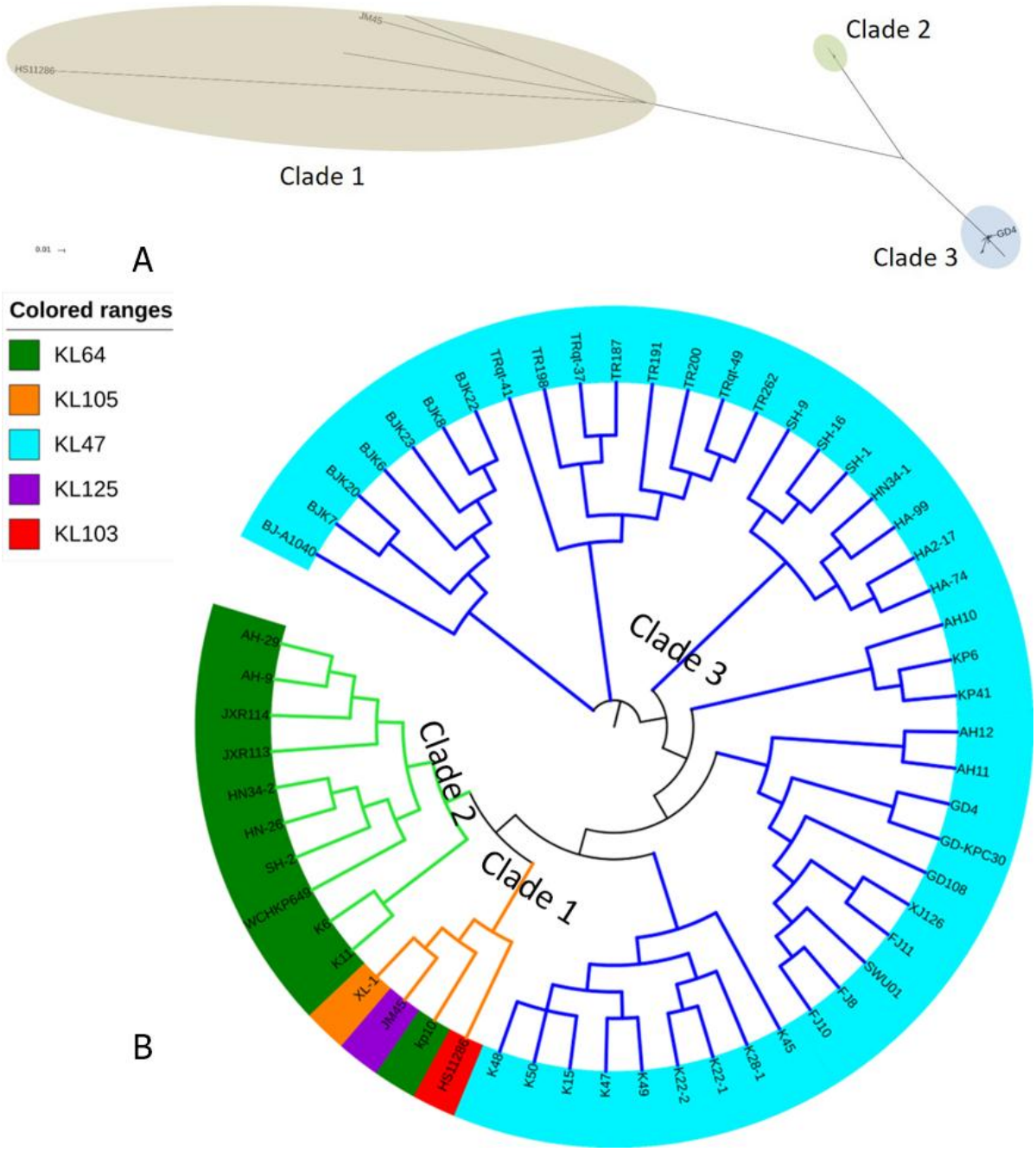


Figure 2.2. Approximately maximum-likelihood phylogeny estimated with the parsnp software [151] based on a total of 6749 unique concatenated SNPs in the core genome of 58 clinical ST11 *K. pneumoniae* strains from China. (A) Unrooted phylogenetic analysis of 58 *K. pneumoniae* clinical isolates. GD4, HS11286, JM45 are ST11 strains with completed genome sequences. Bar, 0.01 substitutions per nucleotide position. (B) Circular phylogenetic tree of the 58 strains. The branch lengths are not proportional to the

evolutionary distances in the cladogram. Different background colors underneath each strain name indicate the K locus type of the corresponding strain detected by Kaptive [45].

Using the ST38 *K. pneumoniae* isolate MGH78578 as an outgroup, the CG258 isolates grouped into a two branches clade which shows extensive variations compared with the ST38 clade (Figure 2.3). The ST258 lineage harbors a ~1.1Mbp region divergent from the ST11 lineage which have been proposed to be originated from an ST442-like clone [135]. This finding is in line with the previous study which indicated that members of CG258 descend from a common ancestor (mostly of ST11) and then diversified into distinct lineages [27]. The ST11 lineage, which grouped into three clades in this study, were mainly classified by the *cps* locus it harbors as displayed in the variation regions in Figure 2.3.

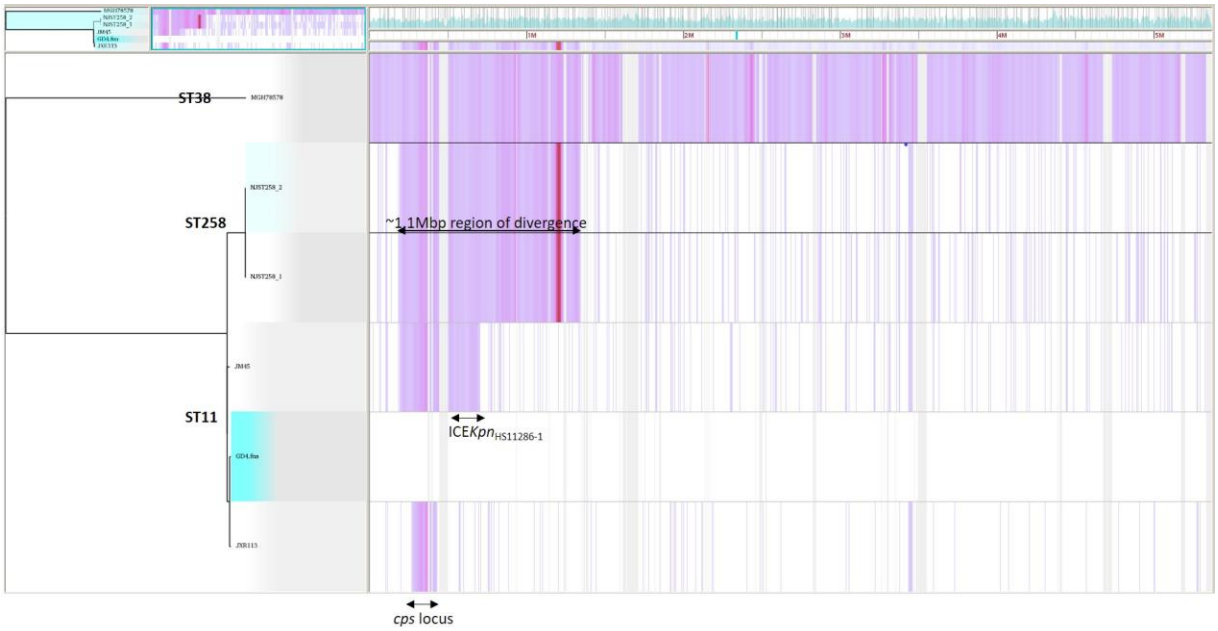


Figure 2.3 Phylogenetic tree and SNPs of representative isolates from CG258.

2.4.3. Comparison of the *cps* cluster

The genomes of ST258 and ST11 *K. pneumoniae* strains were reported to differ mainly in the composition of a ~1.1-Mbp region in which the *cps* locus is located [138]. SNPs analysis showed that the most diversified area in this region was the *cps* operon, which encoded the capsule polysaccharide (CPS), a known key virulence determinant in *K.*

pneumoniae [1]. Large-scale recombination events and capsule switches are known to contribute to the strain variation within CG258 [27]. Traditional serological typing method has identified more than 78 distinct capsular types (K types) in *K. pneumoniae* [1, 26, 27, 44]. Recently a novel program (Kaptive) for identifying the CPS synthesis loci (K loci) was developed by using the full locus information extracted from the genome data, the use of which resulted in an increasing in the total number of known K loci to 134 [45]. By adopting the Kaptive program in our study, the K types of 58 ST11 were identified (Figure 2.2B). In line with the previous finding that two distinct *cps* gene clusters were involved in segregating the ST258 clinical isolates into two distinct clades [27], the *cps* loci of the ST11 strains could also segregated into phylogenetically defined sublineages. The 10 strains in clade 2 were KL64 and all the 44 strains in clade 3 were found to belong to KL47. On the other hand, the four strains (HS11286, JM45, kp10 and XL-1) within clade 1 were found to belong to different K types including KL103, KL125, KL64 and KL105. This is not surprising as the four strains did not exhibit significant genetic relatedness, even though we manually grouped them into one cluster (Figure 2.2A). Based on these observations, we speculated that the ST11 "strain" has undergone a number of capsular exchanges since its emergence as a major nosocomial outbreak agent. Notably, the serotype of strain HS11286 was previously characterized as K74 through *wzi* typing [27]. However, the 78 different *wzi* loci could be clustered into four homology groups, suggesting that *wzi* typing has lower discrimination power compared to *cps* loci typing [44]. By using the complete locus sequence, it is reasonably to redefine HS11286 as the novel type, KL103 based on the genetic sequence of its *cps* locus. Some new capsular types were reported to be predominant among clinical carbapenem-resistant *K. pneumoniae* strains in Italy and the USA [26, 63, 155].

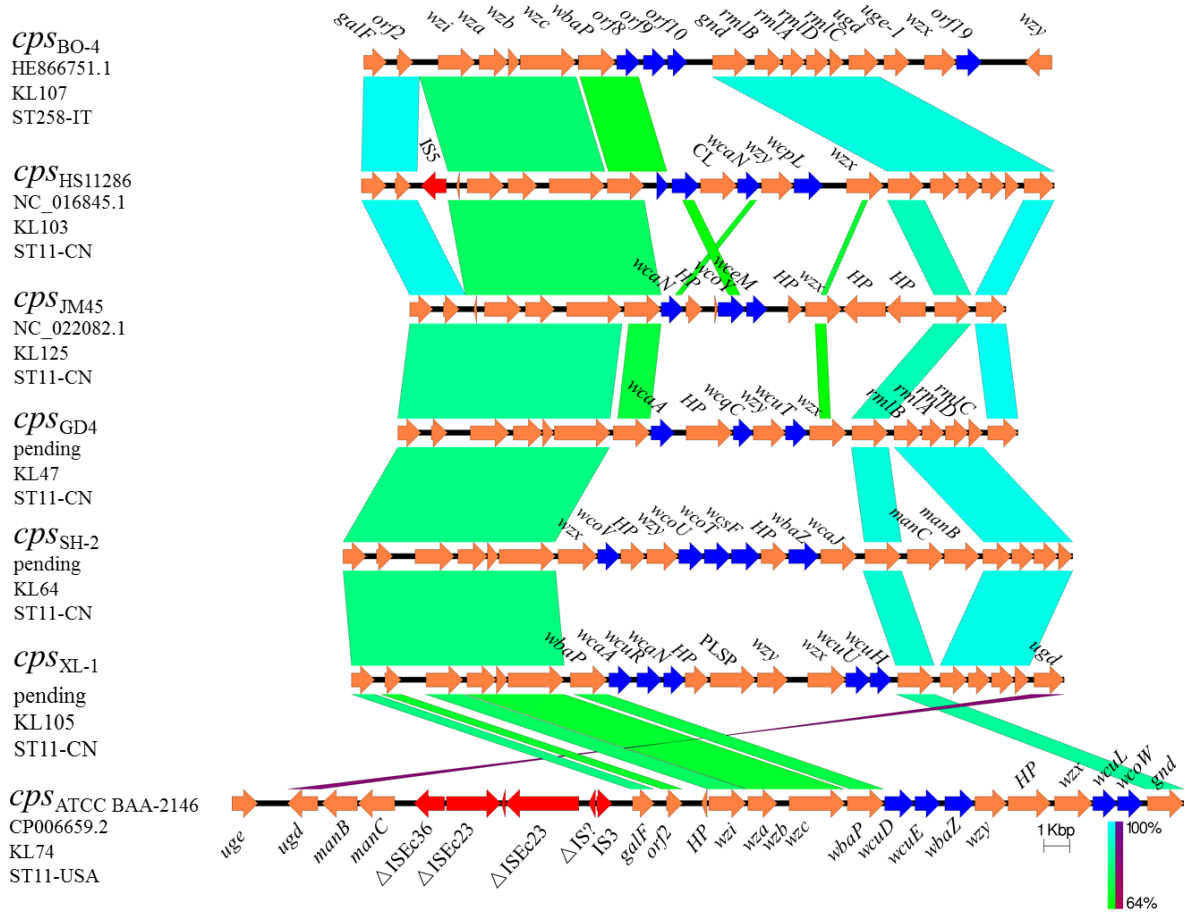


Figure 2.4 Comparison of the cps gene clusters from *K. pneumoniae* strains of CG258. The order of the pairwise comparisons are defined by the phylogenetic relationships. Locus types KL47 and KL64 correspond to serotypes K47 and K64, respectively. K types KL103, KL125 and KL105 are locus types which have not been phenotypically defined and are defined from DNA sequence data on the basis of gene content [123]. Sequence origins, accession numbers and STs for the respective strains are indicated. Arrows indicate the direction, relative length and function of open reading frames (ORFs). The *cps* loci of *K. pneumoniae* strains ATCC BAA-2146 and BO4 were downloaded from the NCBI database and used as references for the comparison. ORFs encoding transposases are colored in red, while those encoding non-initial glycosyltransferases are colored in blue. HP, CL and PLSP are short for hypothetical protein, carbohydrate lyase and Pectate lyase superfamily protein, respectively. Homologous regions are connected by areas of different colors reflecting the degree of nucleotide identity (from 64% to 100%).

Table 2.5 *cps* loci profile of representative *K. pneumoniae* strains of CG258.

Strain	STs	origin	Genetic lineage	*cps* locus				
				type	name	length (bp)	# of ORFs (start and end genes)	Reference/Accession numbers
GD4	11	China	Clade 3	KL47	cps_{GD4}	23,953	19 (*galF* to *ugd*)	CP025951
SH-2	11	China	Clade 2	KL64	cps_{SH-2}	28,193	23 (*galF* to *ugd*)	PJPE00000000
HS11286	11	China	Clade 1	KL103	$cps_{HS11286}$	26,763	21 (*galF* to *ugd*)	NC_016845.1
JM45	11	China	Clade 1	KL125	cps_{JM45}	23,035	18 (*galF* to *ugd*)	NC_022082.1
XL-1	11	China	Clade 1	KL105	cps_{XL-1}	27,528	22 (*galF* to *ugd*)	GCA_001939845.1
KKBO-4	258	Italy	-	KL107	cps_{BO-4}	26,587	20 (*galF* to *wzy*)	[156]/ HE866751
ATCC BAA-2146	11	USA	-	KL74	$cps_{ATCC\ BAA-2146}$	36,774	26 (*uge* to *gnd*)	[157]/ CP006659.2

The common genetic features of the *cps* clusters in *K. pneumoniae* strains have been determined previously [1]. Generally, the *cps* locus harbored a highly conserved 5' end region of six genes (*galF*, *orf2*, *wzi*, *wza*, *wzb* and *wzc*) whose products are responsible for CPS assembly and translocation with sequence variations often observable in the central and 3' end regions (from *gnd* to *ugd*) [44, 158]. The structures of the five distinct loci detected in this study as well as two reference loci types (cps_{BO-4} and $cps_{ATCC\ BAA-2146}$) reported in previous studies [156, 157] were compared and shown in Figure 2.4 and Table 2.5. Briefly, the *cps* loci of the Chinese ST11 strains ranged from approximately 23Kpb~28Kbp with 18~23 ORFs (Table 2.5). Synthesis of the capsular repeat in *K. pneumoniae* is mediated by the initial glycosyltransferase (GT) WbaP or WcaJ, which catalyze transferal of galactose-1-phosphate or glucose-1-phosphate to undecaprenol phosphate, respectively [44]. The gene *wcaJ* was found only in the clade 2 strains and one strain (kp10) in clade 1, and the initial GTs gene for other ST11 strains were all found to be *wbaP*, indicating that different saccharides might be present in the repeat unit of capsule. Addition of sugars in the capsule is catalyzed by specific non-initial GTs [159]. In the case of ST11 *K. pneumoniae*, strains within distinct clades were found to harbor different combinations of non-initial GT genes (Figure 2.4, blue arrows) in the central *cps* region. For instance, clade 3 strains (see GD4) were found to harbor *wcaA*, *wcqC* and *wcuT* genes in the central region, whereas clade 2 strains such as SH-2

contained genes *wcoV, wcoU, wcoT, wcsF* and *wbaZ*. The 3' end *gnd- ugd* region of the *cps* locus which harbored the gene clusters *manCB* or *rmlBADC* are known to be responsible for the synthesis of GDP-D-mannose or dTDP-L-rhamnose in the majority of capsular types [44]. In this work, the finding that the *cps* types KL107 (cps_{BO-4}), KL103 ($cps_{HS11286}$), KL47 (cps_{GD4}) and KL105 (cps_{XL-1}) harbored the *rmlBADC* gene cluster, is highly consistent with the phenomenon of the presence of rhamnose in the repeat units of capsule [44]. On the other hand, the presence of *manCB* genes does not always correlate with the existence of mannose in capsule [44]. Both *manCB* and *rmlBADC* gene clusters were detected in the KL64 *cps* type (cps_{SH-2}) and none of the two clusters was found in the KL125 type ($cps_{HS11286}$). Additionally, genes for capsule modification was detected in the *cps* loci of some strains, such as those encoding carbohydrate lyase in HS11286 and the pectate lyase superfamily protein-encoding gene in XL-1 (**Fig 3**), suggesting that distinct modifying systems were responsible for production of different capsule structures. It should be noted that, when T11 and ST258 were compared to each other, the region which exhibited the largest degree of genetic variation was the middle section of *cps*; the presence of three putative ORFs in this region in ST258 type of strains suggests that the sugar content in the capsule of ST11 might be high different from that of ST258. Such difference may account for the discrepancy in the level of prevalence of these genetically related strains in different parts of the world. Future studies should focus on investigating the underlying mechanisms governing their adaptability to different environmental conditions.

2.4.4 Antimicrobial Resistance (AMR) genes in ST11 *K. pneumoniae*

Dissemination of resistance determinants has been recognized as a major challenge in treatment of bacterial infections worldwide [160]. A targeted survey performed within the 58 ST11 genomes revealed a total of 62 known AMR genes (Figure 2.5). The bla_{SHV} and *fosA3* genes were shown to be the core chromosomal genes that were present in all the ST11 strains, suggesting that these two AMR gene were likely present in the ST11 *K. pneumoniae* ancestor [8, 161]. The *oqxA* and *oqxB* genes were both detected in 34 different strains. Being the core genes in *K. pneumoniae*, their role in conferring resistance to fluoroquinolones is not well defined [8, 145]. Other AMR genes that were

found to be present in more than 30 strains included *bla*$_{KPC-2}$ (detectable in 57 strains, and only absent in strain kp10), *aadA2* (51), *bla*$_{TEM-1b}$ (45), *rmtB* (38), *bla*$_{CTX-M-65}$ (33) and *fosA*-14 (31), conferring resistance to carbapenems, aminoglycosides, β-lactams or fosfomycin, respectively. Besides, the test strains were also found to harbor different combinations of the following resistance genes *arr*-3, *qnrB*, *qnrS*, *sul*, *tet*, *str*, *msr*, *mph*, *floR*, *dfrA*, *cat*, *bla*$_{VEB}$, *bla*$_{TEM}$, *bla*$_{NDM}$, *bla*$_{DHA}$, *armA*, *aph*, *aac*, with the number of such genes harbored by each strain ranging from 4~17. Undoubtedly, the presence of resistance determinants allows the ST11 strains to survive the barrage of antibiotics used in treatment of hospital infections. The distribution of these genes varied dramatically among strains and was not with lineage (Figure 2.5), indicating the AMR genes were acquired through horizontal transfer. Due to the constraints of using short-read illumina data [8], we were not able to link each resistance locus to a specific plasmid reliably. However, a search against the PlasmidFinder database [91] allowed us to identify 26 plasmid replicons in the 58 strains, including 6 colicin replicons and 20 associated with large conjugative AMR plasmids. Importantly, 2 to 7 distinct types of plasmid replicon were detectable in each strain. The *bla*$_{KPC-2}$ genes were found to be frequently associated with the IncFII/IncR plasmid replicons and have a similar core structure, IS*Kpn27*-*bla*KPC-2-IS*Kpn2*, as reported in our previous nationwide surveillance study [90]. The completely sequenced plasmid pKPGD4 was found to be an IncFII/IncR-like plasmid, carrying the *bla*$_{KPC-2}$, *bla*$_{CTX-M-65}$, *bla*$_{TEM-1b}$, *rmtB*, *catA2*, and *fosA14* genes. The sequence organization of pKPGD4 was similar to that of the unnamed plasmid (accession number: CP018455) in strain *K. pneumoniae* SWU01 (coverage 91%, identity 99%) and plasmid pCT-KPC (accession number: KT185451.1, coverage 86%, identity 99%) (Figure 2.6).

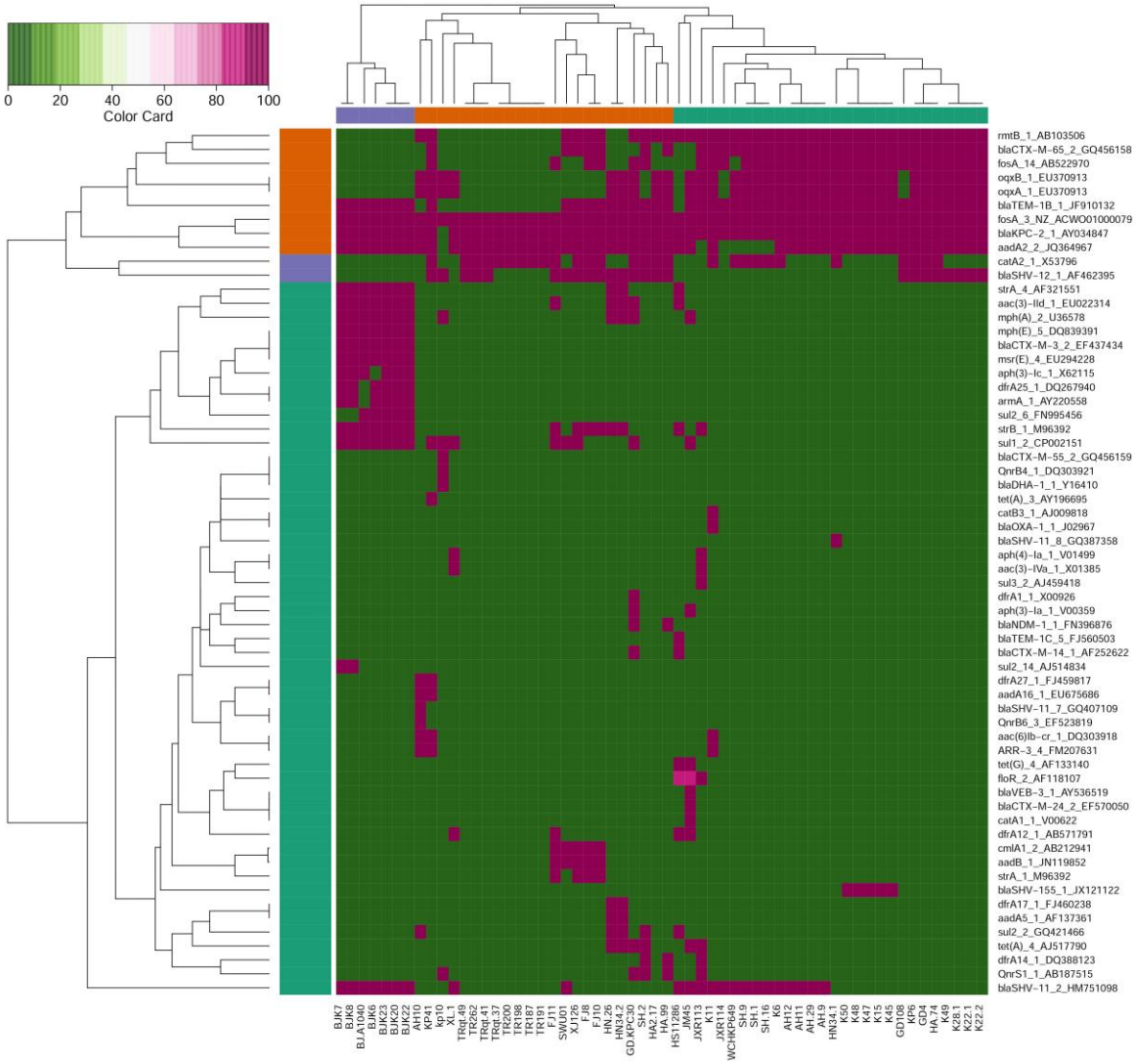


Figure 2.5 Heatmap of the antimicrobial resistance genes harbored by clinical ST11 *K. pneumoniae* strains from China.

Color bar indicated the presence/absence and similarity of the resistance determinants. The presence of resistance genes in a specific genome is represented by the red box and the absence of resistance genes is represented by a purple box.

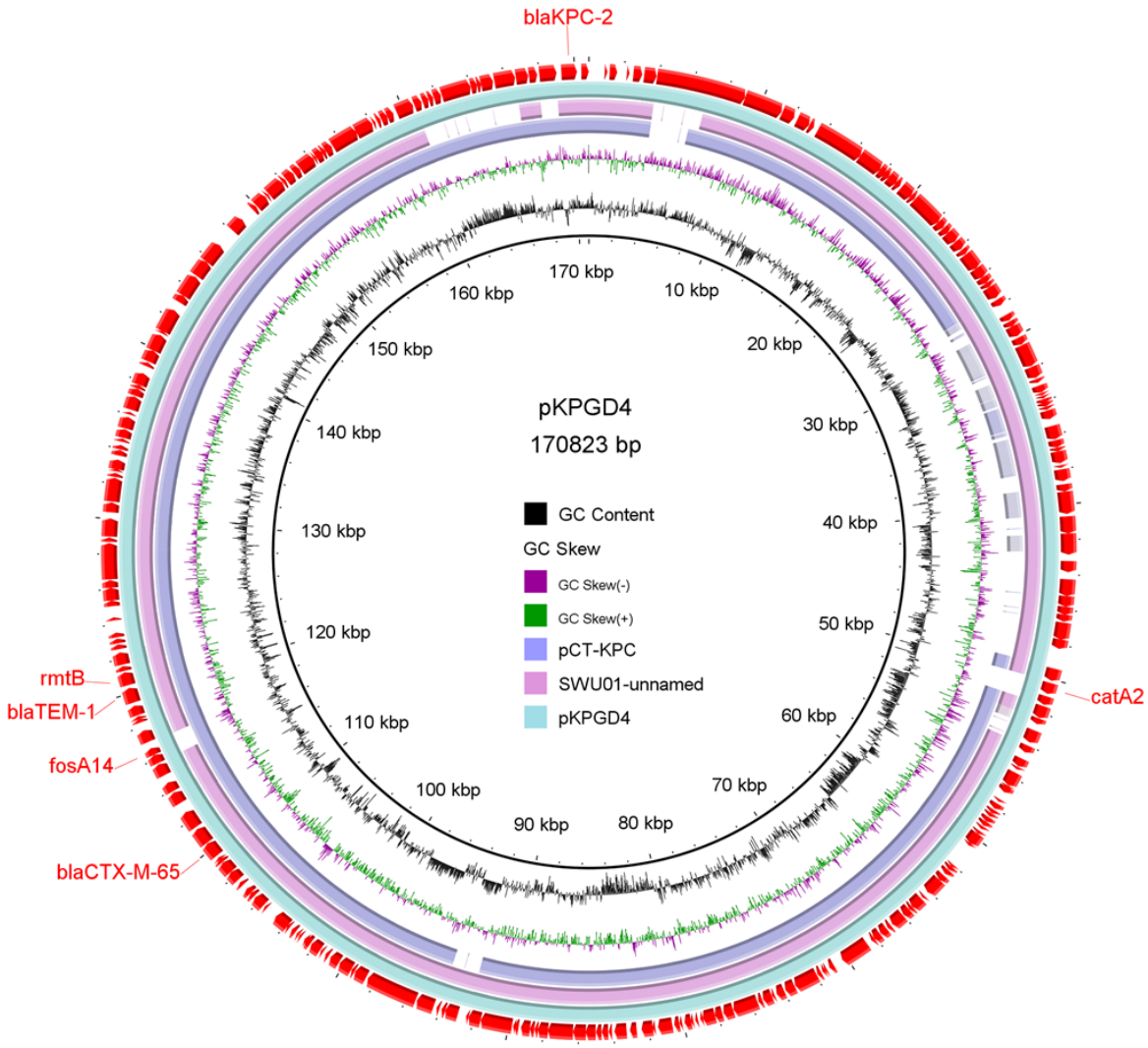


Figure 2.6 Circular map of plasmid pKPGD4 harbored by *K. pneumoniae* strain GD4. Resistance determinants are indicated in the outmost circle. Plasmids pCT-KPC (accession: KT185451.1) and SWU01 unnamed plasmid (accession: CP018455.1) with similar sequence organizations were aligned against the plasmid pKPGD4.

2.4.5 Prevalence of virulence factors in ST11 *K. pneumoniae*

As an opportunistic pathogen, *K. pneumoniae* relies on an array of virulence factors to colonize and propagate in a host, which include at least (a) surface antigen, especially capsular polysaccharide (CPS, K antigen); (b) siderophores that are responsible for binding ferric iron secreted by the iron-binding proteins of the host; and (c) adherence factors that are responsible for attachment to host cell surfaces, such as type 1 and type 3

fimbriae, and non-fimbrial adhesion proteins [1]. Virulence gene analysis of these ST11 strains showed that some of the virulence genes were commonly present in ST11 strains. The *FimA, B, C, D, E, F, G, H, I* and *K* genes encoding type 1 fimbriae, which have been reported to be involved in enhancing bacterial virulence during urinary tract infection, were detected in almost all the ST11 strains and ST258 strains. The *mrkA, B, C, D, F, J, H* and *I* genes encoding type 3 fimbriae, which were shown to mediate biofilm formation on both abiotic and biotic surfaces, as well as *kpn* (coding for FimH-like adhesins) and *ycfM* (coding for outer membrane lipoproteins (OMLs)), both of which were reported to be involved in bacterial adhesion processes, were also detected in almost all the ST11 strains and ST258 strains [162]. Expression of these genes might therefore enhance the adhesive capacity of *K. pneumoniae* towards respiratory epithelial cells and surfaces of medical device like ventilators, thus enhancing their ability to cause ventilator associated infections. Besides, findings of two previous studies revealed a strong relationship between antibiotic resistance and the prevalence of biofilms and OMLs in bacteria, as biofilms and OMLs are known to actively protect bacteria from drug exposure [163, 164]. Carriage of all these virulence determinants might be associated with the high-level antimicrobial resistance phenotype of ST11 *K. pneumoniae*. Another outer membrane lipoprotein TraT, encoded by the plasmid-borne transfer gene *traT*, was previously demonstrated to be able to mediate resistance to bacterial killing by serum. The *traT* gene was found to be frequently associated with production of K1 capsule [165]. Interestingly, virulence factor analysis showed that some of the ST11 strains also harbored this gene.

Several types of siderophores are known to be differently expressed in ST11 *K. pneumoniae*, with genes encoding enterobactin synthesis such as *entB* being present in the majority of ST11 strains [1]. Genes encoding yersiniabactin (the *irp* genes) and its transporters (the *ybt* and *fyu* genes) were detected in majority of the ST11 *K. pneumoniae* genomes in this work, with only one exception (strain JM45, Figure 2.7). Interestingly, these genes were not detectable in ST258 type of *K. pneumoniae*, constituting a major difference between these two types of strains. The *ybt* locus was reported to be located in a self-transmissible ICE in the *K. pneumoniae* genome and was significantly associated with infections [8, 145]. Carriage of yersiniabactin-encoding genes in the ST11 strains may pose significant threat to public health since multidrug resistant and virulent *K.*

pneumoniae strains are causing an increasing number of fatal hospital infections in China. Surprisingly, genes encoding salmochelin (*iroBCDN*), aerobactin (*iucABCD*) and the *rmpA* gene were also detected in a few ST11 genomes. Notably, three strains (HN-26, HN34-2 and SH-2) in phylogenetic clade 2, all of which are capsular locus type KL64, were found to harbor the *iucABCD* and *rmpA* genes, and three strains (SH-16, SH-1 and SH-9) in clade 3 of the capsular locus type KL47 were found to contain the gene cluster *iroCDN* in addition to *iucABCD* and *rmpA* (Figure 2.7). Combined carriage of the *iro* and *iuc* gene clusters, as well as the *rmpA* gene, is frequently, but not always linked to the publicly known *K. pneumoniae* virulence plasmid pLVPK, which is uniquely associated with hvKP strains [1, 8, 130]. These strains may belong to the newly emerged ST11 carbapenem-resistant, hypervirulent *K. pneumonia* (ST11-CR-HvKP) suggesting the wide spread of this new superbug in different parts of China [166].

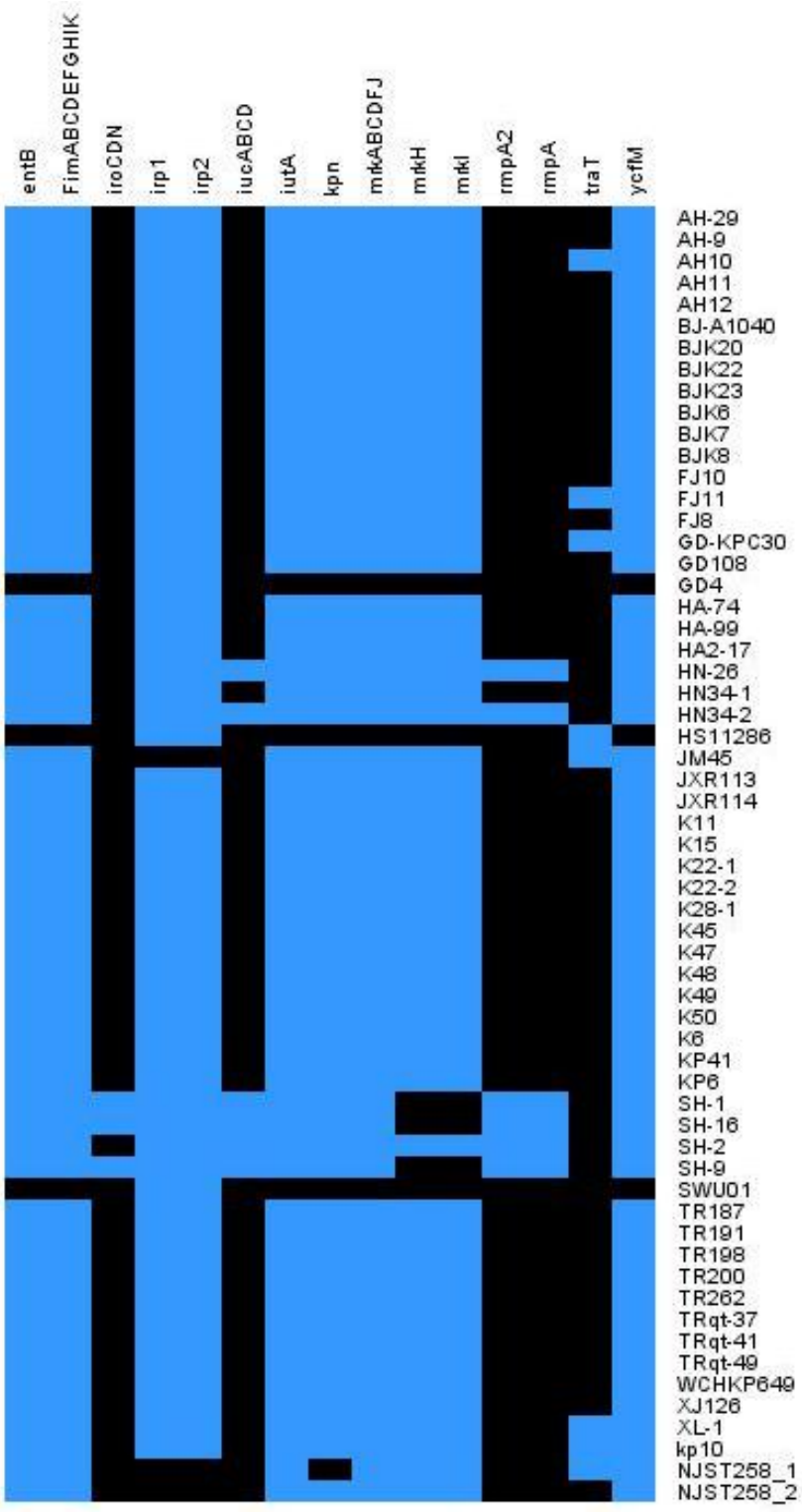


Figure 2.7 Heatmap of virulence determinants harbored by clinical ST11 *K. pneumoniae* strains from China. The presence of virulence genes in a specific genome is represented by the blue box and the absence of virulence genes is represented by a black box.

2.5 Conclusions

ST11 *K. pneumoniae* has emerged as the dominant KPC-producing clone in China [136]. Comprehensive genomic analysis was performed on representative ST11 strains collected in various location in China. The genome of these ST11 strains were found to be highly heterogeneous and could be grouped into three major genetic lineages and 5 different capsular types through phylogenetic analysis. Multiple resistance and virulence determinants were found in the ST11 genomes; such transposable elements are apparently responsible for rendering these strains a severe threat to human health. Genomic analysis indicated that ST11 CR-hvKp has potential emerged in China. These data therefore provide insights into the development of prevention, diagnosis and treatment of strategies to combat infections caused by ST11 *K. pneumoniae* strains.

CHAPTER 3. A fatal outbreak of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital

The content in this chapter has been published in following paper:

Gu, D.[#], Dong, N. [#], Zheng, Z., Lin, D., Huang, M., Wang, L., ... & Chen, S. (2018). A fatal outbreak of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital: a molecular epidemiological study. The Lancet Infectious Diseases, 18(1), 37-46. ([#]Contributed equally).

The other first author of this paper provided the clinical isolates and clinical data from the patients. All the major research work was done by the candidate. This paper will not be used for other theses.

3.1 Abstract

Hypervirulent *Klebsiella pneumoniae* strains often cause life-threatening community-acquired infections in young and healthy hosts, but are usually sensitive to antibiotics. In this study, we investigated a fatal outbreak of ventilator-associated pneumonia caused by a new emerging hypervirulent *K. pneumoniae* strain. The outbreak occurred in the integrated intensive care unit of a new branch of the Second Affiliated Hospital of Zhejiang University (Hangzhou, China). We collected 21 carbapenem-resistant *K. pneumoniae* strains from five patients and characterized these strains for their antimicrobial susceptibility, multilocus sequence types, and genetic relatedness using VITEK-2 compact system, multilocus sequence typing, and whole genome sequencing. We selected one representative isolate from each patient to establish the virulence potential using a human neutrophil assay and *Galleria mellonella* model and to establish the genetic basis of their hypervirulence phenotype. All five patients had undergone surgery for multiple trauma and subsequently received mechanical ventilation. The patients were aged 53–73 years and were admitted to the intensive care unit between late

February and April 2016. They all had severe pneumonia, carbapenem-resistant *K. pneumoniae* infections, and poor responses to antibiotic treatment and died due to severe lung infection, multiorgan failure, or septic shock. All five representative carbapenem-resistant *K. pneumoniae* strains belonged to the ST11 type, which is the most prevalent carbapenem-resistant *K. pneumoniae* type in China and originated from the same clone. The strains were positive on the string test, had survival of about 80% after 1 h incubation in human neutrophils, and killed 100% of wax moth larvae (*G. mellonella*) inoculated with 1×10^6 colony-forming units of the specimens within 24 h, suggesting that they were hypervirulent *K. pneumoniae*. Genomic analyses showed that the emergence of these ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains was due to the acquisition of a roughly 170 kbp pLVPK-like virulence plasmid by classic ST11 carbapenem-resistant *K. pneumoniae* strains. We also detected these strains in specimens collected in other regions of China. The ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains pose a substantial threat to human health because they are simultaneously hypervirulent, multidrug resistant, and highly transmissible. Control measures should be implemented to prevent further dissemination of such organisms in the hospital setting and the community.

3.2 Introduction

Klebsiella pneumoniae is a major Gram-negative bacterial pathogen that can cause invasive hospital-acquired infections among immunocompromised patients [167]. Pyogenic liver abscesses caused by *K. pneumoniae* have become a serious clinical challenge in Asia. In Taiwan, more than 3000 new cases of pyogenic liver abscesses occur each year [168]. Compared with the classic *K. pneumoniae* strains that cause other types of opportunistic infections, pyogenic liver abscess-associated *K. pneumoniae* strains often have substantially higher virulence and are therefore designated hypervirulent *K. pneumoniae*. Hypervirulent *K. pneumoniae* has the ability to cause life-threatening, community-acquired infections such as liver abscesses, pneumonia, meningitis, and endophthalmitis in young and healthy individuals and is therefore associated with high morbidity and mortality [19]. These strains can efficiently acquire iron and produce an increased amount of capsular substance compared with classic *K. pneumoniae*, which

confers a hypermucoviscous phenotype that is detectable as a positive result on the string test (a viscous string of >5 mm in length is produced when touched with an inoculation loop) [19]. The hypervirulent phenotype of *K. pneumoniae* is thought to be attributable to the carriage of a virulence plasmid harboring two capsular polysaccharide (CPS) regulator genes (*rmpA* and *rmpA2*) and several siderophore gene clusters that contribute to the hypermucoviscous phenotype [34, 169]. Correlation between carriage of the virulence plasmid and the hypervirulence phenotype has also been reported [170]. To date, most hypervirulent *K. pneumoniae* strains have been K1 or K2 types and have remained antibiotic-sensitive [48].

In reports from 2013, the US Centers for Diseases Control and Prevention (CDC) described the emergence of carbapenem-resistant Enterobacteriaceae strains, which commonly cause untreatable or hard-to-treat infections among patients in hospitals, as an urgent public health threat. Carbapenem-resistant *K. pneumoniae* strains account for roughly 70–90% of clinical carbapenem-resistant Enterobacteriaceae infections in the European Union and China [89, 170]. The most common clinical carbapenem-resistant *K. pneumoniae* strains are those of the clonal group (CG) 258, with ST258 and ST11 being the most prevalent multilocus sequence types in different parts of the world [25]. ST258 is a hybrid clone composed of 80% of ST11 genome and 20% of ST442 genome [133]. ST258 has disseminated worldwide since its emergence in the early 2000s and has become particularly prevalent in North America, Latin America, and several European countries [25]. However, in Asia, the dominant clone is ST11 carbapenem-resistant *K. pneumoniae*, which accounts for up to 60% of carbapenem-resistant *K. pneumoniae* in China [89]. Consequently, in recent years, ST11 has been regarded as the most transmissible clone contributing to the increasing prevalence of carbapenem-resistant *K. pneumoniae* in China. In this study, we investigate a fatal ventilator-associated pneumonia outbreak in an intensive care unit (ICU) in a Chinese hospital with the aim of identifying the molecular basis for the hypervirulence of the emerging ST11 carbapenem-resistant *K. pneumoniae* strains responsible for this outbreak.

3.3 Materials and Methods

3.3.1 Outbreak investigation

In late March and April 2016, we identified several cases of severe pneumonia in the integrated ICU of a new branch of the Second Affiliated Hospital of Zhejiang University (Hangzhou, China). This new integrated ICU consists of three units with a total of 26 wards and 40 beds and has been open since November 2015. Before the outbreak described, no patients in this ICU had reported severe symptoms of pneumonia or had poor outcomes with antibiotic treatments. We therefore initiated an outbreak investigation that included five patients. The first patient in this outbreak, who was suspected of being the index patient, had previously been admitted to a local hospital (Fuyang People's Hospital in the Fuyang district of Hangzhou) for multiple trauma caused by a car accident and was transferred to the ICU of our hospital on Feb 28, 2016. The other four patients were admitted in March and April. All five patients stayed in different wards of the ICU, but all had overlapping stays in the ICU during this outbreak. All clinical data were collected in accordance with the ORION checklist. Bacterial strains were isolated from sample specimens at the clinical laboratory of the hospital. We subjected the isolated strains to phenotypic and genotypic characterisation to identify the causative agent and understand the molecular basis of the high mortality of these causative agents. We obtained permission to report the cases from the patients' families.

3.3.2 Phenotypic characterization

We used a VITEK-2 compact system (bioMérieux, Marcy-l'Étoile, France) to establish the strain identity and antimicrobial susceptibilities of the isolates and we interpreted these in accordance with the guideline document M100-S26 established by Clinical and Laboratory Standards Institute [139]. We confirmed the species identity of all isolates via matrix-assisted laser desorption/ionisation mass spectrometry (Bruker Daltonics, Billerica, MA, USA). We identified capsular antigens by PCR targeting genes encoding for K1, K2, K5, K20, K54 and K57 antigens as previously described [171]. We screened for β-lactamase genes by PCR as previously reported [172].

We did conjugation experiments, pulsed-field gel electrophoresis (PFGE) multilocus sequence typing (MLST), S1-PFGE, and Southern hybridisation as reported previously

[172]. We also did plasmid curing experiments, which were done as previously described [173], to test whether the putative virulence plasmid contributed to the hypervirulence phenotype of these strains.

For the string test to identify the hypermucoviscous phenotype, all isolates were inoculated onto agar plates containing 5% sheep blood and incubated at 37 ℃ overnight. The string test was deemed positive when a viscous string longer than 5 mm could be generated by touching and pulling a single colony upwards with a standard inoculation loop [19].

As a test of virulence, we did the human neutrophil assay as described previously [26]. Briefly, we isolated neutrophils from the venous blood of healthy volunteers, who had provided written informed consent before participation in the studies. We measured the bactericidal activity of neutrophils by incubating 1×10^6 neutrophils with 1×10^6 colony-forming units (CFU) of opsonised *K. pneumoniae* in RPMI/H medium at 37 ℃ with intervals of 15 min, 30 min, and 60 min, with gentle rotation. 1% saponin was added to each tube, mixed, and then chilled on ice for 15 min before diluting and plating on Luria broth agar. Survival was expressed as the percentage of CFUs recorded after neutrophil treatment compared with the control. We analysed the data using a one-way ANOVA and Tukey's post-hoc test on GraphPad Prism version 6.0b.

We also tested virulence in wax moth (*Galleria mellonella*) larvae weighing about 300 mg (purchased from Tianjin Huiyude Biotech Company, Tianjin, China) and maintained on woodchips in the dark at 15 ℃ until being used. Overnight cultures of *K. pneumoniae* strains were washed with phosphate-buffered saline (PBS) and further adjusted with PBS to concentrations of 1×10^4 CFU/mL, 1×10^5 CFU/mL, 1×10^6 CFU/mL, and 1×10^7 CFU/mL. We infected the *G. mellonella* with the bacteria as described previously (10 larvae in each group) and we recorded the survival rate of the *G. mellonella* [174, 175]. All experiments were done in triplicate.

3.3.3 Genomic characterization

We sequenced genomes using the NextSeq 500 sequencing platform (Illumina, San Diego, CA, USA). We trimmed or filtered raw reads to remove low-quality sequences and adaptors. At least 500 Mb raw sequencing reads were obtained for each isolate with the illumina sequencer. *De novo* assembly of the illumina reads was conducted using SPAdes Genome Assembler version 3.9.1 [140]. Genome of strain *K. pneumoniae* 4 was also sequenced with the nanopore MinION sequencing using rapid Sequencing Kit (SQK219 RBK001) and flowcell type R9.4, generating a total of 500 Mbp passed basecalled reads [176]. Hybrid assembly of both illumina and nanopore sequencing reads for genome of *K. pneumoniae* 4 was conducted with Unicycler [177]. We annotated the draft genome sequences with the RAST tool version 2 and Prokka 1.12 [142, 143]. We compared genome sequences using the BLAST Ring Image Generator (BRIG) version 0.95 [153]. We did the pangenome analysis with Roary: the pangenome pipeline (version 3.6.0) using the Prokka annotation (method described in Chapter 2) [152]. We analyzed single nucleotide polymorphisms (SNPs) with the CSI Phylogeny 1.4 pipeline available from the Center for Genomic Epidemiology with default settings and whole genome sequence raw reads for the analysis. We did capsular typing on the assembled sequences using Kaptive [45]. We deposited the genome sequences in GenBank under the accession numbers SAMN07259328 (*K. pneumoniae* 1), SAMN07259327 (*K. pneumoniae* 2), SAMN07259329 (*K. pneumoniae* 3), SAMN07259326 (*K. pneumoniae* 4), SAMN07259325 (*K. pneumoniae* 5), SAMN07259330 (FJ8), SAMN07259331 (FJ9), SAMN07259332 (hypervirulent *K. pneumoniae* 1088), and SAMN07259333 (carbapenem-resistant *K. pneumoniae* SH1). The antibiotic resistance genes, virulence loci were identified using methods described in Chapter 2 [107, 114]. We generated a heatmap of the virulence loci with Genesis software version 1.7.7 [178].

To check for the presence of the virulence plasmid in clinical ST11 carbapenem-resistant *K. pneumoniae* isolates collected from patients in major hospitals located in 25 provinces and municipalities in China (provided by our collaborators) [89], we screened the isolates with four sets of primers (Table 3.1) targeting the *rmpA*, *rmpA2*, *iucA*, and *iroN* genes.

Table 3.1 Primers used to screen for the presence of virulence plasmid in clinical ST11 CRKP strains.

target gene	primer	Primer sequence	product size	reference
rmpA[*]	rmpA-F	TACATATGAAGGAGTAGTTAAT	505bp	[179]
	rmpA-R	GAGCCATCTTTCATCAAC		
rmpA2[*]	rmpA2-F	TGTGCAATAAGGATGTTACATTAGT	609bp	[179]
	rmpA2-R	TTTGATGTGCACCATTTTTCA		
iucA[#]	iucA-F	ATAAGGCAGGCAATCCAG	2927bp	[61]
	iucA-R	TAACGGCGATAAACCTCG		
iroN[#]	iroN-F	GTCCGGCGGTAACTTCAGCC	829bp	[180]
	iroN-R	TCAGAATGAAACTACCGCCC		

*primers targeting only the plasmid-borne genes.

#primers targeting to both plasmid-borne and chromosomally-encoded genes.

3.4 Results

3.4.1 Patient information

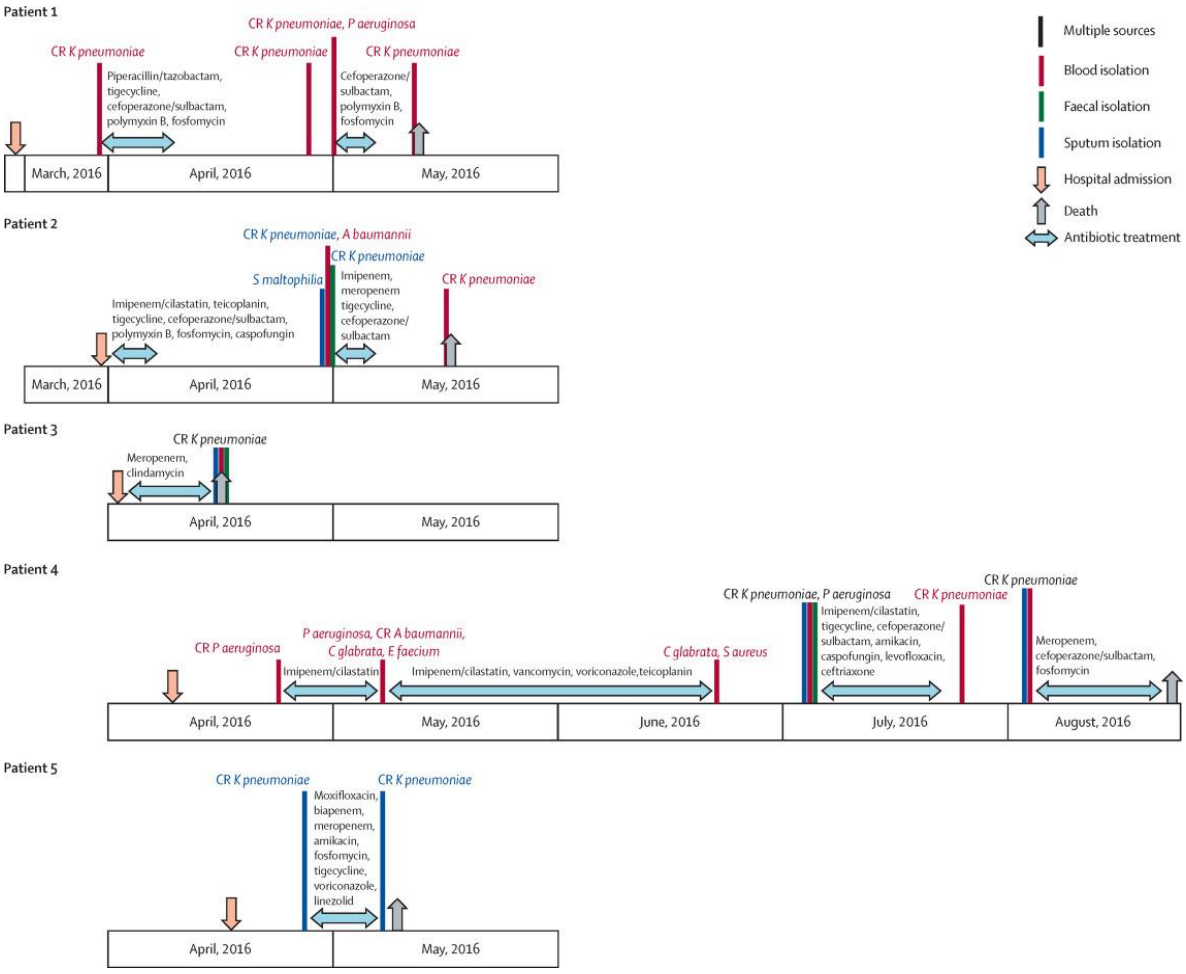


Figure 3.1 Epidemiology of the *K. pneumoniae* outbreak cases.

Coloured text and bars represent the source that the bacterial species was isolated from. CR = carbapenem-resistant; *K. pneumoniae* = *Klebsiella pneumoniae*; *P. aeruginosa* = *Pseudomonas aeruginosa*; *S. maltophilia* = *Stenotrophomonas maltophilia*; *A. baumannii* = *Acinetobacter baumannii*; *C. glabrata* = *Candida glabrata*; *E. faecium* = *Enterococcus faecium*; *S. aureus* = *Staphylococcus aureus*.

Five patients aged 53–73 years were admitted to the ICU between late February and April 2016. These five patients were admitted for various forms of trauma due to car accidents,

falling objects, or atlantoaxial subluxation. They all underwent surgery, followed by antimicrobial treatment and mechanical ventilation (Figure 3.1, Table 3.2). The five patients developed pneumonia, and all showed the typical symptoms of pulmonary oedema, pleuraleffusion, excessive sputum, and shortness of breath. Multiple species of bacteria could be isolated from blood, sputum, and fecal samples of the patients, with carbapenem-resistant *K. pneumoniae* being consistently isolated in all patients and present in different types of specimen before the patients died, suggesting that carbapenem-resistant *K. pneumoniae* might be the causative agent of the outbreak. They responded poorly to antibiotic treatment. The duration of the infections lasted from 10 days to 4 months for these patients. All five patients died of severe lung infection, multiorgan failure, or septic shock after carbapenem-resistant *K. pneumoniae* could be recovered from their blood or sputum samples.

Detailed information about the patients is listed as follows:

Patient 1, a 58-year-old man, was transferred to our hospital on Feb. 28, 2016. He was previously admitted to other hospitals due to multiple trauma caused by a car accident. He was in coma and diagnosed with multiple injuries, brain contusion, traumatic epidural hematoma, subarachnoid hemorrhage, pelvic fracture, hypertension and cerebral infarction. He was then admitted to ICU. and received mechanical ventilation and other supporting treatments. He had constant diarrhea and high body temperature since admission to the hospital. Blood culture revealed positive isolation of *K. pneumoniae* and *Acinetobacter baumannii* on March 29, 2016. The patient was then subjected to antibiotic treatment including piperacillin/tazobactam, tigecycline, cefoperazone/sulbactam, polymyxin B and fosfomycin. Blood culture was cleared of organisms on April 04, 2016. On April 26, 2016, however, blood culture yielded *K. pneumoniae* again; the body temperature of the patient remained at around 38 ℃ and antibiotic treatment continued. On April 28, 2016, the patient was found to exhibit signs of pneumonia and increased infection indexes, developing high temperature on April 30, 2016. Blood culture again resulted in positive isolation for *K. pneumoniae* and *A. baumannii* and the patient was subjected to treatment with polymyxin B and cefoperazone/sulbactam. However, the temperature and infection indexes remained high upon treatment. Chest CT indicated that pneumonia worsened (Figure 3.2, 1A and 1B), followed by signs of deteriorating liver

and kidney function and abnormality in bone marrow and coagulation system. On May 04, 2016, in addition to treatment with polymyxin E and cefoperazone/sulbactam, fosfomycin was added to the regimen, along with other supportive treatment. The temperature and infection indexes slightly reduced, but spiked again on May 08, 2016, followed by multi-organ failure and internal bleeding. *K. pneumoniae* was shown to persist in blood culture until the patient died on May 10, 2016.

Patients 2 was a 62-year-old man admitted to the Emergency Department due to liver and spleen rupture caused by falling objects on March 28th, 2016. He received tracheostomy and endotracheal intubation and was moved to ICU where he further received mechanical ventilation, followed by anti-infection treatment with imipenem and cilastatin. He was then treated with imipenem, teicoplanin, tigecycline, cefoperazone/sulbactam, fosfomycin and caspofungin due to high body temperature. On April 06, the patient was moved out of ICU due to improved conditions. On April 27, 2016, the patient experienced severe diarrhea and breathing problem and was transferred back to ICU. On April 29, 2016, the patient showed signs of abdominal, pulmonary and bloodstream infections. *K. pneumoniae*, *P. aeruginosa* and *Stenotrophomonas maltophilia* were isolated from sputum; *K. pneumoniae* and *A. baumannii* were isolated from blood sample. *K. pneumoniae* was also recoverable from stool and urine culture for several days. The patient was then treated with imipenem, meropenem, tigecycline and cefoperazone/sulbactam. Pneumonia worsened in the following days with chest CT scan showing increased lung markings and patchy lesions on both lungs (Figure 3.2, 2A and 2B). Abdominal infection, liver abscess, multi organ failure and severe sepsis were subsequently observed and *K. pneumoniae* was shown to be persistent until the patient died on May 10, 2016.

Patients 3, a 65-year-old man, was admitted to the hospital on April 03, 2016, after a car accident. He was diagnosed with cervical spine fracture and cervical spinal cord injury with high paraplegia. He was admitted to ICU and given mechanical ventilation, which was removed on the morning of April 08, 2016 due to improvement in conditions. In the afternoon, the patient produced excessive sputum, which affected breathing. He was then put on mechanical ventilation again and given meropenem and clindamycin. On April 09, 2016, brain CT of the patient was normal, but excessive sputum persisted. Anti-infection

treatment with meropenem and clindamycin was continued. Carbapenem-resistant *K. pneumoniae* strains were recoverable from sputum sample on April 11, 2016, from sputum and blood culture on April 12, and from fecal samples on April 13, 2016. The chest X-ray demonstrated the presence of patchy opaque areas in both lungs, the borders of which were also unclear (Figure 3.2, 3A ~5D). The patient died of septic shock and multiple organ failure on April 13, 2016.

Patient 4, a 73-year-old man, was admitted to the hospital on April 10th, 2016, with a diagnosis of cerebral infarction, hypertension and diabetes. He received mechanical ventilation and other treatments including antibiotic imipenem / cilastatin sodium. On April 27, 2016, the patient received tracheotomy, followed by anti-infection treatments using imipenem/cilastatin and cefoperazone/sulbactam, which was effective in controlling lung infection. From April 20 till July 06, 2016, however, signs of lung infection continued and multiple pathogens were isolated from different specimens. On April 29, 2016, carbapenem-resistant *A. baumannii* was detectable in blood culture; the patient was then treated with polymyxin E. On May 07, 2016, *P. aeruginosa* and *A. baumannii* were detected from blood culture, prompting treatment with the same antibiotic regimen. On May 06~09, 2016, *Candida glabrata*, *P. aeruginosa* and *Enterococcus faecium* were recovered from blood culture, and polymyxin B, voriconazole and vancomycin were administered. On June 20, 2016, *C. glabrata* and *Staphylococcus aureus* were detectable, treatment with vancomycin and voriconazole continued. *P. aeruginosa* was isolated from sputum samples for several times during this period, when the lung infections could be regarded as barely controllable, with only slight sign of pleural effusion. On July 06, 2016, however, 6 carbapenem-resistant *K. pneumoniae* strains were isolated from the sputum, blood, urine and fecal samples of the patient simultaneously. *P. aeruginosa* and fungi were also detectable in his blood sample. The patient was then treated with imipenem, cefoperazone/sulbactam, amikacin, caspofungin, tigecycline and linezolid successively, which turned out to be ineffective. Infection indexes of the patient were above normal, and the body temperature reached 39.4^{o} C, with large amount of fluid detectable in the chest. On July 27, carbapenem-resistant *K. pneumoniae* continued to be recovered in the blood and sputum cultures. The patient was then treated with meropenem and ceftriaxone, which led to the improvement of lung infection and

reduction of chest fluid. On August 04, 2016, carbapenem-resistant *K. pneumoniae* was isolated from blood and sputum culture and chest CT scan depicted reduced transparency and patchy shadows in both lungs, inferring occurrence of bilaterally pleural effusion. The trachea and bronchus swelled significantly and turned into a cobblestone-like structure (Figure 3.2, 4A and 4B). The patient was then treated with fosfomycin, meropenem and cefoperazone/sulbactam. The lung infection worsened, followed by multi-organ failure. The patient died on August 30, 2016.

Patient 5, a 53-year-old woman, was admitted into the hospital on April 17th, 2016 due to high paraplegia caused by atlantoaxial subluxation. The patient underwent cervical vertebra surgery and was admitted to ICU. She received mechanical ventilation due to short of breath. On April 24, 2016, pneumonia was observed, along with signs of pleural effusion and incomplete expansion of lower lung. On April 26, 2016, carbapenem-resistant *K. pneumoniae* was isolated from the fiberoptic bronchoscope suction tube samples and sputum. The patient was then treated with multiple antibiotics including biapenem, meropenem, amikacin, fosfomycin, tigecycline, voriconazole and linezolid. However, the patient did not respond to antibiotic treatment. Infection 7 indexes of the patient including white blood cell (WBC) count, procalcitonin (PCT), C-reactive protein (CRP) were above the normal values. Chest CT scan showed extensive, patchy, high density shadows in both lungs with obscure boundaries, bilaterally pleural effusion, and atelectasis of bilaterally inferior lobes (Figure 3.2, 5A and 5B). On May 05, 2016, the lung infection and pleural effusion worsened, along with development of a high temperature of 39.8 ^{o}C. On May 8th, the patient exhibited signs of lung infection and respiratory failure and died on the same day. All five patients stayed in different wards in the ICU, while they all showed overlapped stay in the same ICU.

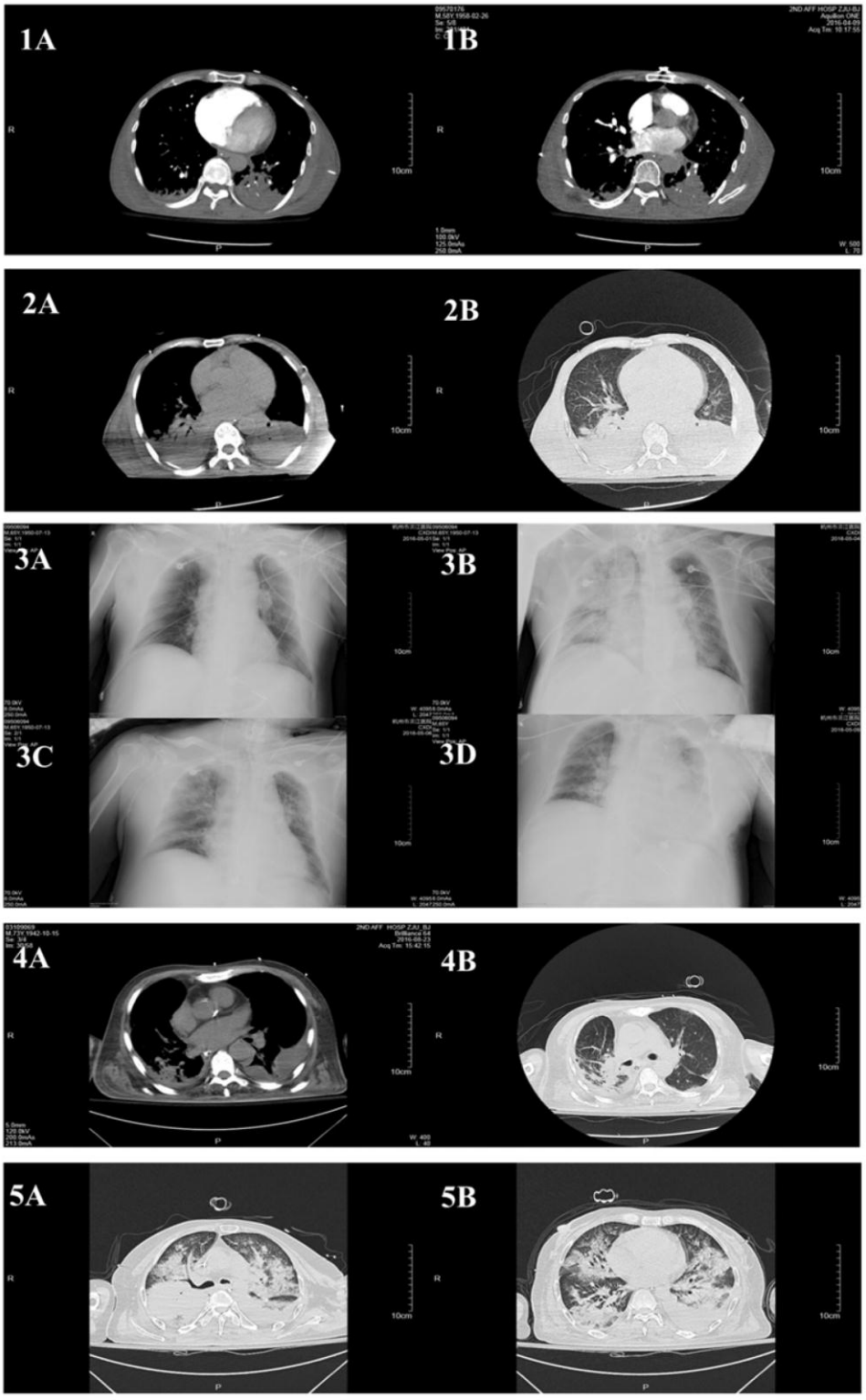


Figure 3.2 Chest scan images of five patients.

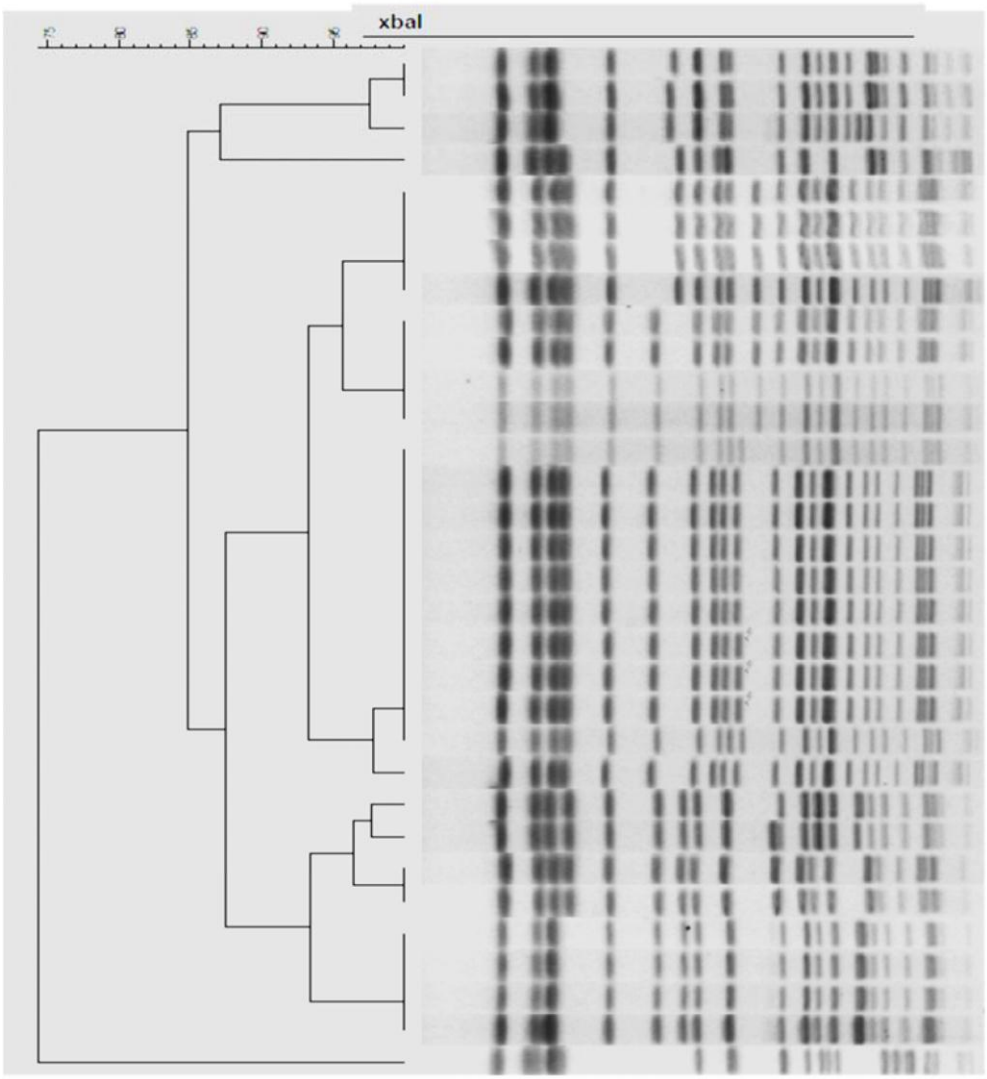
Chest scan images of five patients. Photos 1~2 A, B and 4~5 A, B are chest computed tomography (CT) scans of Patient 1, 2, 4 and 5 respectively; Photos 3A through 3D

depicted the dynamic changes in chest X-rays of Patient 3. All imageological examinations indicated pulmonary infections in these five patients. 1A, 1B) CTPA image of 1mm thickness showing segmental consolidation located in the apical and posterior basal segment of bilateral lower lobe, with the artery branches passing through the shadow, displaying evidence of partial air bronchograms and signs of pleural effusion; 2A, 2B) CT axial image of lung window of 1.5mm thickness depicting consolidation of bilateral lower lobes and discontinued air bronchograms. Both upper lobes exhibited signs of interlobular septal thickening and small amount of pleural effusion. These signs indicated interstitial pulmonary edema; 3A~3D) Chest X-ray plain films showing patchy consolidation of bilateral lung, which appeared and disappeared quickly within nine days. The cardiac shadow was enlarged with both hilar shadows being blurred and dilated. These signs indicated pulmonary edema due to infections; 4A, 4B) CT axial images of lung window of 5mm thickness showing dense opacity and fibrosis of clear margin located in the apical segment of the right lower lobe, broken air bronchograms, major fissure thickening and adhesion, shrinkage of lower lobe, and encapsulated effusion in both pleural cavity; 5A) CT axial image of lung window of 1.5mm thickness showing well-defined heterogeneous “bat-wing” pattern consolidation composed of ground glass opacity, with reticular shadow in bilateral lung fields and pleural effusion in both sides (heavier in the left pleural cavity), indicating alveolar edema and cardiac dysfunction; 5B) Patchy opacity with blurred margin located in the posterior segment of bilateral upper lobe; discontinued air bronchograms were seen within the area of consolidation, which might indicate aspiration pneumonia.

Table 3.2 Clinical record of patients of the five outbreak cases.

Patient ID	**Gender**	**Age (years)**	**Diseases for hospitalization**	**Admission Date**	**First CRKP isolation**	**Date of death**	**Chemotherapy**	**Outcome**
Patient 1	Male	58	Multiple trauma	28/02/16	29/03/16	10/05/16	TZP, TIG, C/S, AMI, PXB	Died
Patient 2	Male	62	Liver and spleen rupture	28/03/16	29/04/16	10/05/16	I/C, TEC, TIG, FOS, CAS, C/S	Died
Patient 3	Male	65	Spinal cord injury	03/04/16	11/04/16	13/04/26	MRP, CLI	Died
Patient 4	Male	73	Cerebral infarction	10/04/16	06/07/16	30/08/16	I/C, C/S, CAS, AMI, PXB, CLI, VRC, TEC, VAN, LEV, CRO, MRP, FOS	Died
Patient 5	Female	53	High paraplegia	17/04/16	26/04/16	08/05/16	MXF, BIA, MRP, AMI, FOS, TIG, VRC, LZD	Died

CRKP, carbapenem-resistant *K. pneumoniae*; TZP, Piperacillin/tazobactam; TIG, tigecycline; C/S, cefoperazone / sulbactam; AMI, amikacin; PXB, polymyxin B; CAS, caspofungin; I/C, Imipenem / cilastatin; TEC, teicoplanin; FOS, fosfomycin; MRP, meropenem; CLI, clindamycin; VRC, voriconazole; VAN, vancomycin; LEV, levofloxacin; CRO, ceftriaxone; MXF, Moxifloxacin; BIA, biapenem; LZD, linezolid.



Strain ID	ST type	Date of Isolation	Site of Isolation
SH1	11	2015	
SH16	11	2015	
SH9	11	2015	
SH12	11	2015	
K. pneumoniae 1	11	10/05/16	Blood
K. pneumoniae 1.1	11	29/03/16	Blood
K. pneumoniae 1.2	11	26/04/16	Blood
K. pneumoniae 1.3	11	30/04/16	Stool
K. pneumoniae 4	11	04/08/16	Blood
K. pneumoniae 4.1	11	06/07/16	Blood
K. pneumoniae 4.2	11	06/07/16	Sputum
K. pneumoniae 4.3	11	27/07/16	Blood
K. pneumoniae 3	11	12/04/16	Blood
K. pneumoniae 3.1	11	11/04/16	Sputum
K. pneumoniae 3.2	11	12/04/16	Sputum
K. pneumoniae 3.3	11	13/04/16	feces
K. pneumoniae 5	11	05/05/16	Sputum
K. pneumoniae 5.1	11	26/04/16	Secretion
K. pneumoniae 5.2	11	26/04/16	Sputum
K. pneumoniae 5.3	11	05/05/16	Secretion
K. pneumoniae 2	11	09/05/16	Blood
K. pneumoniae 2.1	11	29/04/16	Blood
K. pneumoniae 2.2	11	29/04/16	Sputum
SH2	11	2015	
SH3	11	2015	
K. pneumoniae 4.4	11	04/08/16	Sputum
K. pneumoniae 4.5	11	06/07/16	Feces
HA2-23	11	2015	
HA2-26	11	2015	
HA2-36	11	2015	
HA2-38	11	2015	
HB25-1	11	2015	

Figure 3.3 PFGE analysis of ST11 CRKP strains.

PFGE patterns of 21 ST11 CRKP outbreak strains, *K. pneumoniae* 1~1.3, 2~2.2, 3~3.3, 4~4.5 and 5~5.3, and 11 clinical ST11 CRKP strains carrying virulence plasmid. Strains *K. pneumoniae* 1~1.3 were isolated from patient 1; strains *K. pneumoniae* 2~2.2 were isolated from patient 2; strains *K. pneumoniae* 3~3.3 were isolated from patient 3; strains *K. pneumoniae* 4~4.5 were isolated from patient 4; strains *K. pneumoniae* 5~5.3 were isolated from patient 5. Other strains were isolated from various parts of China, the detailed information of which is provided in Table 3.6. **Interpretation of data** Four different PFGE patterns were observed among the 21 CRKP strains isolated from 5 patients. CRKP strains from the same patient exhibited identical PFGE profile except for strains recovered from patient 4, among which 4 strains were found to belong to one pattern, whereas the other two belonged to another pattern. Strains from patients 2, 3 and 5 exhibited identical PFGE pattern. PFGE analysis of 11 ST11 CRKP strains carrying virulence plasmids obtained from various regions of China revealed that strains collected from the same region were genetically close to each other, whereas those collected from different regions exhibited a higher degree of genetic diversity. Both clonal and non-clonal spread were observed among ST11 strains collected from a hospital in Shanghai (SH). Four strains, HA2-23 to HA2-38, were isolated from different patients within a short period of time in a hospital in Henan province, suggesting that an outbreak caused by this strain had occurred. Strain HB25-1, isolated from a hospital in Hubei province, was genetically distinct from other ST11 CRKP strains. Interestingly, strains *K. pneumoniae* 4.4 and 4.5 isolated from patient 4 exhibited similar PFGE pattern to ST11 CRKP strains collected in Shanghai, namely SH2 and SH3, suggesting that this type of ST11 CRKP strains, which harbored the virulence plasmid, was transmissible in this area as both Hangzhou and Shanghai are geographically close to each other, with distance of about 100 miles.

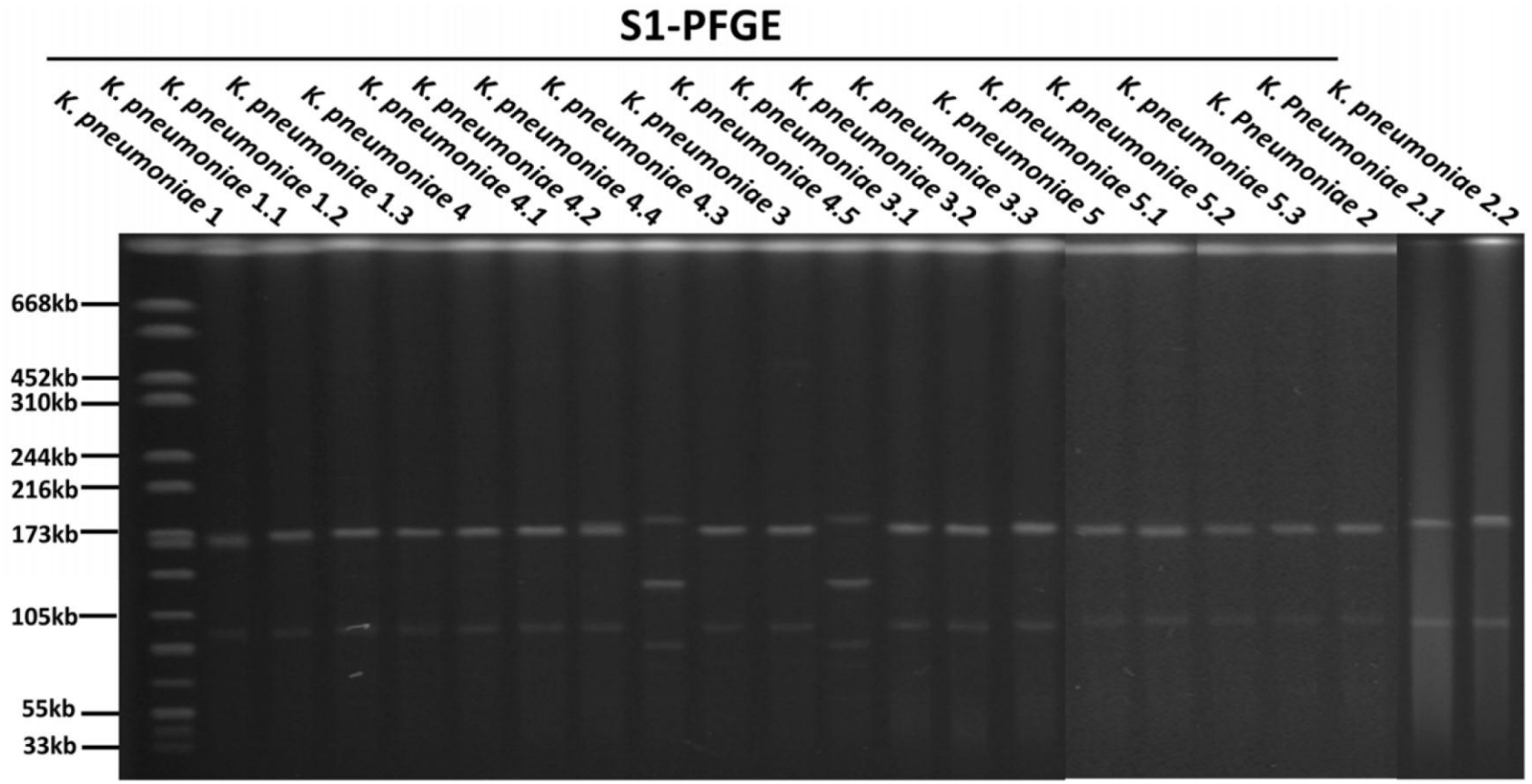


Figure 3.4 S1-PFGE profile of 21 CRKP strains isolated from 5 patients.

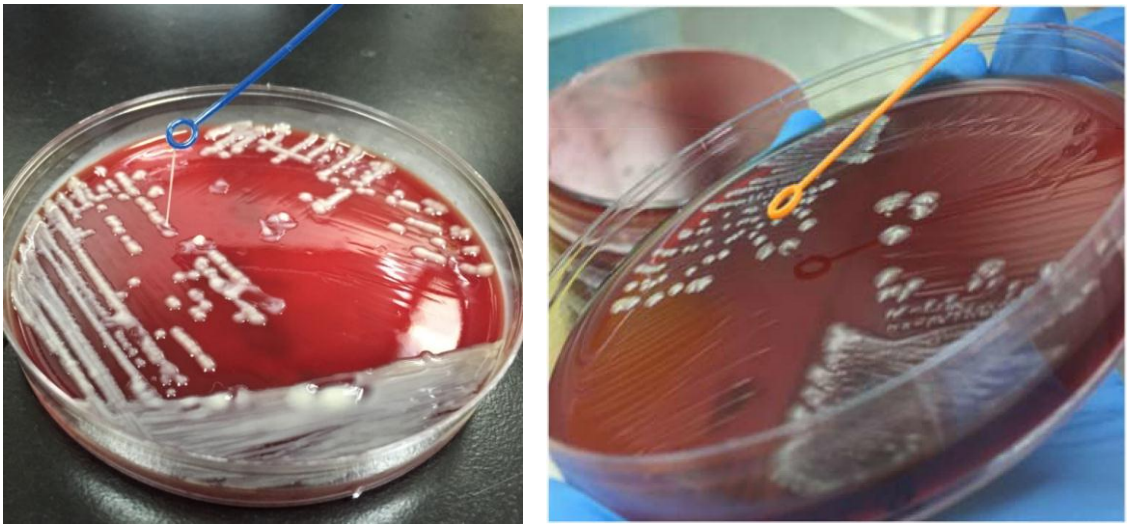
Nineteen CRKP strains exhibited identical plasmid profile except for two strains, *K. pneumoniae* 4.4 and *K. pneumoniae* 4.5, which were recovered from patient 4 and exhibited different plasmid profiles from other strains. The distinct plasmid profiles of these two strains was consistent with their distinct PFGE profiles which differed markedly from those of other strains, suggesting that these two strains might have originated from a different clone.

3.4.2 Strain source，antimicrobial resistance and virulence potential

21 non-repeated carbapenem-resistant *K. pneumoniae* strains were recovered from various clinical specimens of the five patients. These strains had almost identical antibacterial susceptibility profiles, belonged to ST11 and shared highly similar PFGE patterns (Figure 3.3, Figure 3.4). Strains recovered from the same patient had identical PFGE profiles, except for patient 4, who had four strains that belonged to the major PFGE pattern and two strains (*K. pneumoniae* 4.4 and 4.5) with different PFGE patterns and plasmid profiles, suggesting that patient 4 was simultaneously infected by two different clones of carbapenem-resistant *K. pneumoniae* (Figure 3.3). We selected one representative carbapenem-resistant *K. pneumoniae* strain from each patient (*K. pneumoniae* 1–5; for patient 4, we selected the strain isolated from blood samples) for further genetic and phenotypic characterisation (Table 3.3). The corresponding transconjugants are termed C1–C5, respectively. The carbapenem and cephalosporin resistance phenotypes of these five strains could be transferred to the *E. coli* strain EC600. All five isolates carried the $bla_{\text{KPC-2}}$, $bla_{\text{CTX-M-65}}$, and $bla_{\text{TEM-1}}$ genes, which were located in conjugative plasmids (Table 3.3). The MLST using the draft genome data showed that these five strains, *K. pneumoniae* 1–5, belonged to ST11 and serotype K47 (*wzi* 209). Pairwise SNP analysis for these five strains based on their raw sequencing reads showed that their core genome differed only by a few SNPs ($n \leq 4$), suggesting that these strains originated from a single clone (Table 3.4).

Compared with other ST11 carbapenem-resistant *K. pneumoniae* infections, the carbapenem-resistant *K. pneumoniae* strains in this outbreak caused much more severe pneumonia and higher mortality [138]. We speculated that these ST11 carbapenem-resistant *K. pneumoniae* strains might be more virulent than previously reported ST11 carbapenem-resistant *K. pneumoniae* strains, which prompted us to investigate the virulence potential of these isolates. The string test was positive for all five ST11 carbapenem-resistant *K. pneumoniae* outbreak strains, which each produced string longer than 20 mm (Figure 3.5). We used a human neutrophil assay to test the virulence potential of two representative outbreak strains, *K. pneumoniae* 4 and 5, two classic ST11 strains, FJ8 and FJ9, which produced a negative string test result, and two K1

hypervirulent *K. pneumoniae* strains, 1088 and 91, which were reported previously [39]. The *K. pneumoniae* 4 and 5 strains had survival of about 80% after incubation with the human neutrophils for 60 min, which was slightly higher than that of the K1 hypervirulent *K. pneumoniae* strains 1088 and 91 (Figure 3.6). However, the survival of strains FJ8 and FJ9, at 25% after 15 min, 10% after 30 min, and 3% after 60 min incubation respectively, was significantly lower than that of *K. pneumoniae* 4 and 5 (p<0.0001 by one-way ANOVA; Figure 3.6).



(**A**) (**B**)

Figure 3.5 String tests on an ST11 CRKP strain (*K. pneumoniae* 4, **A**) and the corresponding plasmid curing mutant (**B**).

All 5 ST11 CRKP strains, *K. pneumoniae* 1~5, exhibited similar hypermucoviscous phenotype. The plasmid curing mutant of *K. pneumoniae* 4, PC-*K. pneumoniae* 4, was also shown. The data showed that curing of the virulence plasmid from ST11 CRKP strains, *K. pneumoniae* 4, resulted in loss of the hypermucoviscous phenotype.

Table 3.3 MLST type and antibiotic resistance characteristics of *K. pneumoniae* outbreak strains and their corresponding transconjugants.

	Source of isolate	MLST	Antimicrobial resistance genes present	Minimum inhibitory concentration (μg/mL)												
				Imipenem	Ertapenem	Cefepime	Ceftriaxone	Cefazolin	Aztreonam	Amoxicillin plus clavulanic acid	Amikacin	Ciprofloxacin	Gentamicin	Tobramycin	Tigecycline	Piperacillin plus tazobactam
*K. pneumoniae*1	Patient 1	ST11	Yes	>16	>8	>64	>64	>64	>64	>32	>64	>4	>16	>16	0·5	>128
*K. pneumoniae*2	Patient 2	ST11	Yes	>16	>8	>64	>64	>64	>64	>32	>64	>4	>16	>16	0·5	>128
*K. pneumoniae*3	Patient 3	ST11	Yes	>16	>8	>64	>64	>64	>64	>32	>64	>4	>16	>16	1	>128
*K. pneumoniae*4	Patient 4	ST11	Yes	>16	>8	>64	>64	>64	>64	>32	>64	>4	>16	>16	0·5	>128
*K. pneumoniae*5	Patient 5	ST11	Yes	>16	>8	>64	>64	>64	>64	>32	>64	>4	>16	>16	0·5	>128
Transconjugant C1	..	..	Yes	>16	>8	32	>64	>64	>64	>32	>64	≤0·25	>16	>16	≤0·5	>128
Transconjugant C2	..	..	Yes	>16	>8	32	>64	>64	>64	>32	>64	≤0·25	>16	>16	≤0·5	>128
Transconjugant C3	..	..	Yes	>16	>8	32	>64	>64	>64	>32	>64	≤0·25	>16	>16	≤0·5	>128
Transconjugant C4	..	..	Yes	>16	>8	32	>64	>64	>64	>32	>64	≤0·25	>16	>16	≤0·5	>128
Transconjugant C5	..	..	Yes	>16	>8	32	>64	>64	>64	>32	>64	≤0·25	>16	>16	≤0·5	>128
EC600	..	..	No	≤1	≤0·5	≤1	≤1	≤4	≤1	≤2	≤2	≤0·25	≤1	≤1	≤0·5	2

Antimicrobial genes were deemed to be present if the isolate had three antimicrobial resistance genes, including $bla_{\text{KPC-2}}$, $bla_{\text{CTX-M-65}}$ and $bla_{\text{TEM-1}}$. MLST=multilocus sequence type. EC600=*Escherichia coli* 600.

Table 3.4 Pairwise SNP comparison between ST11 CRKP strains in this study using the complete chromosome sequence of HS11286 as a reference. The numbers depict differences in SNPs exhibited by each strain pair.

	K. pneumoniae 5	*K. pneumoniae* 4	*K. pneumoniae* 2	*K. pneumoniae* 1	*K. pneumoniae* 3	HS11286	JM45	FJ8	FJ10
K. pneumoniae 5	0	2	3	3	3	1232	1290	39	41
K. pneumoniae 4	2	0	3	1	1	1232	1290	39	41
K. pneumoniae 2	3	3	0	4	4	1233	1291	40	42
K. pneumoniae 1	3	1	4	0	2	1233	1291	40	42
K. pneumoniae 3	3	1	4	2	0	1233	1291	40	42
HS11286	1232	1232	1233	1233	1233	0	1468	1247	1249
JM45	1290	1290	1291	1291	1291	1468	0	1305	1307
FJ8	39	39	40	40	40	1247	1305	0	2
FJ10	41	41	42	42	42	1249	1307	2	0

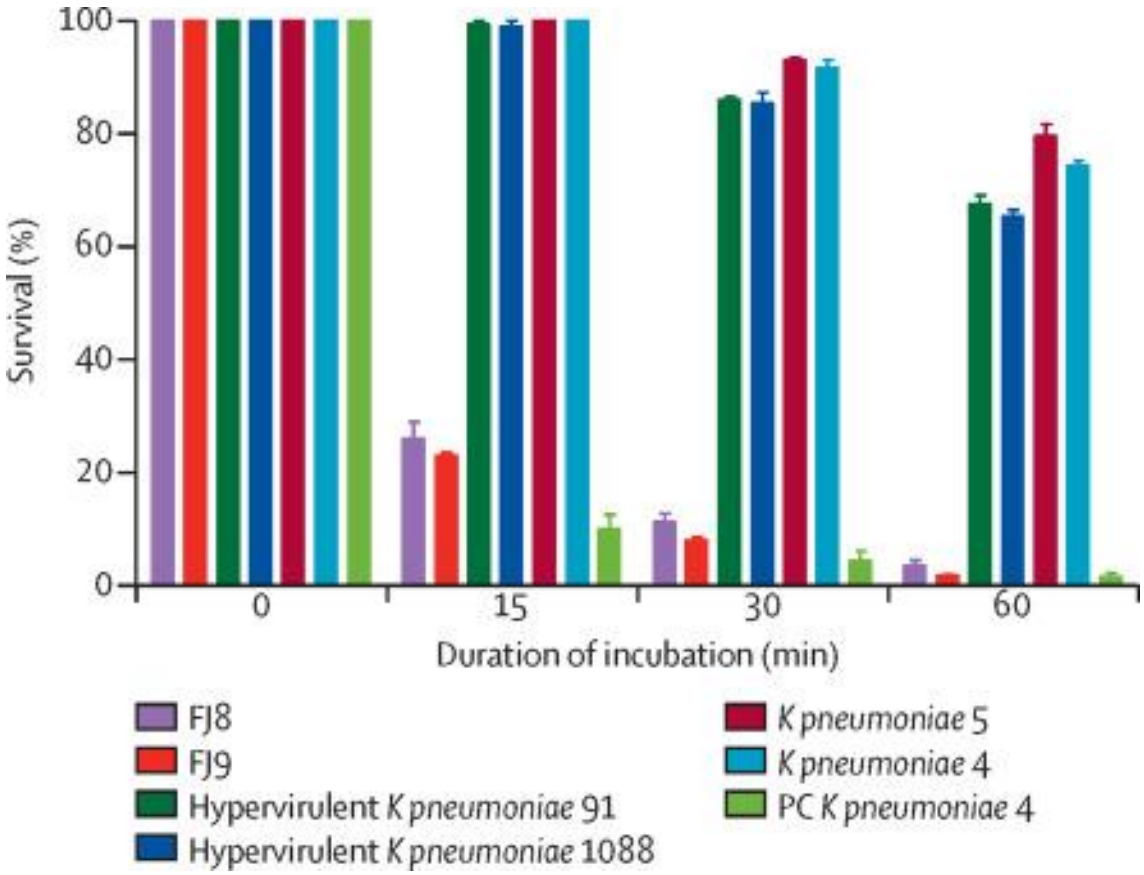


Figure 3.6 Human neutrophil assays of *Klebsiella pneumoniae* strains.

FJ8 and FJ9 are classic ST11 *K. pneumoniae* strains that did not harbour the virulence plasmid. Hypervirulent *K. pneumoniae* 1088 and 91 are two ST23 K1 hypervirulent *K. pneumoniae* strains reported in a previous study [39]. *K. pneumoniae* 4 and 5 are two ST11 hypervirulent *K. pneumoniae* strains tested in this study. Strain PC *K. pneumoniae* 4 is a mutant of strain *K. pneumoniae* 4, in which the virulence plasmid has been removed in plasmid curing experiments.

We infected *G. mellonella* larvae with selected *K. pneumoniae* strains: with an inoculum of 1×10^6 CFU, the classic ST11 strain FJ8 survival was 80% at 48 h after infection; survival was 0% with the K1 hypervirulent *K. pneumoniae* strain 1088 at 48 h and 40% with the K1 hypervirulent *K. pneumoniae* strain 91 at 36 h post-infection. The ST11 carbapenem-resistant *K. pneumoniae* outbreak strains *K. pneumoniae* 1, 3, 4, and 5 resulted in 0% survival by 12 h; 0% survival was reached with *K. pneumoniae* 2 after 24 h, suggesting that strains *K. pneumoniae* 1–5 were more virulent than the K1

hypervirulent *K. pneumoniae* strains 1088 and 99, as well as the classic ST11 *K. pneumoniae* strains, FJ8 and FJ9 (Figure 3.7). Data on the effects of the other inoculums of these strains are available in Figure 3.8. The consistency between the clinical data and the results of the phenotypic assays supported the notion that the ST11 carbapenem-resistant *K. pneumoniae* strains, *K. pneumoniae* 1–5, were ST11 carbapenem-resistant hypervirulent *K. pneumoniae*.

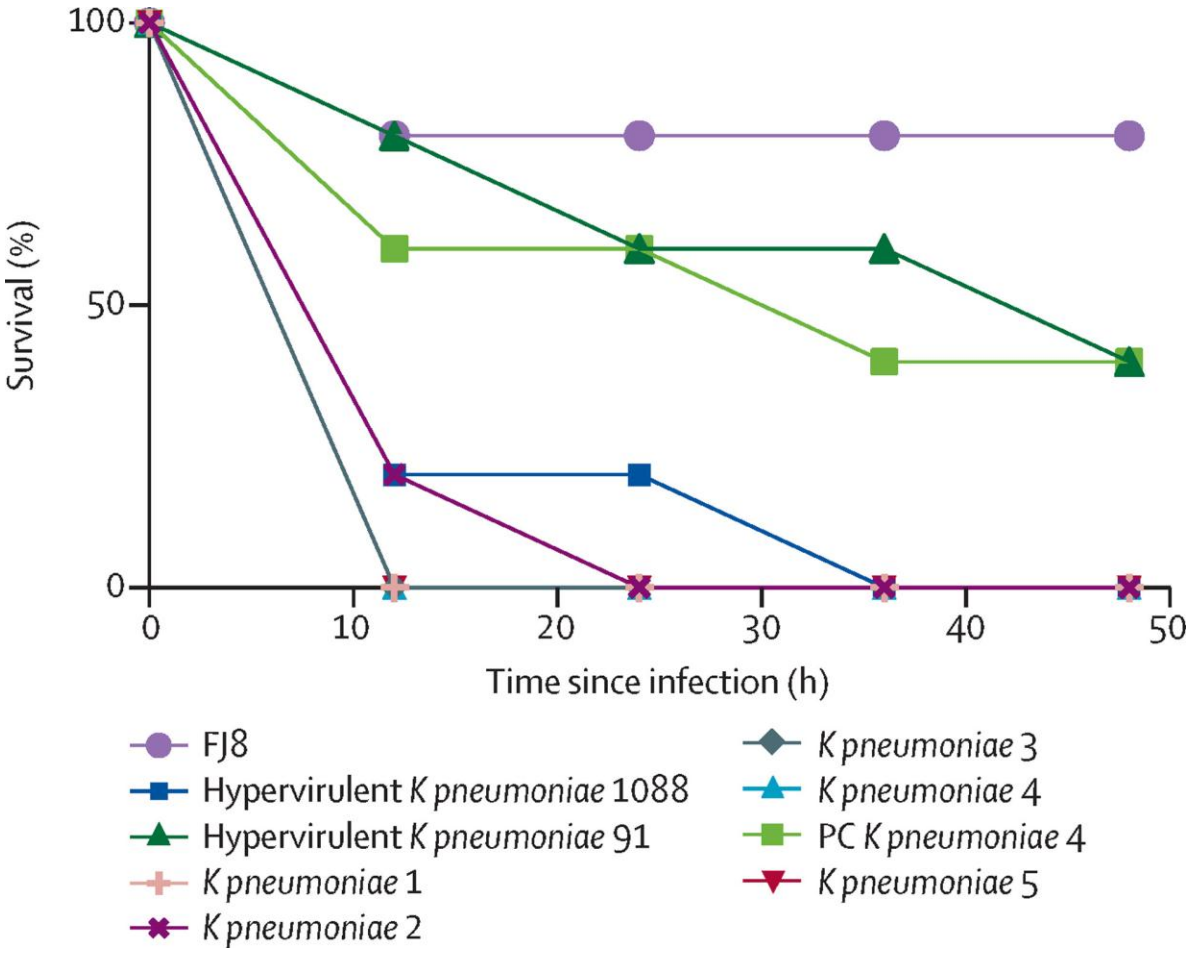


Figure 3.7 Virulence potential of *Klebsiella pneumoniae* strains in a *Galleria mellonella* infection model. The effect of 1×10^6 colony-forming units of each *K. pneumoniae* strain on survival was assessed in *G. mellonella*. The results for other doses of each *K. pneumoniae* strain are shown in the Figure 3.8. FJ8 is a classic ST11 *K. pneumoniae* strain that did not harbor virulence plasmid. Hypervirulent *K. pneumoniae* 1088 and 91 are two ST23 K1 hypervirulent *K. pneumoniae* strains reported in a previous study [39]. *K. pneumoniae* 1–5 are two ST11 hypervirulent *K. pneumoniae* strains tested in this study. Strain PC *K. pneumoniae* 4 is a mutant of strain *K. pneumoniae* 4, in which the virulence plasmid has been removed in plasmid curing experiments.

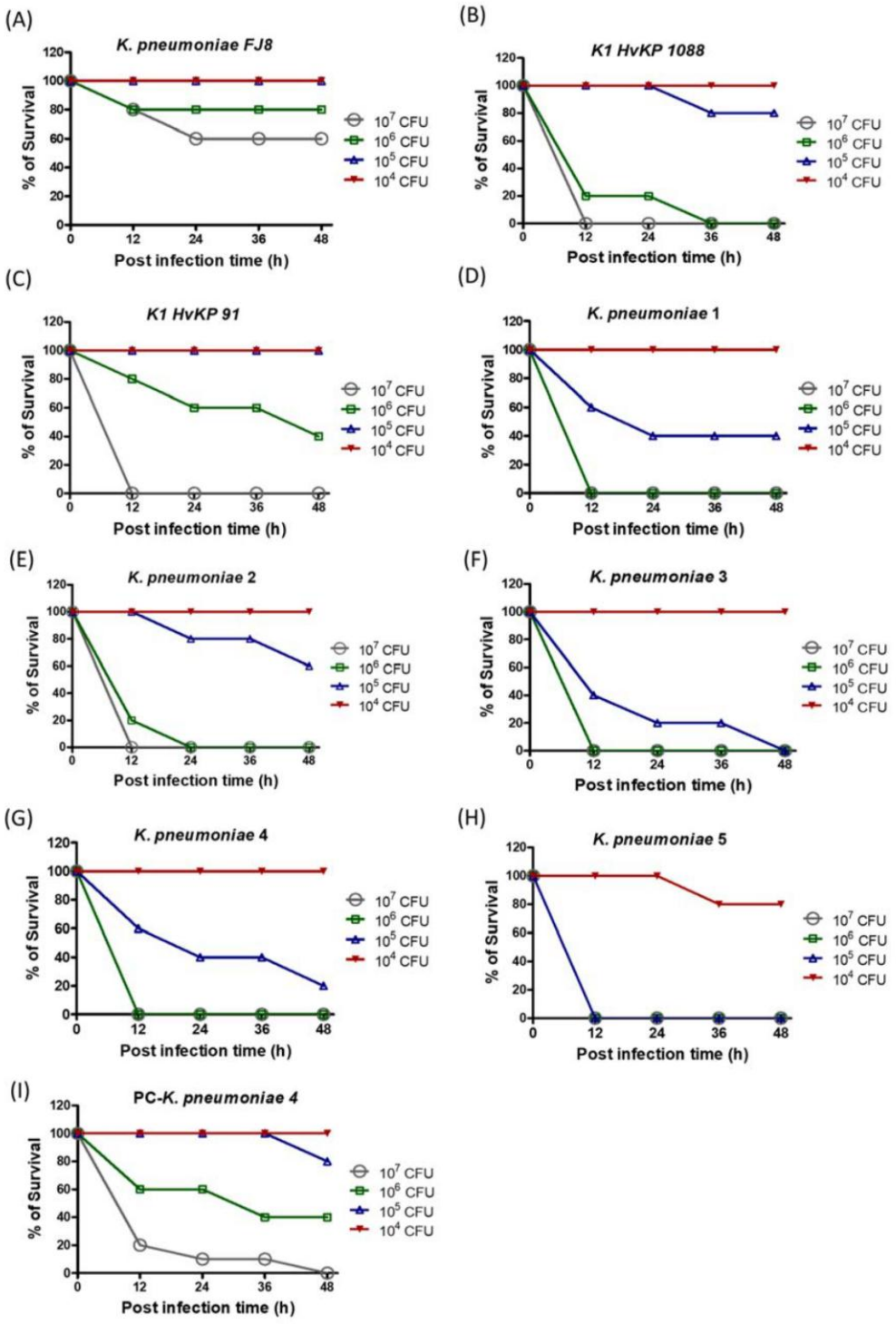


Figure 3.8 Assessment of virulence potential of various *K. pneumoniae* strains in *G. mellonella* infection model.

(A) virulence assay of clinical ST11 CRKP strains, FJ8 and FJ9, which exhibited similar results, hence only the result for FJ8 was shown; (B, C) virulence assays of two ST23 K1 hypervirulent *K.*

pneumoniae strains, 1088 and 91; (E~H) virulence assays of the ST11 CRKP outbreak strains, *K. pneumoniae* 4 and *K. pneumoniae* 5.; (I) virulence assays of plasmid curing strain of *K. pneumoniae* 4, PC-*K. pneumoniae* 4. Classic ST11 strain, BJ9, produced very low mortality rate in *G. mellonella*, leading to about 60% survival of *G. mellonella* at 48 h after being challenged with 10^7 CFU of bacteria. K1 HvKP strains, 1088 and 91 were shown to be much more virulent than classic ST11 stains, resulting in 20% *G. mellonella* survival at 12 h and 40% survival at 48 h respectively after challenge with 106 CFU of bacteria. The five ST11 CRKP strains exhibited even higher virulence potential than two K1 HvKP strains, 1088 and 91. Strain *K. pneumoniae* 1 displayed the highest virulence level, with 0% survival in *G. mellonella* at 12 h when challenged with 10^5 CFU of bacteria. Upon curing of the virulence plasmid, the plasmid-cured mutant of *K. pneumoniae* 4, PC-*K. pneumoniae* 4, exhibited a dramatically reduced virulence level compared to the parental strain, *K. pneumoniae* 4.

3.4.3 Genomic analysis of the *K. pneumoniae* strains

Pangenome analysis of nine ST11 strains (SH-1, *K. pneumoniae* 1–5, FJ8, HS11268, and JM45) identified a unique set of genes in the five ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains, which aligned well with the virulence plasmid pLVPK (AY378100), a 219,385 bp IncHI1B/IncFIB plasmid recovered from *K. pneumoniae* strain CG43 (Figure 3.9). In the virulence gene analysis, we found that all ST11 strains tested, except for HS11286, had type 3 fimbriae (*mrkABCDF*) and type 1 fimbrial adhesion genes (*fimA–H*), as well as the *iroE*, *iutA*, *kpn*, and *ycfM* genes, which encode various virulence factors, suggesting that these virulence genes might be typical of ST11 *K. pneumoniae* (Figure 3.10, Table 3.5). The K1 hypervirulent *K. pneumoniae* strains NTUH-K2044 and 1088 lacked these virulence genes typical of ST11 strains, but harboured a different set of virulence genes including *iroBCDN*, *iucABCD*, *rmpA* and *rmpA2*, and *irp1* and *irp2*, which were located in the virulence plasmid pLVPK (Figure 3.10, Table 3.5) [34]. In addition to harbouring the typical virulence genes for ST11 strains, the five outbreak strains also contained the *iucABCD*, *rmpA2*, and *iutA* genes. This finding was consistent with the results of the pangenome analysis in that virulence plasmid-related genes were unique to ST11 carbapenem-resistant hypervirulent *K. pneumoniae*, but not the classic ST11 carbapenem-resistant *K. pneumoniae* strains (Figure 3.9, Figure 3.10).

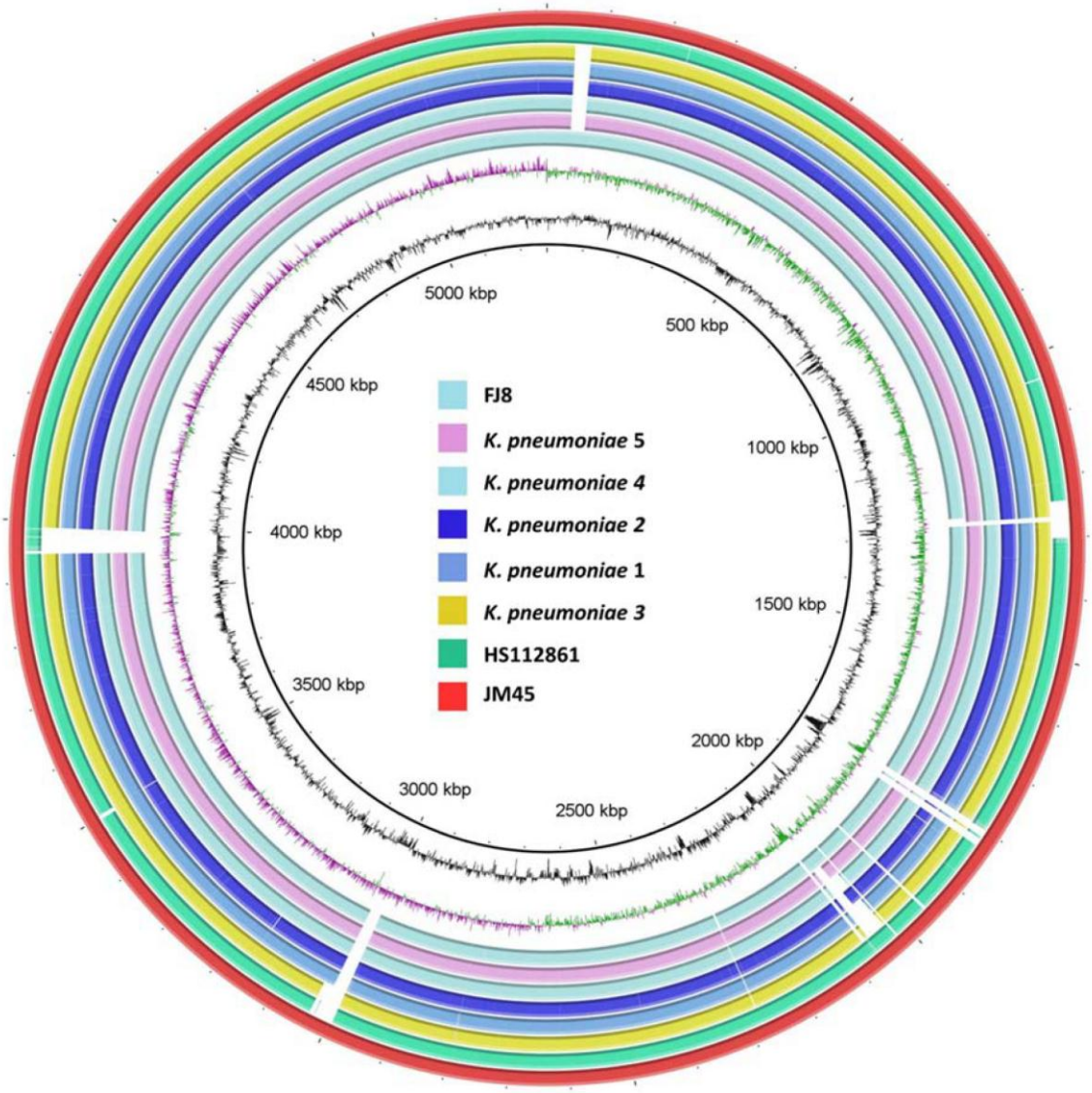


Figure 3.9 Pangenome analysis of 9 ST11 CRKP strains.

Comparative chromosome maps of five ST11 CR-HvKP strains, two classic ST11 CRKP strains (only FJ8 was shown) and two publicly available CRKP strains (HS11286 and JM45) using JM45 as a reference. The circular map was generated using the BLAST Ring Image Generator (BRIG). All five ST11 CR-HvKP strains, *K. pneumoniae* 1~5, exhibited highly similar genome content, with several genome fragments missing when compared to JM45. However, pangenome analysis identified a set of genes unique to the five ST11 CR-HvKP strains, which aligned well with the virulence plasmid, pLVPK (AY378100), a 219,385 bp pLVPK (AY378100) recovered from *K. pneumoniae* strain CG43.

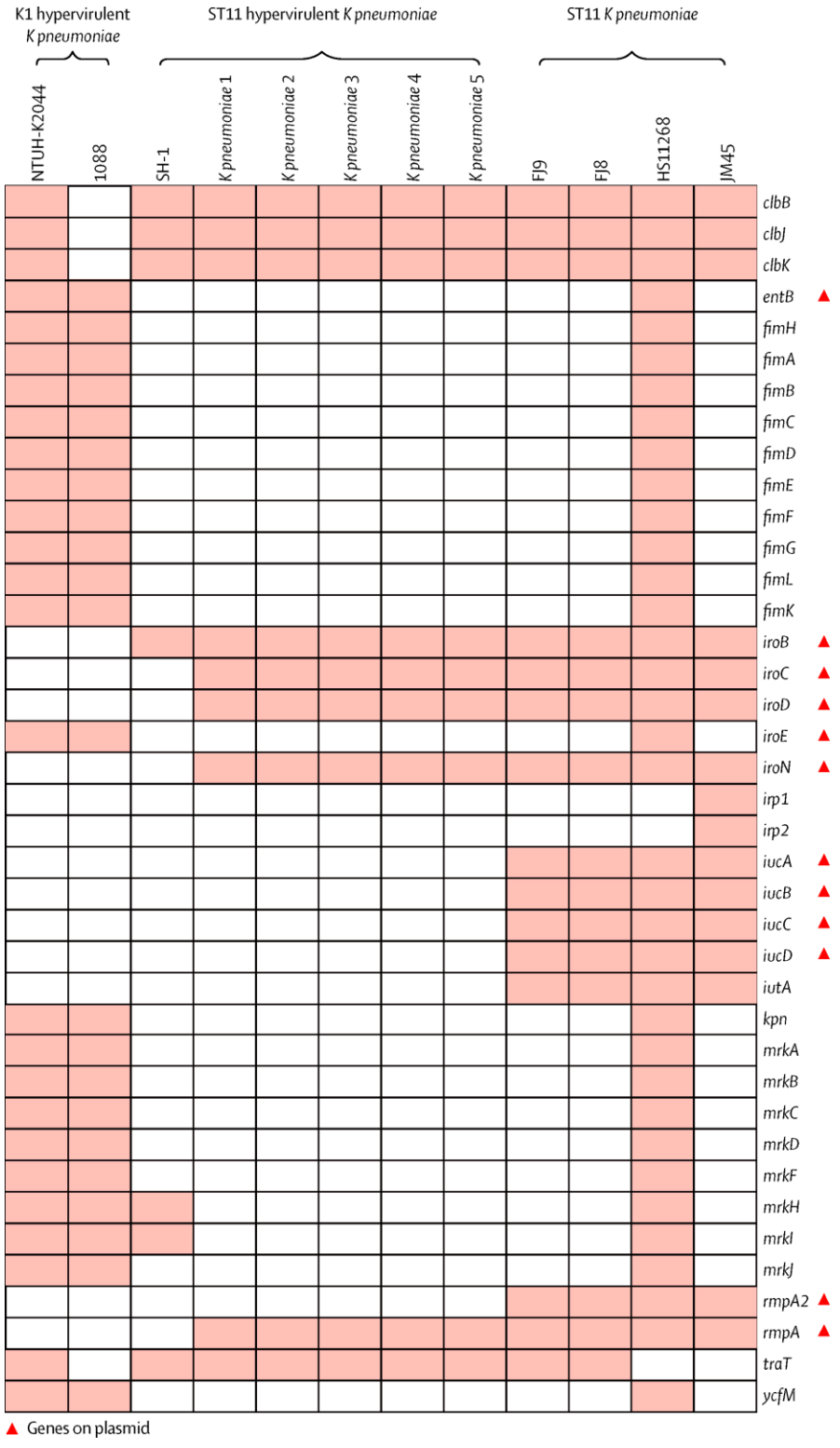


Figure 3.10 Virulence gene analysis of *Klebsiella pneumoniae* strains.

Heatmaps were generated by aligning the draft genome sequence of each strain to the virulence gene database. The presence of virulence genes in a specific genome is represented

by the white box and the absence of virulence genes is represented by a pink box. Red triangles show virulence genes located in a plasmid. FJ8 and FJ9 are classic ST11 *K. pneumoniae* strains that did not a harbour virulence plasmid. Hypervirulent *K. pneumoniae* 1088 is an ST23 K1 hypervirulent *K. pneumoniae* strain reported in a previous study [39]. *K. pneumoniae* 1–5 are ST11 hypervirulent *K. pneumoniae* strains tested in this study. NTUH-K2044 is a publicly available K1 hypervirulent *K. pneumoniae* strain; HS11268 and JM45 are two publicly available classic ST11 carbapenem-resistant *K. pneumoniae* strains; and SH-1 is a clinical carbapenem-resistant *K. pneumoniae* strain isolated from a hospital in Shanghai in 2015 that carried the virulence plasmid. *clbBJK*=colibactin synthesis loci. *entB*=enterobactin-related genes. *fimA–K=t*ype 1 fimbriae genes. *iroBCDEN*=iron acquisition system. *irp1*/2=yersiniabactin-related genes. *iucABCD*=aerobactin-related genes. *iutA*=ferric aerobactin receptor. *mrkA–J*=type 3 fimbriae genes. *rmpA/rmpA2*=cps transcriptional activator. *kpn*=FimH-like adhesion gene. *ycfM*=outer membrane lipoproteins. *traT*=outer membrane lipoprotein.

Table 3.5 Presence of homologues of virulence genes in classic ST11 *K. pneumoniae*, ST11 CR-HvKP and ST23 HvKP strains.

	NTUH-K2044	**1088**	**SH-1**	**CRHvKP1**	**CRHvKP2**	**CRHvKP3**	**CRHvKP4**	**CRHvKP5**	**FJ10**	**FJ8**	**HS11286**	**JM45**
clbB	0	100	0	0	0	0	0	0	0	0	0	0
clbJ	0	99.98	0	0	0	0	0	0	0	0	0	0
clbK	0	100	0	0	0	0	0	0	0	0	0	0
entB	0	0	82.44	82.44	82.44	82.44	82.44	82.44	82.44	82.44	0	82.44
fimA	0	0	91.07	91.07	91.07	91.07	91.07	91.07	91.07	91.07	0	91.07
fimB	0	0	91.89	91.89	91.89	91.89	91.89	91.89	91.89	91.89	0	91.89
fimC	0	0	94.33	94.33	94.33	94.33	94.33	94.33	94.33	94.33	0	94.33
fimD	0	0	94.23	94.23	94.23	94.23	94.23	94.23	94.23	94.23	0	94.27
fimE	0	0	89.6	89.6	89.6	89.6	89.6	89.6	89.6	89.6	0	89.6
fimF	0	0	93.19	93.19	93.19	93.19	93.19	93.19	93.19	93.19	0	93.19
fimG	0	0	97.41	97.41	97.41	97.41	97.41	97.41	97.41	97.41	0	97.41
FimH	0	0	95.81	95.81	95.81	95.81	95.81	95.81	95.81	95.81	0	95.81
fimI	0	0	91.48	91.48	91.48	91.48	91.48	91.48	91.48	91.48	0	91.48
fimK	0	0	92.43	92.43	92.43	92.43	92.43	92.43	92.43	92.43	0	92.43
iroB	100	100	0	0	0	0	0	0	0	0	0	0
iroC	100	99.97	100	0	0	0	0	0	0	0	0	0
iroD	100	100	100	0	0	0	0	0	0	0	0	0
iroE	0	0	99.36	99.36	99.36	99.36	99.36	99.36	99.36	99.36	0	99.36
iroN	100	100	100	0	0	0	0	0	0	0	0	0
irp1	100	100	100	99.99	99.99	99.99	99.99	99.99	100	100	100	0
irp2	100	100	100	100	100	100	100	100	100	100	100	0
iucA	100	99.94	100	100	100	100	100	100	0	0	0	0

iucB	100	100	100	100	100	100	100	100	0	0	0	0
iucC	100	100	100	100	100	100	100	100	0	0	0	0
iucD	100	100	100	100	100	100	100	100	0	0	0	0
iutA	100	100	100	100	100	100	100	100	99.5	99.5	0	99.5
kpn	0	0	99.82	99.82	99.82	99.82	99.82	99.82	99.82	99.82	0	99.82
mrkA	0	0	100	100	100	100	100	100	100	100	0	100
mrkB	0	0	100	100	100	100	100	100	100	100	0	100
mrkC	0	0	100	100	100	100	100	100	100	100	0	100
mrkD	0	0	100	100	100	100	100	100	100	100	0	100
mrkF	0	0	100	100	100	100	100	100	100	100	0	100
mrkH	0	0	0	100	100	100	100	100	100	100	0	100
mrkI	0	0	0	100	100	100	100	100	100	100	0	100
mrkJ	0	0	100	100	100	100	100	100	100	100	0	100
rmpA2	84.89	84.91	100	99.69	100	0	100	100	0	0	0	0
rmpA	0	0	100	0	0	0	0	0	0	0	0	0
traT	0	96.63	0	0	0	0	0	0	0	0	96.5	100
ycfM	0	0	99.85	99.85	99.85	99.81	99.85	99.85	99.85	99.85	0	99.85

*Number in the Table represents the percentage of homology of virulence gene in ST11 CR-HvKP strains and ST23 HvKP strains when compared to the reference virulence genes in the database. “0” means absence of this gene in the specific strain analyzed; “100” infers presence of identical gene in the specific strain.

Alignment of contigs showed that all five ST11 carbapenem-resistant hypervirulent *K. pneumoniae* outbreak strains carried a plasmid that aligned well to most parts of the pLVPK plasmid, including the region in which the *rmpA2*, *iucABCD*, and *iutA* genes were located, but not to a roughly 50 kbp region carrying the *rmpA* and *iroBCDN* (salmochelin) genes. Southern hybridisation confirmed the presence of a roughly 170 kbp virulence plasmid in all five strains (Figure 3.11). The complete sequence of the virulence plasmid from strain *K. pneumoniae* 4, pVir-CR-HvKP4 (178,154 kbp), aligned well to pLVPK (Figure 3.11). pVir-CR-HvKP4 is an IncHI1B/IncFIB-type plasmid with a length of 178,154 bp and an average GC content of 50.5%, which encodes 216 predicted open reading frames. It shares 99% identity with pRJF999 (GenBank CP014011), pK2044 (GenBank AP006726), pRJA166b (GenBank CP019049), pRJF293 (GenBank CP014009), and pLVPK (GenBank AY378100), with query coverages ranging from 90% to 97%. We also obtained the complete sequence of a $bla_{\text{KPC-2}}$-carrying plasmid from *K. pneumoniae* 4, pKPC-CR-HvKP4 (MF437312), and found it to be a similar size (177 585 bp) to pVir-CR-HvKP4 (MF437313, Figure 3.12). To test whether acquisition of the roughly 170 kbp virulence plasmid contributed to the hypervirulence phenotype of the ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains, we used the plasmid curing approach to remove the virulence plasmid pVir-CR-HvKP4 from *K. pneumoniae* 4, to produce the strain PC-*K. pneumoniae* 4 (Figure 3.13). Subsequently, we found that strain PC-*K. pneumoniae* 4 had a negative string test phenotype and substantially reduced survival in human neutrophils (Figure 3.6) and virulence in *G. mellonella* (Figure 3.7), supporting the notion that pVir-CR-HvKP4 contributed to the hypervirulence phenotype of *K. pneumoniae* 4 (Figure 3.8) and, by extension, the other newly emerged ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains.

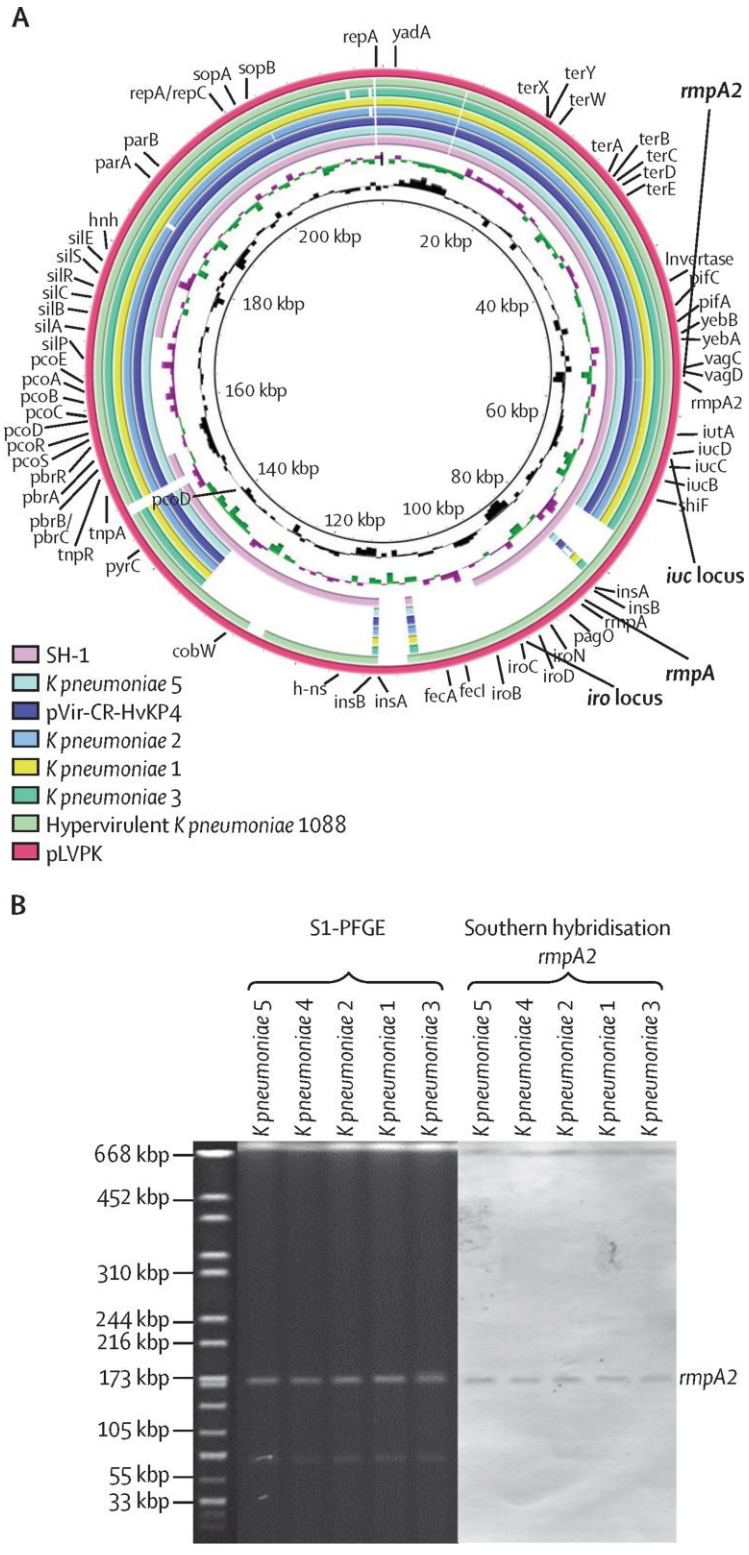


Figure 3.11 Gene map of virulence plasmid harboured by *K. pneumoniae* outbreak strains

(A) Alignment of the roughly 170 kbp plasmid recovered from the five ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains, a plasmid recovered from strain K1

hypervirulent *K. pneumoniae*1088, and a plasmid harboured by strain ST11 *K. pneumoniae* SH-1, against the known virulence plasmid pLVPK from the *K. pneumoniae* strain CG43 (AY378100). The circular map was generated with the BLAST Ring Image Generator. Plasmids, pLVPK and pVir-CR-HvKP4 (accession number MF437313) were mapped with complete sequences, whereas others were generated by aligning the draft genome sequences to pLVPK. Plasmid pVir-CR-HvKP4 is the virulence plasmid from ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strain *K. pneumoniae* 4. (B) S1-PFGE and Southern hybridisation of the marker gene of the virulence plasmid *rmpA2*, which was hybridised to the roughly 170 kbp plasmid to confirm the presence of the virulence plasmid in the five ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains. PFGE=pulsed-field gel electrophoresis.

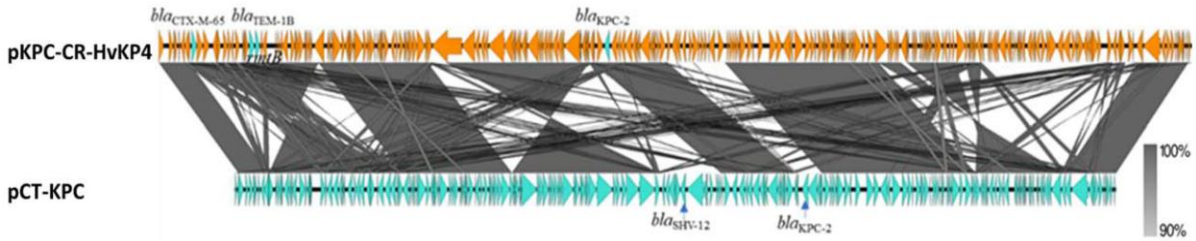


Figure 3.12 Alignment of the bla_{KPC-2}-bearing plasmid recovered from ST11 CR-HvKP strain, *K. pneumoniae* 4.

Plasmid pKPC-CR-HvKP4 (accession number MF437312) was the bla_{KPC-2}-bearing plasmid with complete sequence recovered from a ST11 CR-HvKP strain, *K. pneumoniae* 4. Plasmid sequencing was performed as previously described [181]. It is an IncFII/IncR type hybrid plasmid with 177,585bp in length and an average G+C content of 53.1% and encodes 268 predicted ORFs. It shares 99% identities with unnamed plasmid from *K. pneumoniae* strain SWU01 (CP018455), plasmid pCT-KPC (KT185451) from *K. pneumoniae* strain LJ04 and plasmid pKP1034 (KP893385) from *K. pneumoniae* strain KP1034, with a query coverage of 87%, 81% and 73%, respectively.

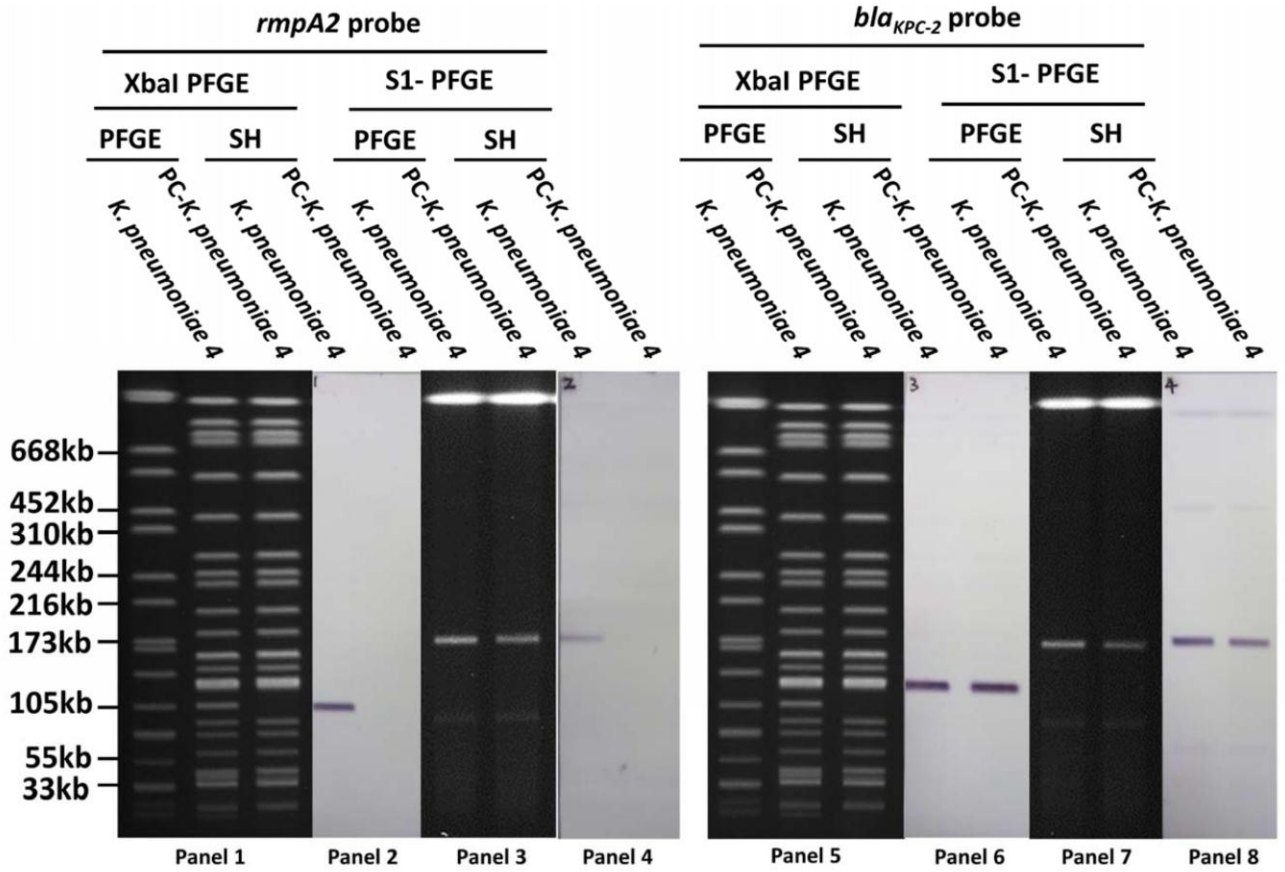


Figure 3.13 XbaI-PFGE, S1-PFGE and Southern Hybridization analysis of ST11 CR-HvKP strain *K. pneumoniae* 4 and PC- *K. pneumoniae* 4, the corresponding mutant in which the virulence plasmid was removed in plasmid curing experiment.

Plasmid curing was performed using SDS method as described previously. Briefly, strain *K. pneumoniae* 4 was incubated in LB broth supplemented with various concentrations of SDS ranging from 0.5~5% at 25℃. Bacteria culture were collected every 12 h for up to

120 h, diluted and spread on MacConkey Agar plates. Single colonies were picked up and confirmed by PCR targeting the *rmpA2* and *iucA* genes using primers described in Table 3.1. The colonies that were negative for both *rmpA2* and *iucA* were further selected to test for the hypermucoviscous phenotype by string test. Colonies that were negative for string test were subjected to XbaI-PFGE, S1-PFGE and Southern Hybridization analysis using the *rmpA2* and bla_{KPC-2} probes. Our data showed that the mutant PC-*K. pneumoniae* 4 was negative to *rmpA2* and *iucA* in PCR assay and exhibited negative string test result (Figure 3.5). Compared to *K. pneumoniae* 4, PC-*K. pneumoniae* 4 exhibited a loss of one band in XbaI PFGE (Panel 1), which was shown to hybridize to the *rmpA2* probe (Panel 2). S1-PFGE failed to reveal the loss of the ~170 kb virulence in PC-*K. pneumoniae* 4, which was due to the presence of similar size of plasmid carrying bla_{KPC-2} (Panel 3). The ~170 kb plasmid in *K. pneumoniae* 4 could be successfully hybridized with *rmpA2* probe, whereas that of PC- *K. pneumoniae* 4 could not, confirming that PC- *K. pneumoniae* 4 had lost the virulence plasmid (Panel 4). Probing the XbaI and S1-PFGE membrane with the bla_{KPC-2} probe revealed that the bla_{KPC-2} probe could hybridize to a band in the XbaI PFGE membrane of both *K. pneumoniae* 4 and PC- *K. pneumoniae* 4 (Panel 5 and 6) as well as the ~170 kb plasmid of these two strains (Panel 7 and 8). These data confirmed that *K. pneumoniae* 4 carried two plasmids with the similar size of ~170 kb, one of which was virulence plasmid, while the other one was the one which contained bla_{KPC-2}.

3.4.4 Screening of ST11 CR-hvKP strains

We retrospectively screened 387 clinical ST11 carbapenem-resistant *K. pneumoniae* strains collected from 25 provinces and municipalities in China in 2015 for the presence of the virulence plasmid. 11 (3%) of the 387 ST11 carbapenem-resistant *K. pneumoniae* strains carried the virulence plasmid. Of these 11 isolates, two carried the *rmpA*2 and *iucA* genes. The other nine isolates had two additional virulence genes, *rmpA* and iroN, suggesting that these isolates could harbour the full length of virulence plasmid pLVPK. Whole genome sequencing of SH-1, one of the 11 strains, confirmed that it had virulence plasmid highly similar to pLVPK (Figure 3.11, Table 1.6). These 11 carbapenem-resistant *K. pneumoniae* strains, all of which carried the bla_{KPC-2} gene, were collected from three different provinces. Strains from within the same region were more closely genetically related than those recovered from different regions (Figure 3.3). Retrospective analysis of the clinical records showed that these 11 ST11 *K. pneumoniae* were recovered from blood and sputum culture of the patients, and that five of the 11 patients died, and the other six patients were discharged with critical illnesses, suggesting that these 11 isolates could cause substantial morbidity and mortality.

Table 3.6 Genetic and phenotypic characteristics of ST11 *K. pneumoniae* strains recovered from clinical specimens in 2015 in various parts of China that were found to harbour virulence plasmids.

Strain ID	Isolation location	Resistance genes*	*rmpA*	*rmpA2*	*iucA*	*iroN*	ST	MIC (mg/L)									
								CTX	CAZ	FEP	ETP	IMP	MEM	AK	CIP	AMX	PE
HB25-1	Hubei	bla_{KPC-2}		+	+		ST11	128	32	1	≤0.25	≤0.25	32	＞256	128	≤ 0.5	≤0.25
HA2-23	Henan	bla_{KPC-2}	+			+	ST11	＞256	＞256	128	＞128	32	128	≤0.5	32	＞256	≤0.25
HA2-26	Henan	bla_{KPC-2}	+	+	+	+	ST11	＞256	256	128	＞128	32	64	≤0.5	32	＞256	≤0.25
HA2-34	Henan	bla_{KPC-2}	+	+	+	+	ST11	＞256	＞256	256	＞128	16	128	1	32	＞256	≤0.25
HA2-38	Henan	bla_{KPC-2}	+	+		+	ST11	＞256	＞256	128	＞128	32	64	≤0.5	32	＞256	≤0.25
SH1	Shanghai	bla_{KPC-2}	+	+	+	+	ST11	＞256	256	256	＞128	64	128	＞256	≤0.5	＞256	1
SH2	Shanghai	bla_{KPC-2}	+	+	+	+	ST11	＞256	64	256	＞128	64	＞128	＞256	64	≤ 0.5	1
SH3	Shanghai	bla_{KPC-2}	+	+			ST11	＞256	256	＞256	＞128	16	64	＞256	64	＞256	≤0.25
SH9	Shanghai	bla_{KPC-2}	+	+	+	+	ST11	＞256	128	128	＞128	16	128	＞256	64	＞256	≤0.25
SH12	Shanghai	bla_{KPC-2}		+	+		ST11	32	64	16	16	4	16	＞256	64	＞256	≤0.25
SH16	Shanghai	bla_{KPC-2}	+	+	+	+	ST11	＞256	128	128	128	32	64	＞256	64	＞256	1

*presence of carbapenemase genes; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ETP, ertapenem; IPM, imipenem; MEM, meropenem; AMK, amikacin; CIP, ciprofloxacin; AMX, amoxicillin; CLS, colistin.

3.5 Discussion

Our results show the emergence of new ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains that caused fatal hospital infections. Due to acquisition of a virulence plasmid by classic ST11 carbapenem-resistant *K. pneumoniae* strains, these new strains are simultaneously hypervirulent, multidrug resistant, and transmissible, and should therefore be regarded as a real superbug that could pose a serious threat to public health.

Known virulence factors of *K. pneumoniae* that are responsible for disease progression include, but are not limited to surface antigens, especially CPS (K antigen), siderophores that are responsible for binding ferric iron secreted by the iron-binding proteins of the host, and adherence factors that are responsible for attachment to host cell surfaces, such as type 1 and type 3 fimbriae and non-fimbrial adhesion proteins [1]. Consistently, K1 hypervirulent *K. pneumoniae* strains such as NTUH-K2044 and 1088 have been shown to produce virulence factors associated with surface antigen and siderophores, which are encoded by genes located in a virulence plasmid. ST11 *K. pneumoniae* strains, however, have been shown to express various adherence factors. The putative functional roles of fimbriae and outer membrane lipoproteins in biofilm formation and the development of antibiotic resistance might explain why the ST11 *K. pneumoniae* strains were more resistant to antibiotics than hypervirulent *K. pneumoniae* [182]. It has previously been common for ST11-type *K. pneumoniae* to be resistant to carbapenems, but not hypervirulent. The acquisition of a roughly 170 kbp virulence plasmid represents an important evolution event underlying the emergence of ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains that have virulence factors from both K1 and K2 hypervirulent *K. pneumoniae* and ST11 carbapenem-resistant *K. pneumoniae* strains. ST11 hypervirulent *K. pneumoniae* strains are therefore not only highly resistant to antibiotics, but also hypervirulent. Clinically, the ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains caused more severe disease than classic ST11 strains and high mortality. Despite being susceptible to tigecycline in in-vitro minimum inhibitory concentration tests, long-term treatment with this antibiotic (alone or in combination with several other antibiotics) or even polymyxin B, was not able to

eradicate such organisms from the bloodstream, consistently resulting in a fatal outcome. Unfortunately, ceftazidime and avibactam combination is not available in China, so we could not test their efficacy in treating these ST11 carbapenem-resistant hypervirulent *K. pneumoniae*.

Since none of the available antibiotics was effective in treating infections caused by ST11 carbapenem-resistant hypervirulent *K. pneumoniae*, we have implemented a new infection prevention and control (IPC) policy to control the outbreak in the hospital. Current consensus IPC policy on multidrug-resistant bacterial pathogens in China includes hand hygiene management, isolation of patients, periodic environmental cleansing and equipment disinfection, investigation of outbreak agents, and cautious use of antibiotics. We tried to map route or routes of transmission of these ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains, but we were unable to do so. We speculated that patients transferred from a small-scale local hospital, where the IPC standard was low, might be the sources of carbapenem-resistant *K. pneumoniae* infections. In view of this risk, we modified the IPC policy to control the outbreak. First, we introduced pre-screening of carbapenem-resistant Enterobacteriaceae in fecal and sputum samples of patients before their admission to the ICU. Second, we implemented stringent isolation procedures for carbapenem-resistant Enterobacteriaceae-bearing patients. Third, it was made necessary for medical personnel who came into contact with patients infected with carbapenem-resistant Enterobacteriaceae to go through a disinfection procedure. Finally, the ICU wards where the carbapenem-resistant Enterobacteriaceae-positive patients stayed were completely disinfected after the discharge of the patients and left unoccupied for more than 2 weeks after disinfection and before the admission of new patients. Since the implementation of these IPC procedures in this ICU, no fatal infections due to ST11 carbapenem-resistant hypervirulent *K. pneumoniae* have occurred as of April, 2017. More evidence is needed to confirm that this IPC policy is effective in preventing carbapenem-resistant Enterobacteriaceae infections in hospitals.

The main limitation of this study is that only a small number of cases have been investigated. A large-scale study on the relationship between the clinical outcomes of infections caused by carbapenem-resistant *K. pneumoniae* strains with or without the

virulence plasmid is underway to provide more evidence on the clinical significance of these newly emerged strains.

Importantly, these ST11 carbapenem-resistant hypervirulent *K. pneumoniae* were found to have disseminated in other parts of China, signifying that their threat to human health is imminent. Worldwide surveillance of these carbapenem-resistant hypervirulent *K. pneumoniae* strains and implementation of stricter control measures are needed to prevent these novel strains from further disseminating in hospital settings and the community.

3.6 Conclusions

In summary, results in this section showed that carbapenem-resistant ST11 hypervirulent *K. pneumoniae* (ST11 carbapenem-resistant hypervirulent *K. pneumoniae*) strains have emerged and can cause fatal ventilator-associated pneumonia in patients in hospital. Such strains have disseminated across various regions of China, accounting for as much as 3% of clinical ST11 carbapenem-resistant *K. pneumoniae* infections in the country. ST11 carbapenem-resistant hypervirulent *K. pneumoniae* has a hypervirulence phenotype characterised by a positive string test result, extremely high survival on exposure to human neutrophils, and high virulence in a wax moth (*G. mellonella*) larva infection model. The emergence of ST11 carbapenem-resistant hypervirulent *K. pneumoniae* strains was due to the acquisition of a roughly 170 kbp virulence plasmid carrying the *rmpA2* and aerobactin biosynthesis genes by classic ST11 carbapenem-resistant *K. pneumoniae* strains.

CHAPTER 4. Carriage of *bla*$_{KPC-2}$ by a virulence plasmid in hypervirulent *Klebsiella pneumoniae*

The content in this chapter has been published in following paper:

Dong, N.[#], Lin, D.[#], Zhang, R., Chan, E. W. C., & Chen, S. (2018). Carriage of *bla*$_{KPC-2}$ by a virulence plasmid in hypervirulent *Klebsiella pneumoniae*. Journal of Antimicrobial Chemotherapy. doi: 10.1093/jac/dky358. ([#]Contributed equally).

The other first author of this paper provided the clinical isolates and clinical data from the patients. All the major research work was done by the candidate. This paper will not be used for other theses.

4.1 Abstract

This study characterized a plasmid encoding both hypervirulence and carbapenem resistance phenotypes in strain KP70-2 which is a K1 hypervirulent *Klebsiella pneumoniae* (HvKP). The plasmid was subjected to whole-plasmid sequencing using both the Illumina NextSeq 500 sequencing platform and Nanopore MinION sequencer platforms. A hybrid virulence- and resistance-encoding plasmid of 240 kb, harbouring both the virulence gene *rmpA2* and the carbapenemase gene *bla*$_{KPC-2}$, designated pKP70-2, was recovered from strain KP70-2. The plasmid was found to be almost structurally identical to various known hypervirulence-encoding plasmids harboured by other HvKP strains, except for an extra MDR-encoding region located within the genetically conserved plasmid backbone. This MDR region was flanked by two copies of IS*26* in the same orientation, one at each end and linked to an external 8 bp (CTAAAATT) product of target site duplications, suggesting that an insertion event was responsible for the integration of the MDR region into the virulence plasmid. The MDR region was also found to harbour mobile elements that in turn contain the antibiotic resistance genes *dfrA14* and *bla*$_{KPC-2}$. Based on the genetic composition of pKP70-2, we postulate that the multiple insertion elements that it harbours were responsible for mediating the plasmid recombination events that underlie continuous emergence and genetic adaptation of novel resistance- and virulence-encoding mobile elements in *K. pneumoniae*.

4.2 Introduction

Klebsiella pneumoniae, a key member of the human gut flora, has evolved into two major types of clinically significant pathogens, namely carbapenem-resistant *K. pneumoniae* (CRKP) and hypervirulent *K. pneumoniae* (HvKP). CRKP has become one of the most important carbapenem-resistant Enterobacteriaceae, commonly causing untreatable and hard-to-treat infections among hospitalized patients and is considered an urgent public health threat according to the US CDC reports in 2013. This strain is also known to account for 70%–90% of clinical carbapenem-resistant Enterobacteriaceae infections in the EU and China [89, 170]. The most dominant clinical CRKP strains belong to clonal group (CG) 258, with ST258 and ST11 being the dominant multilocus STs that prevail in different parts of the world [25]. HvKP, on the other hand, is known to exhibit the ability to cause life-threatening, community-acquired infections such as liver abscess, pneumonia, meningitis and endophthalmitis in young and healthy individuals, resulting in a high morbidity and mortality [19]. These strains are able to acquire efficiently iron and produce an increased amount of capsular substance, which confers a hypermucoviscous phenotype detectable as a positive 'string test' result on blood agar plates [19]. The hypervirulent phenotype of *K. pneumoniae* was thought to be attributed to carriage of a virulence plasmid that harbours two CPS regulator genes (*rmpA* and *rmpA2*) and a number of siderophore gene clusters that contribute to the hypermucoviscous phenotype [183]. To date, ~70%–80% of reported HvKP strains belonged to the K1 and K2 types of *K. pneumoniae* [48]. These HvKP strains could further evolve to become carbapenem-resistant HvKP (CR-HvKP) through acquisition of a carbapenemase-encoding plasmid [39]. Alternatively, CR-HvKP strains may emerge as a result of acquisition of a hypervirulence plasmid by existing CRKP strains that do not necessarily belong to K1 or K2 [166]. Nevertheless, CR-HvKP strains are still rarely reported, presumably due to the compatibility of these plasmids with specific ST types of *K. pneumoniae*. In this study, we report for the first time (to the best of our knowledge) that a $bla_{\text{KPC-2}}$-encoding element can be integrated into a virulence plasmid, which then possesses the ability to mediate expression of both hyper-resistance and hypervirulence phenotype in *K. pneumoniae*. Transmission of this new plasmid among *K.*

pneumoniae strains will result in a significant increase in the prevalence of the CR-HvKP infections.

4.3 Materials and Methods

4.3.1 Bacterial strains

CR-HvKP strain KP70-2 was recovered from the sputum sample of a patient who died of septic shock and characterized with MLST and S1-PFGE hybridization as previously described [39]. This strain was subjected to further genetic characterization in this study. HvKP strain KP70-1, which was the parental carbapenem-susceptible strain isolated from the same patient before carbapenem treatment, was also included as a control strain for the virulence study.

4.3.2 String test and *Galleria mellonella* infection model

String test and virulence of *K. pneumoniae* strains in wax moth larvae (*G. mellonella*) were conducted with the method described in Chapter 3 [19, 175]. The survival rates of *G. mellonella* were recorded over 48 h post-infection. All experiments were carried out in triplicate. The susceptible HvKP strain 70-1 and the classic carbapenem-resistant *K. pneumoniae* strain FJ8 from previous studies were used as controls of high and low virulence strains, respectively [39, 166]. Statistical analyses were performed and visualized with GraphPad Prism v.7.00 (GraphPad Software Inc., La Jolla, CA, USA).

4.3.4 Whole-plasmid sequencing and analysis

Plasmids harboured by strain KP70-2 were extracted using Qiagen Midi prep kit and sequenced using the Illumina NextSeq 500 sequencing platform and Nanopore MinION sequencer platforms with methods described previously [176]. Raw Illumina reads were trimmed or filtered to remove low-quality sequences and adaptors. A total of 500 Mb raw illumina sequencing reads and 300 Mb raw nanopore sequencing reads were obtained. Hybrid assembly of both short Illumina reads and long MinION reads was constructed using Unicycler v 0.3.0 with the Pilon (v1.22) option on for the modification of the assembled reads [177, 184]. The plasmid sequences were annotated with the RAST tool and Prokka [142, 143]. Plasmid sequence comparison was performed using the using

BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the BLAST Ring Image Generator [153]. Incompatibility types, antimicrobial resistance genes and ISs were searched for using PlasmidFinder 1.3, ResFinder 2.1 and ISfinder tools [137]. Virulence-associated genes were searched for manually using the BIGSdb *Klebsiella* genome database [107]. Comparison between the drug resistance region in pKP70-2 and similar sequences in the NCBI database was conducted with EasyFig [154].

4.3.5 Accession number

The sequence of plasmid pKP70-2 was deposited in GenBank with accession number MF398271.

4.4 Results and Discussion

4.4.1 Virulence potential of strain KP70-2

In the previous study, a *K. pneumoniae* ST23 and serotype K1 strain known as KP70-2 was isolated and characterized as previously described [39]. Strain KP70-2 was found to exhibit resistance to β-lactams (imipenem, meropenem, cefotaxime and ceftriaxone) [39], as well as the hyperviscous phenotype in string test. At the inoculum of 1×10^6 cfu at 48 h after infection, survival of *G. mellonella* was 0% with *K. pneumoniae* strains KP70-1 and KP70-2, while survival was 80% with FJ8 (Figure 4.1), suggesting strain KP70-2 was hypervirulent and this virulence potential was not attenuated after the acquisition of MDR phenotypes when compared with KP70-1.

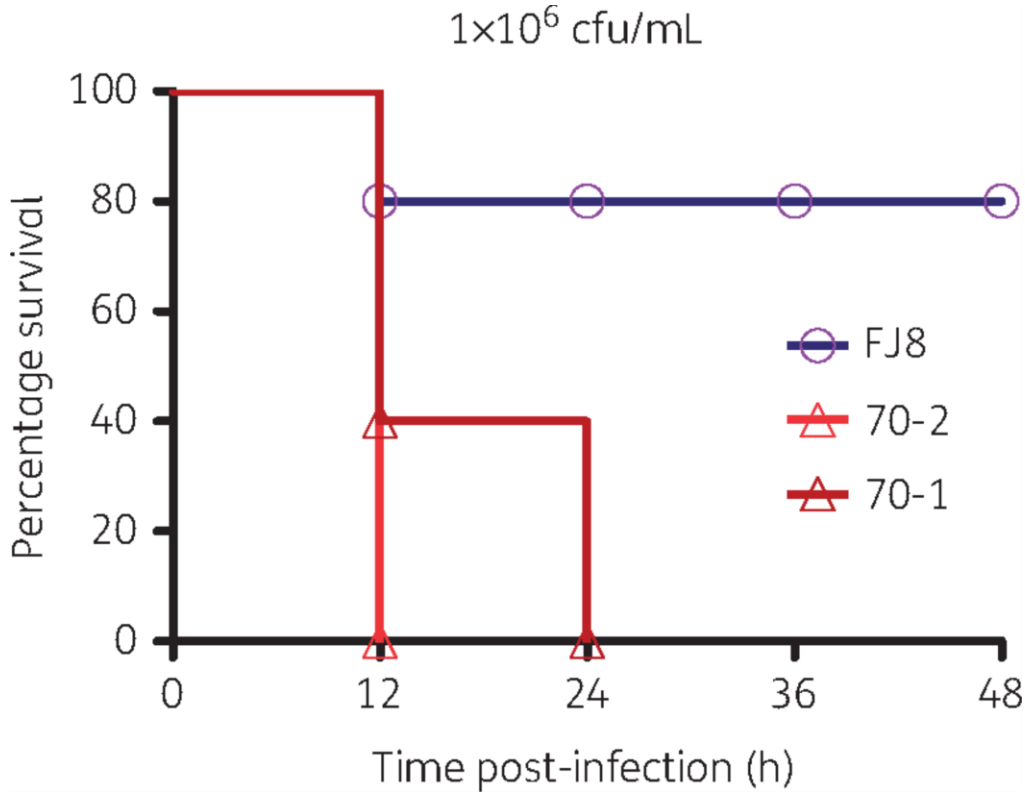


Figure 4.1 Virulence potential of *K. pneumoniae* strains in the *G. mellonella* infection model. Survival rates of *G. mellonella* (10 larvae in each group) with infection of 1×10^6 CFU of indicated *K. pneumoniae* isolates. Previously reported ST23 carbapenem-susceptible HvKP isolate KP70-1 was used as a control of hypervirulence and ST11 carbapenem-resistant classic *K. pneumoniae* isolate FJ8 was used as a control of low virulence.

4.4.2 Genetic characterization of plasmid pKP70-2

Unlike other CR-HvKP strains that normally harbour two plasmids, one encoding hypervirulence and the other hyper-resistance, S1-PFGE and Southern hybridization showed that the virulence gene *rmpA2* and the carbapenemase gene bla_{KPC-2} were both located in a plasmid of ~240 kb that was non-transferable to *Escherichia coli* [39]. Consistent with the S1-PFGE data, only one plasmid, designated as pKP70-2, was identified by hybrid assembly of whole-plasmid sequencing data. Plasmid pKP70-2 is 238 153 bp in length, with an average G + C content of 50.3%, and comprises 290 predicted ORFs. A plasmid comparison based on a full-plasmid BLASTn query showed that it exhibited 99% identity with each of the following plasmids: pRJF999 (GenBank CP014011), pK2044 (GenBank AP006726), pRJA166b (GenBank CP019049), pRJF293 (GenBank CP014009), pLVPK

(GenBank AY378100), an unnamed plasmid in strain ED23 (GenBank CP016815), pKCTC2242 (GenBank CP002911) and pKp_Goe_414-2 (CP018338), with query coverage ranging from 93% to 77% (Figure 4.2a). Apart from pKp_Goe_414-2, which belongs to the incompatibility group FIB (IncFIB), pKP70-2 and seven other related plasmids were all multi-replicon (IncHI1B/IncFIB) plasmids. Importantly, pKP70-2 was found to harbour a unique region of ~15 kb that was not present in all other plasmids. A further BLAST search of the 223 373 bp backbone sequence of pKP70-2, excluding the unique region, reveals that it is highly similar to pRJF999 (GenBank CP014011) and pK2044 (GenBank AP006726), both from *K. pneumoniae* subsp. *pneumoniae* isolates in Asia, with 99% identity and 99% coverage. The backbone of these plasmids was found to harbour genes responsible for plasmid replication, virulence traits of the strain and heavy metal resistance [34].

4.4.2 Comparison analysis of the MDR region in plasmid pKP70-2

Similar MDR genetic structures were detected in the ~65 kb IncN plasmids pECN580 (GenBank KF914891) and pKo6 (GenBank KC958437), which were recovered from *E. coli* and *K. pneumoniae* strains, respectively, with 100% nucleotide identity and 97% coverage (Figure 4.2b) [185]. There was a total of four copies of IS*26* in pKP70-2, but only two in pECN580 and pKo6. These observations provided evidence of insertion of this region into the backbone of this virulence plasmid, presumably because of intermolecular replicative transposition mediated by IS*26* [186]. This theory is further supported by the observation of an 8 bp target site duplication at the end of each of the two IS*26* elements flanking the MCR region. It should also be noted that there are two other structural differences between the MDR region in pKP70-2 and other closely related plasmids. First, an additional 230 bp is present in pKP70-2 between the two copies of IS*26*, in the middle of the class 1 integron-like element and the NTE_{KPC}-Id element. Second, the orientation of the entire NTE_{KPC}-Id element in pKP70-2 was opposite that of other gene loci in this element, when compared with pECN580 and pKo6. The multiple copies of IS*26* in pKP70-2 may facilitate occurrence of recombination events between plasmids, generating hybrid elements such as pKP70-2.

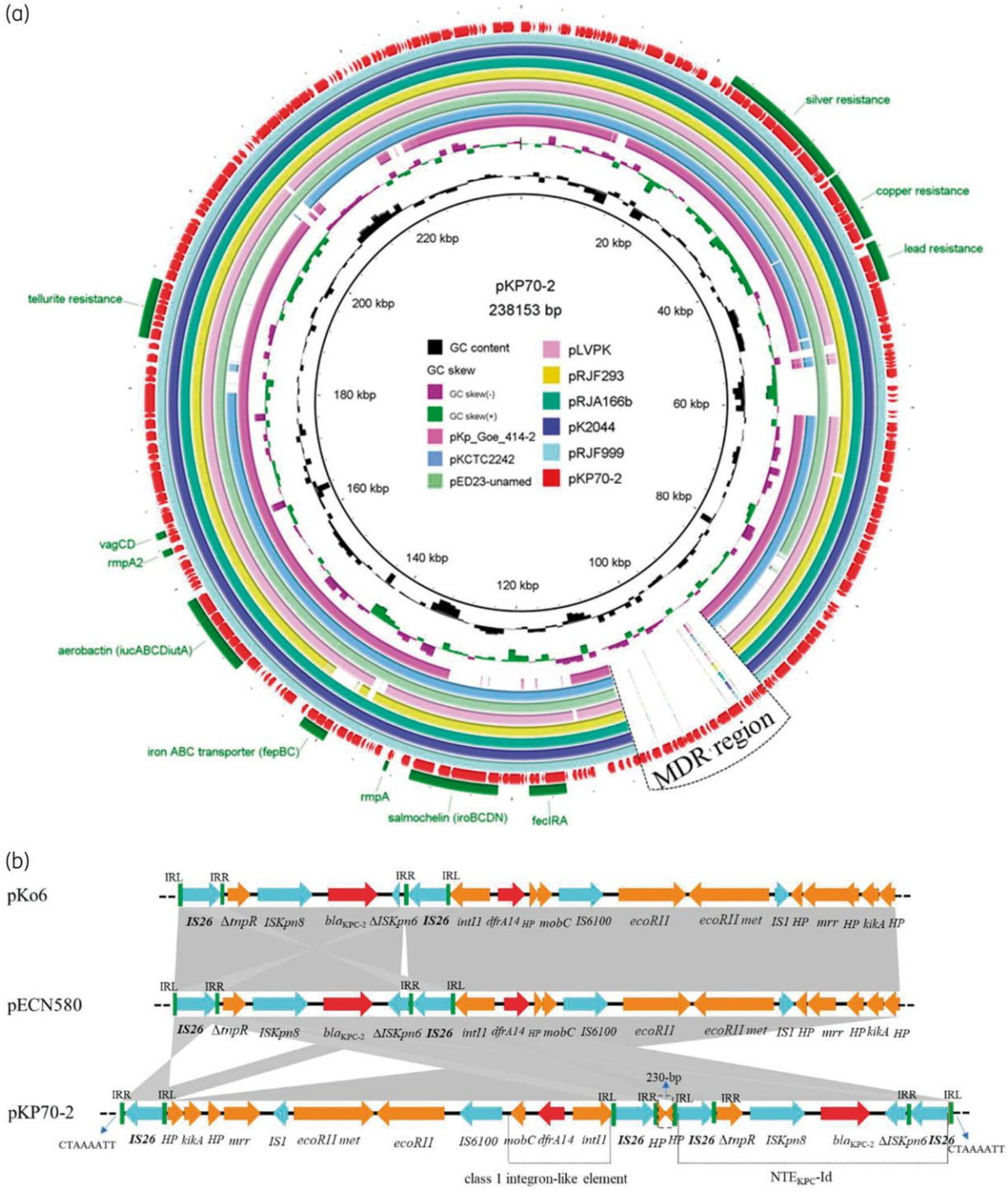


Figure 4.2 Detailed information regarding a virulence plasmid carrying an MDR region that encodes KPC-2 and DfrA14.

(a) Sequence alignment of nine virulence plasmids in *K. pneumoniae* using the complete sequence of plasmid pKP70-2 (outermost red circle) as a reference. Genetic regions associated with virulence and heavy metal resistance are highlighted in green. (b) Alignment of the

14 780 bp MDR region in plasmid pKP70-2 with similar regions in plasmids pKo6 and pECN580. Red, light blue and orange arrows denote antimicrobial resistance genes, IS elements and other protein-encoding genes, respectively. Green boxes refer to the left and right IRs (IRL and IRR) of the IS*26* element.

4.5 Conclusions

In conclusion, we reported the genetic structure of a hybrid plasmid in an ST23 carbapenem-resistant, hypervirulent *K. pneumoniae* which was formed by IS*26*-mediated insertion of the bla_{KPC-2} and *dfrA* drug resistance mobile elements into the virulence plasmid. To the best of our knowledge, this is the first report of a bla_{KPC-2}-bearing virulence hybrid plasmid in *K. pneumoniae*, whose emergence poses a serious public health concern.

CHAPTER 5. An IncR Plasmid harbored by a hypervirulent, carbapenem resistant *Klebsiella pneumoniae* strain possesses five tandem repeats of the *bla*$_{KPC-2}$::NTE$_{KPC-Id}$ fragment

Part of the content in this chapter is reproduced from the published paper:

Dong, N., Liu, L., Zhang, R., Chen, K., Xie, M., Chan, E.W., Chen, S. An IncR Plasmid harbored by a hypervirulent, carbapenem resistant *Klebsiella pneumoniae* strain possesses five tandem repeats of the *bla*$_{KPC-2}$::NTE$_{KPC-Id}$ fragment. Antimicrobial agents and chemotherapy.

5.1 Abstract

This study aimed at determining the virulence level and antimicrobial susceptibility profile of a carbapenem-resistant, hypervirulent *Klebsiella pneumoniae* isolate, SH9, and investigating the genetic features of the phenotype-encoding plasmids that it harbored. Phenotyping of strain SH9, including antimicrobial susceptibility tests, string test and virulence assay using the *Galleria mellonella* infection model, was performed. Genetic profiles of three plasmids recovered from SH9 were determined by whole genome sequencing. Plasmid sequences were analyzed by various bioinformatic tools. Strain SH9 was confirmed to be carbapenem-resistant, string test positive and hypervirulent. Complete sequences of three plasmids were obtained, including a pLVPK like virulence-conferring plasmid (pVir-CR-HvKP_SH9) and two MDR plasmids (pKPC-CR-HvKP4_SH9 and pCTX-M-CR-HvKP4_SH9) which comprised various resistance genes. The two MDR plasmids were predicted to originate from a single pKPC-CR-HvKP4-like multi-replicon plasmid through homologous recombination. The *bla*$_{KPC-2}$ gene was detectable in five tandem repeats in a non-conjugative plasmid, pKPC_CR-HvKP4_SH9, which exhibited the format of a NTEKPC-Id-like transposon (IS*26*-ΔTn*3*-IS*Kpn8*-*bla*$_{KPC-2}$-ΔIS*Kpn6*-*korC*-*orf*-IS*26*). Such fragment was presumably created due to Tnp*26*-catalyzed conservative reaction through activity of translocatable units. Carriage of a hypervirulence plasmid and two multidrug resistance-encoding plasmids renders *K.*

pneumoniae strain SH9 highly hypervirulent and pandrug-resistant. Recombination events were found play a role in mediating active evolution of the resistance-encoding plasmids harbored by this strain, the future evolution trends of which should be closely monitored.

5.2 Introduction

The notorious nosocomial pathogen *Klebsiella pneumoniae* has evolved into two clinically significant clades, namely hypervirulent *K. pneumoniae* (hvKP) and carbapenem-resistant *K. pneumoniae* (CR-*Kp*), both of which could cause severe infections [1]. The hvKP strains generally present a hypermucoviscous phenotype on agar plates and often cause infections in otherwise healthy individuals [19]. A pLVPK-like virulence plasmid which encodes regulators of mucoid phenotype and siderophores is known to play a pivotal role in hvKP pathogenesis [1, 34]. On the other hand, CR-*Kp* strains produce carbapenemases, which can hydrolyze a broad variety of β-lactams, rendering the pathogen untreatable by the last line antibiotic carbapenems [187]. Three major types of carbapenemases have been identified in CR-*Kp*, including Ambler class A (KPCs), B (NDMs, IMPs, VIMs) and D (OXA-48) lactamases, among which KPC is the most prevalent [85, 188]. To date, more than 16 KPC variants have been identified, with KPC-2 and -3 being the most common [189]. Recently, convergence of genetic elements encoding hypervirulence and carbapenem resistance (*i.e.*, CR-hvKP) in a single *K. pneumoniae* strain was reported, suggesting that such strains continue to evolve and pose a serious threat to public health [39, 40, 166, 190]. Emergence of CR-hvKP was due to acquisition of the virulence plasmid by a carbapenem-resistant strain, or acquisition of a carbapenemase-producing plasmid by a hypervirulent strain [166, 190].

The KPC-2 encoding gene $bla_{\text{KPC-2}}$ normally exists as a single copy in plasmids. However, by using the Oxford Nanopore sequencing technology, we detected five copies of $bla_{\text{KPC-2}}$ on a single plasmid in an ST11 hvKP isolate. This study characterized the virulence potential and antimicrobial susceptibility of this phenotypically convergent 'superbug' and unveiled the genetic basis of phenotypes conferred by the plasmids that this strain harbored, through comprehensive analysis of the completely resolved plasmid sequences.

5.3 Materials and Methods

5.3.1 Strain source, MLST, String test and *Galleria mellonella* killing assays

The *bla*$_{KPC-2}$-carrying hypervirulent *K. pneumoniae* isolate SH9 was identified in a nationwide program of surveillance of clinical carbapenem resistant *K. pneumoniae* strains in China in 2015 [89]. Multilocus sequence typing (MLST) and string test were conducted as previously described [38, 191]. Virulence in wax moth larvae (*Galleria mellonella*) weighing about 300mg (purchased from Tianjin Huiyude Biotech Company, Tianjin, China) was tested with bacterial infective dosages of 10^3 , 10^4 , 10^5 , 10^6 CFU/ml 202 according to methods described previously [192]. Ten larvae were tested for each inoculum. The survival rates of *G. mellonella* were recorded over 48 h post infection. All experiments were carried out in triplicate.

5.3.2 Antimicrobial susceptibility and conjugation

Antimicrobial susceptibility testing against different antibiotics was conducted for *K. pneumoniae* isolate SH9 using the agar dilution method, and the results were interpreted according to CLSI guidelines [139]. Conjugation experiments were performed as previously described to test the transferability of the *bla*$_{KPC-2}$-bearing plasmids in strain SH9, using an azide-resistant *E. coli* J53 (Az^r) strain as the recipient [193]. Transconjugants carrying the *bla*$_{KPC-2}$-bearing plasmid was selected on MacConkey Agar supplemented with 100 mg/mL sodium azide and 2 mg/mL meropenem.

5.3.3 Plasmid sequencing and bioinformatics analyses

To delineate the genetic content of the plasmids in strain SH9, WGS was conducted using the illumina NextSeq 500 platform and the long-read MinION Sequencer (Nanopore, Oxford, UK), with methods described previously [176]. Genome assembly was conducted using the illumina reads with SPAdes v 3.11.1 [141]. Capsular typing was performed with the assemble genome sequence using Kaptive [45]. Hybrid assembly of both short Illumina reads and long MinION reads was constructed using Unicycler v 0.3.0 [177]. The complete circular plasmid sequences were modified using Pilon v1.22 for several rounds until no change was detected [184]. Complete plasmid sequences were

annotated by the RAST tool [142], edited manually and deposited to the National Center for Biotechnology Information (NCBI) database with accession numbers MH255827 (pKPC-CR-HvKP4_SH9), MH255828 (pVir-CR-HvKP_SH9) and MH255829 (pCTX-M-CR-HvKP4_SH9). The plasmid sequences were annotated in such a way that genes encoding replication initiation proteins were regarded as the first genes. Comparative analysis of plasmids was performed with Easyfig [154] and BRIG [153].

5.4 Results and Discussion

5.4.1 Molecular typing, antimicrobial susceptibility tests and virulence assay in the *G. mellonella* infection model

First recovered from a Chinese hospital in 2015, strains of CR-hvKP are fast evolving as novel 'superbugs' which comprise diverse sequence types including ST23, ST11, ST65, ST25 and ST1797 [39, 40, 194]. One such strain, *K. pneumoniae* SH9, exhibited resistance to cefotaxime (MIC, >128 ug/ml), ceftazidime (128 μg/ml), cefepime (128 μg/ml), ertapenem (>128 μg/ml), imipenem (16 μg/ml), meropenem (128 μg/ml), amikacin (>256 μg/ml) and ciprofloxacin (64 μg/ml). However, the strain remained susceptible to colistin (≤0.25 μg/ml). In this work, SH9 was found to cause time- and dose-dependent death of all larva in a virulence assay using a *G. mellonella* infection model (Figure 5.1). With an infection dose of 10^4 CFU, 60% of the larva were killed by SH9 at 24 hours post infection (hpi), which is much higher than that of the well-known hypervirulent *K. pneumoniae* isolate NTUH-K2044 (25%) recorded at 24 hpi with similar injection dose (10^4 CFU) [195]. Infection with 10^6 CFU of *K. pneumoniae* SH9 resulted in 100% *G. mellonella* mortality at 24 hpi. Consistently, *K. pneumoniae* SH9 was string test positive, generating a viscous string longer than 5 mm in the test. MLST and capsular typing indicated that SH9 belonged to ST11 and serotype K47, a dominant clone of KPC-producing *K. pneumoniae* in China [133, 196]. Capsular typing indicated that SH9 belonged to serotype K47. These findings suggested that *K. pneumoniae* SH9 was an ST11 carbapenem-resistant hypervirulent strain, which carried both a pLVPK-like virulence conferring plasmid as well as MDR plasmids resembling the previously reported ST11 CR-hvKP strains [166, 190]. Analysis of the complete sequences of

plasmids recovered from SH9 revealed the genetic basis of the antibiotic resistance and hypervirulence phenotypes of this strain.

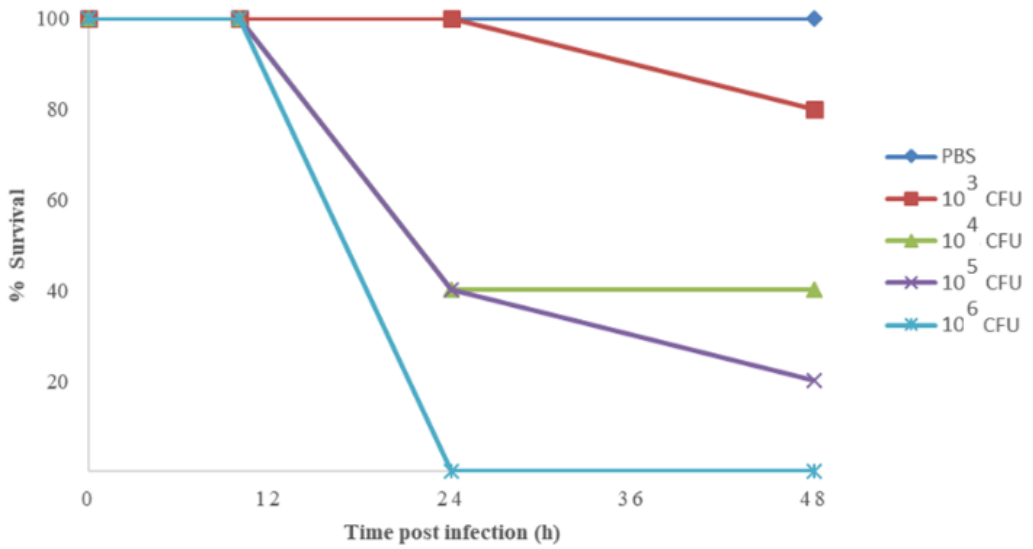


Figure 5.1 *K. pneumoniae* strain SH9 infection of *G. mellonella* induces dose-dependent lethality. Larvae were injected with PBS or with 10^3 ~ 10^6 CFU of *K. pneumoniae* SH9, and survival was monitored over 48 h post infection.

5.4.2 Sequencing and annotation of plasmids from CR-hvKP

Completes sequences of three circular plasmids recovered from *K. pneumoniae* strain SH9, with sizes of 188,648 bp (pVir-CR-HvKP_SH9), 113,941 bp (pKPC-CR105 HvKP4_SH9) and 98,684 bp (pCTX-M-CR-HvKP4_SH9) respectively, were assembled. The plasmids were named by combining the strain name and the representative genes that they carried. A BLASTN search against the NCBI nucleotide database indicated that plasmid pVir-CR-HvKP_SH9 was 99% identical to the pLVPK-like virulence plasmids pVir-CR-HvKP267 (MG053312) and pSGH10 (CP025081), which were previously recovered from clinical *K. pneumoniae* strains, with 97% coverage [128, 197]. Plasmid pVir-CR-HvKP_SH9 was found to contain the IncHI1B and IncFIB(K) replicons, exhibit a GC content of 49.9%, and comprise 225 predicted coding sequences. It also harbors the well-characterized virulence-associated genes *rmpA*, *rmpA2* and *iutAiucABCD* (encoding aerobactin). However, gene cluster encoding salmochelin (*iroBCDN*) in plasmid pLVPK was absent from pVir-CR-HvKP_SH9. By encoding siderophores (aerobactin and/or

salmochelin) and regulators of the mucoid phenotype (RmpA and RmpA2), which were restricted to hvKP isolates, pLVPK-like virulence plasmids were fo`und to play a pivotal role in *K. pneumoniae* hypervirulence [31]. The detailed structure of pVir-CR-HvKP_SH9 is presented in Figure 5.2.

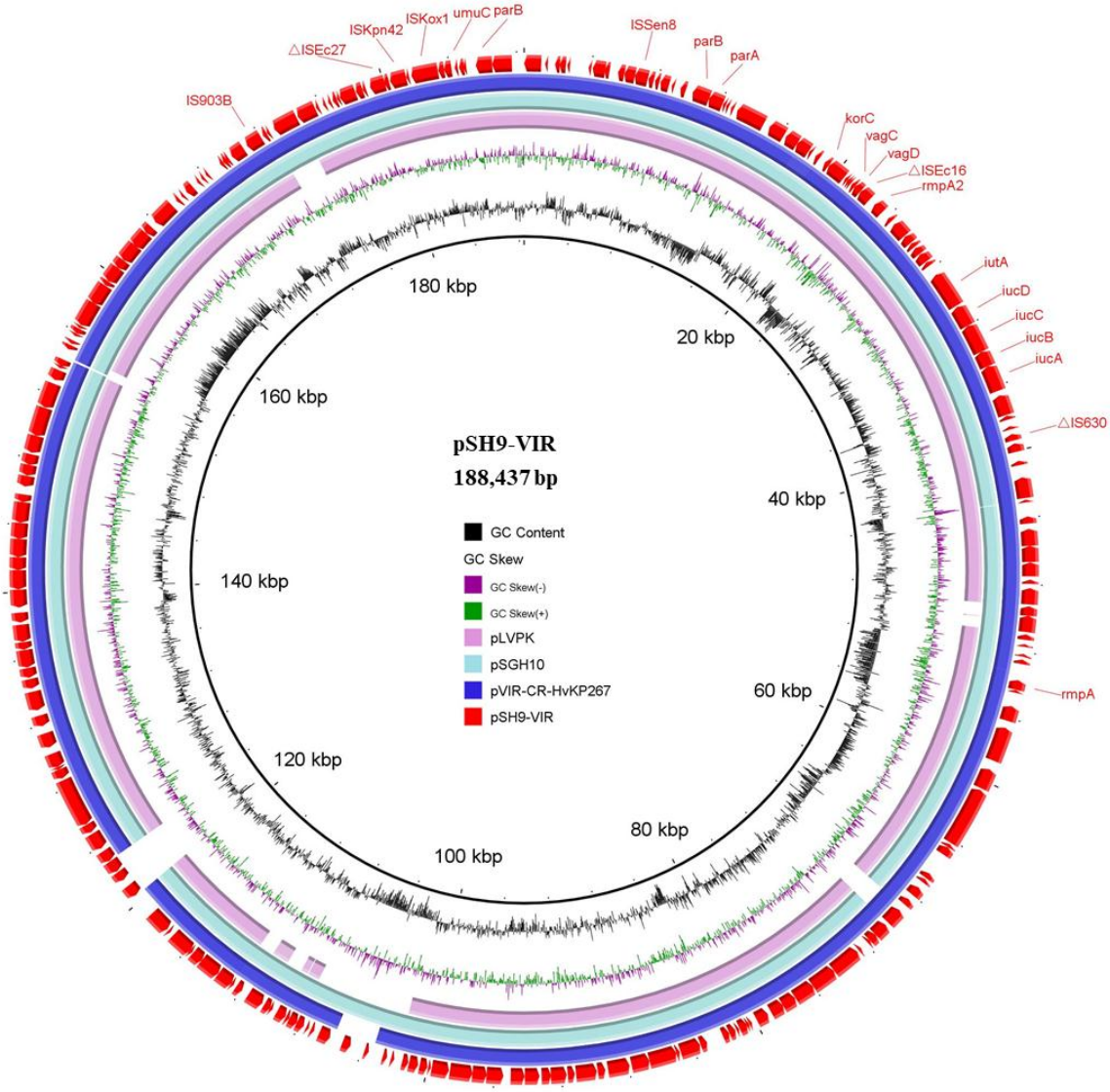


Figure 5.2 Alignment of the virulence-conferring plasmid pSH9-VIR with three homologous plasmids from the NCBI database. Innermost to outermost circles represent sequences of plasmids pLVPK (AY378100), pSGH10 (CP025081), pVir-CR-HvKP267 (MG053312) and pVir-CR-HvKP_SH9.

The carbapenem resistance-encoding plasmid (pKPC-CR-HvKP4_SH9) harbored by strain SH9 was found to exhibit a GC content of 55.0% and comprise 159 predicted ORFs. Interestingly, this plasmid was found to comprise the IncR replicon, the *catA2* gene and five copies of bla_{KPC-2} as confirmed by both the nanopore raw reads and the assembled sequences. The third plasmid harbored by this strain, pCTX-M-CR-HvKP4_SH9, was found to exhibit a GC content of 51.5% and comprise 139 predicted coding sequences, among which were the IncFII replicon and the $bla_{CTX-M-65}$, bla_{TEM-1B}, *rmtB*, *fosA*_14 genes bounded by various insertion sequences. Interestingly, both pKPC-CR-HvKP4_SH9C and pCTX-M-CR-HvKP4_SH9 exhibited more than 99% identities with the IncFII/R type conjugative MDR plasmid pKPC-CR-HvKP4 (MF437312) previously recovered from an ST11 CR-hvKP isolate [166], at 100% and 92% coverage, respectively (Figure 5.3), suggesting that genetic recombination events might be responsible for generation of these plasmids. As many as 12 copies of IS26 were found to be scattered across the complete sequence of plasmid pKPC-CR-HvKP4; however, target site duplications flanking IS*26* were not observed, indicating that homologous recombination events mediated by two IS*26* elements in plasmids of different replicons rather than replicative transposition among the three plasmids occurred (Figure 5.4c) [198, 199]. A previous study indicated that IncR plasmids did not possess conjugational transfer genes but could be mobilizable, broadening their host spectrum by forming multi-replicon cointegrates with plasmids of other incompatible types such as IncA/C and IncN [200]. In this study, plasmid pKPC-CR-HvKP4_SH9 was not transferrable to the recipient *E. coli* strain J53 via conjugation. Also, no pKPC-CR-HvKP4-like cointegrate was detected after conjugation, leading us to hypothesize that homologous recombination occurs at a relatively low frequency, thereby limiting the rate of transmission of bla_{KPC-2} genes located in IncR plasmids.

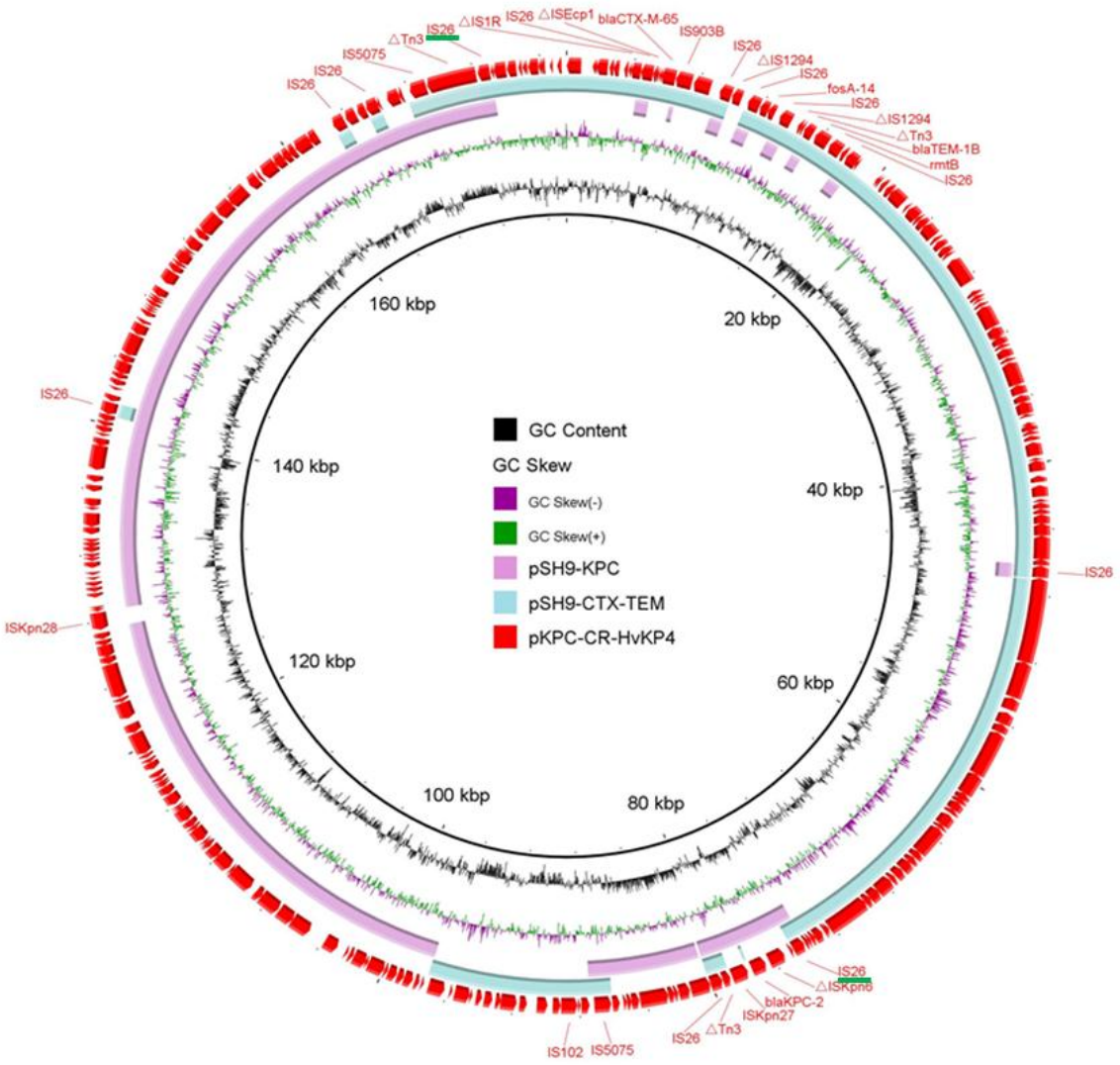


Figure 5.3 Circular maps of two MDR plasmids recovered from *K. pneumoniae* SH9 and a similar plasmid pKPC-CR-HvKP4 recorded in the NCBI database. The two IS*26* elements responsible for the homologous recombination were highlighted by green underlines.

5.4.3 Comparative analysis of the *bla*$_{KPC-2}$ region

Five copies of *bla*$_{KPC-2}$ genes were identified on pKPC-CR-HvKP4_SH9, with each being in one of five identical 5,699-bp regions linked to each other in tandem, suggesting that this region exhibits a high degree of mobility and has been heavily duplicated (Figure 5.4). The most common transmissible element associated with *bla*$_{KPC}$ is the Tn*3*-based transposon, Tn*4401* [25]. However, the 5,699-bp region is located in a non-Tn*4401* element homologous to the NTE$_{KPC-Id}$ fragment in plasmid pKPC-LKEc (KC788405),

with the structure of IS*26*-ΔTn*3*-IS*Kpn8*-*bla*$_{KPC-2}$-ΔIS*Kpn6*-*korC*-*orf* (Figure 5.4) [25, 201]. The first copy of the 5,699-bp NTE$_{KPC-Id}$-like region was located directly upstream of an IS*26* element, generating a 6.5 kb fragment bordered by two IS*26*s. Such 6.5 kb element could effectively act as a composite transposon which can mobilize the intervening genetic components. Detailed sequence analysis of the *bla*$_{KPC-2}$ region enabled us to predict the duplication mechanism that creates the tandem repeats. Firstly, the 5.6 Kb translocatable unit (TU), a circular form of the NTE$_{KPC-Id}$-like fragment, was generated via excision from a preexisting IS*26*-bounded transposon via homologous recombination. The TU was then incorporated to an existing IS*26* using the conservative Tnp*26* catalyzed mechanism or homologous recombination (less frequently). Lastly, repetition of the incorporation process with the same TU should lead to formation of the NTE$_{KPC-Id}$-like tandem repeats in pKPC-CR-HvKP4_SH9 [199]. To our knowledge, co-existence of five *bla*$_{KPC-2}$ genes with the NTE$_{KPC-Id}$ structure within a single plasmid has not been reported before, but carriage of multiple copies of *bla*$_{KPC}$ on Tn*4401* was described previously [202-208]. A previous study reported the carriage of two *bla*$_{KPC-3}$ genes in an IncX plasmid with the structure of Tn*4401a*, which was harbored by a ST512 *K. pneumoniae* isolate that exhibited significant increase in the MICs of carbapenems [202]. Previous study suggested both copy number of the *bla*$_{KPC}$ gene and upstream genetic environment mutations affected the expression level of the KPC carbapenemase [203]. In this study, carriage of multiple copies of *bla*$_{KPC-2}$ genes does not necessarily further enhance carbapenem-resistance level in the host strain when compared with the previously reported ST11 CR-HvKP isolates that carried only one copy of the *bla*$_{KPC-2}$ gene, which already displayed MICs of more than 16 μg/ml towards ertapenem [166]. Factors that determine the level of expression of the plasmid-borne *bla*$_{KPC-2}$ genes require further investigation.

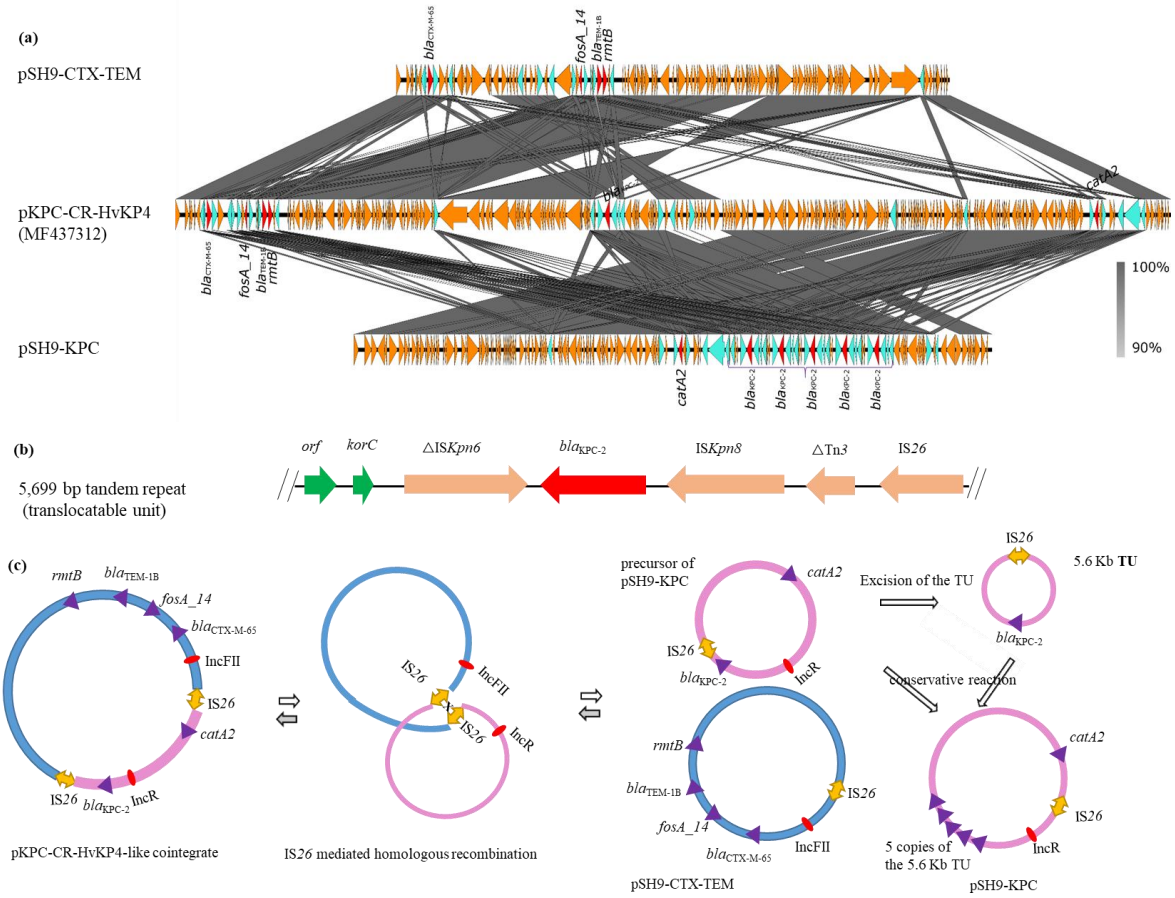


Figure 5.4 Mechanisms of plasmid recombination in strain SH9. (a) Structure alignment of the three plasmids. Yellow, blue and red triangles indicate ORFs, insertion sequences and antimicrobial resistance genes, respectively. (b) Genetic composition of the 5,699 bp *bla*$_{KPC-2}$-bearing tandem repeat region which formed the translocatable unit (TU) that excise from the precursor of plasmid pSH9-KPC. (3) Mechanisms of plasmid recombination. IS26 mediated the homologous recombination leads to the generation of plasmids pKPC-CR-HvKP4-like cointegrate, pSH9-KPC and pSH9-CTX-TEM. Five tandem repeats of *bla*$_{KPC-2}$-bearing fragments were generated via Tnp*26*-catalyzed conservative reaction which incorporated the 5.6 Kb TU next to a preexisting IS*26*. TU, translocatable unit.

5.5 Conclusions

Emergence of carbapenem-resistant, hypervirulent *K. pneumoniae* strains is of great concern because they are simultaneously hypervirulent and multidrug resistant. In this study, a ST11 CR-hvKP isolate of clinical origin was found to harbor three plasmids, namely a pLVPK-like virulence plasmid and two MDR plasmids comprising various

resistance genes including *bla*$_{\text{CTX-M-65}}$, *bla*$_{\text{TEM-1B}}$, *rmtB*, *fosA*_14, *catA2*, and five copies of *bla*$_{\text{KPC-2}}$. The two MDR plasmids were predicted to originate from a single pKPC-CR-HvKP4-like multi-replicon plasmid through homologous recombination. Although the *bla*$_{\text{KPC-2}}$ gene was found to be located on a non-conjugative plasmid, pKPC-CR-HvKP4_SH9, it was shown to exist in five tandem repeats of the NTEKPC-Id-like transposon (IS*26*-ΔTn*3*-IS*Kpn8*-*bla*$_{\text{KPC-2}}$-ΔIS*Kpn6*-*korC*-*orf*-IS*26*), presumably created as a result of Tnp*26*-catalyzed conservative reaction through activity of translocatable units. Findings in this study indicate that plasmids in the ST11 CR-hvKP clone can undergo active genetic recombination events, the evolution trends of which should be closely monitored.

CHAPTER 6. Conclusions and suggestions for future research

In conclusion, this study investigated the genetic and phenotypic characteristics of various strains of the notorious pathogen, *K. pneumoniae*, which has developed both hypervirulence and multidrug resistance phenotypes.

In the first part of this study, we conducted a comprehensive genomic analysis of the dominant carbapenem-resistant clone of *K. pneumoniae* in China, ST11 *K. pneumoniae*. The ST11 genomes were found to be highly heterogeneous and clustered into at least three major lineages distinguished mainly by the capsular types. Multiple resistance and virulence determinants were detectable among the genomes, including the bla_{KPC-2} gene conferring carbapenem resistance, and *rmpA* gene which is the hallmark of hypervirulent *K. pneumoniae*, indicating the potential emergence of ST11 carbapenem-resistant, hypervirulent *K. pneumoniae*. Findings in this part revealed the genetic features of ST11 *K. pneumoniae* and indicates the potential emergence of ST11 carbapenem-resistant, hypervirulent *K. pneumoniae*.

In the second part of this study, we reported a fatal outbreak of ST11 carbapenem-resistant, hypervirulent *K. pneumoniae* in the ICU of a Chinese hospital. Phenotypic characterization indicated all the outbreak strains are carbapenem resistant and hypervirulent. Genomic characterization revealed all strains belonged to a single clone originated from the acquisition of a pLVPK-like virulence plasmid by ST11 carbapenem-resistant *K. pneumoniae*. Our study also indicated that such strains have disseminated across various regions of China, accounting for as much as 3% of clinical ST11 carbapenem-resistant *K. pneumoniae* infections in the country.

The third part of this study focused on the genomic study of two different types of carbapenem-resistant, hypervirulent *K. pneumoniae*. The first belonged to ST23 CR-hvKP whose emergence was due to the IS*26*-mediated insertion of bla_{KPC-2} into a pLVPK-like virulence plasmid in ST23 hypervirulent *K. pneumoniae*. The second case reported the carriage of 5 copies of bla_{KPC-2} by an ST11 CR-hvKP mediated by Tnp*26*-

catalyzed conservative reaction through activity of translocatable units. In the two cases, the carbapenem-resistance and hypervirulence phenotypes were both mediated by the plasmid(s) each strain harbored, indicating an alarming evolutionary event of *K. pneumoniae*. The emergence of diverse CR-hvKp isolates suggest such organisms could cause severe infections in both hospital settings and the community.

Based on the findings of the current research, *K. pneumoniae*, especially the recently reported carbapenem-resistant, hypervirulent *K. pneumoniae*, posed serious threat to public health. CR-hvKp emerged from diverse genetic backgrounds (hvKp or CR-*Kp*), varied significantly in genetic contents and are under constant and active evolution. DNA recombination plays an important role in mediating active plasmid evolution in *K. pneumoniae*. The development of WGS methods and bioinformatics tools shed light on the study of such a pathogen. In this regard, we suggest that future research should focus on the development of intervention measures to prevent further dissemination of such organisms in hospital settings. However, the mechanism which drives transmission of genetic contents such as virulence plasmids and resistance elements among *K. pneumoniae* of different genetic background remains unknown. There is an urgent need to understand this mechanism through which more efficient preventive measures could be taken to slow down the evolution and transmission of such pathogens. Also, more study on the epidemiology and molecular evolution of the multidrug resistant and hypervirulent *K. pneumoniae* strains should be conducted.

REFERENCES

[1] M. K. Paczosa and J. Mecsas, "Klebsiella pneumoniae: going on the offense with a strong defense," *Microbiology and Molecular Biology Reviews,* vol. 80, no. 3, pp. 629-661, 2016.

[2] S. Clegg and C. N. Murphy, "Epidemiology and Virulence of Klebsiella pneumoniae," *Microbiology spectrum,* vol. 4, no. 1, 2016.

[3] S. Brisse, F. Grimont, and P. A. Grimont, "The genus klebsiella," in *The prokaryotes*: Springer, 2006, pp. 159-196.

[4] C. L. Gorrie, "Population structure and carriage-infection dynamics of Klebsiella pneumoniae," 2018.

[5] V. Passet and S. Brisse, "Description of Klebsiella grimontii sp. nov," *International journal of systematic and evolutionary microbiology,* vol. 68, no. 1, pp. 377-381, 2017.

[6] Y. Hu, L. Wei, Y. Feng, Y. Xie, and Z. Zong, "Klebsiella huaxiensis sp. nov., recovered from human urine," *bioRxiv,* p. 335075, 2018.

[7] C. Rodrigues, V. Passet, and S. Brisse, "Identification of Klebsiella pneumoniae complex members using MALDI-TOF mass spectrometry," *bioRxiv,* p. 350579, 2018.

[8] K. E. Holt *et al.*, "Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in Klebsiella pneumoniae, an urgent threat to public health," *Proceedings of the National Academy of Sciences,* vol. 112, no. 27, pp. E3574-E3581, 2015.

[9] M. E. S. de Melo, A. B. Cabral, M. A. V. Maciel, V. M. da Silveira, and A. C. de Souza Lopes, "Phylogenetic groups among Klebsiella pneumoniae isolates from Brazil: relationship with antimicrobial resistance and origin," *Current microbiology,* vol. 62, no. 5, pp. 1596-1601, 2011.

[10] S. Brisse and J. Verhoef, "Phylogenetic diversity of Klebsiella pneumoniae and Klebsiella oxytoca clinical isolates revealed by randomly amplified polymorphic DNA, gyrA and parC genes sequencing and automated ribotyping," *International journal of systematic and evolutionary microbiology*, vol. 51, no. 3, pp. 915-924, 2001.

[11] A. J. Brink *et al.*, "Emergence of New Delhi metallo-beta-lactamase (NDM-1) and Klebsiella pneumoniae carbapenemase (KPC-2) in South Africa," *Journal of clinical microbiology*, vol. 50, no. 2, pp. 525-527, 2012.

[12] K. L. Wyres and K. E. Holt, "Klebsiella pneumoniae population genomics and antimicrobial-resistant clones," *Trends in microbiology*, vol. 24, no. 12, pp. 944-956, 2016.

[13] Y.-Y. Liu *et al.*, "Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study," *The Lancet infectious diseases*, vol. 16, no. 2, pp. 161-168, 2016.

[14] P. Nordmann, L. Poirel, T. R. Walsh, and D. M. Livermore, "The emerging NDM carbapenemases," *Trends in microbiology*, vol. 19, no. 12, pp. 588-595, 2011.

[15] P. Nordmann, G. Cuzon, and T. Naas, "The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria," *The Lancet infectious diseases*, vol. 9, no. 4, pp. 228-236, 2009.

[16] J. Chaves, M. G. Ladona, C. Segura, A. Coira, R. Reig, and C. Ampurdanés, "SHV-1 β-lactamase is mainly a chromosomally encoded species-specific enzyme in Klebsiella pneumoniae," *Antimicrobial agents and chemotherapy*, vol. 45, no. 10, pp. 2856-2861, 2001.

[17] J. Sirot, C. Chanal, A. Petit, D. Sirot, R. Labia, and G. Gerbaud, "Klebsiella pneumoniae and other Enterobacteriaceae producing novel plasmid-mediated β-lactamases markedly active against third-generation cephalosporins: epidemiologic studies," *Clinical Infectious Diseases*, vol. 10, no. 4, pp. 850-859, 1988.

[18] M. S. Ramirez, G. M. Traglia, D. L. Lin, T. Tran, and M. E. Tolmasky, "Plasmid-mediated antibiotic resistance and virulence in gram-negatives: the Klebsiella pneumoniae paradigm," *Microbiology spectrum,* vol. 2, no. 5, p. 1, 2014.

[19] A. S. Shon, R. P. Bajwa, and T. A. Russo, "Hypervirulent (hypermucoviscous) Klebsiella pneumoniae: a new and dangerous breed," *Virulence,* vol. 4, no. 2, pp. 107-118, 2013.

[20] T. A. Russo *et al.*, "Aerobactin mediates virulence and accounts for the increased siderophore production under iron limiting conditions by hypervirulent (hypermucoviscous) Klebsiella pneumoniae," *Infection and immunity,* pp. IAI. 01667-13, 2014.

[21] L. M. Lery *et al.*, "Comparative analysis of Klebsiella pneumoniae genomes identifies a phospholipase D family protein as a novel virulence factor," *BMC biology,* vol. 12, no. 1, p. 41, 2014.

[22] B. Kocsis and D. Szabó, "Antibiotic resistance mechanisms in Enterobacteriaceae," *Microbial pathogens and strategies for combating them: science, technology and education,* vol. 1, 2013.

[23] J. T. Jacob *et al.*, "Vital signs: carbapenem-resistant Enterobacteriaceae," *Morbidity and mortality weekly report,* vol. 62, no. 9, pp. 165-169, 2013.

[24] L. Diancourt, V. Passet, J. Verhoef, P. A. Grimont, and S. Brisse, "Multilocus sequence typing of Klebsiella pneumoniae nosocomial isolates," *Journal of clinical microbiology,* vol. 43, no. 8, pp. 4178-4182, 2005.

[25] L. Chen, B. Mathema, K. D. Chavda, F. R. DeLeo, R. A. Bonomo, and B. N. Kreiswirth, "Carbapenemase-producing Klebsiella pneumoniae: molecular and genetic decoding," *Trends in microbiology,* vol. 22, no. 12, pp. 686-696, 2014.

[26] F. R. DeLeo *et al.*, "Molecular dissection of the evolution of carbapenem-resistant multilocus sequence type 258 Klebsiella pneumoniae," *Proceedings of the National Academy of Sciences,* vol. 111, no. 13, pp. 4988-4993, 2014.

[27] K. L. Wyres *et al.*, "Extensive capsule locus variation and large-scale genomic recombination within the Klebsiella pneumoniae clonal group 258," *Genome biology and evolution,* vol. 7, no. 5, pp. 1267-1279, 2015.

[28] A. S. Shon and T. A. Russo, "Hypervirulent Klebsiella pneumoniae: the next superbug?," *Future microbiology,* vol. 7, no. 6, pp. 669-671, 2012.

[29] Y. Zhang *et al.*, "High prevalence of hypervirulent Klebsiella pneumoniae infection in China: geographic distribution, clinical characteristics and antimicrobial resistance," *Antimicrobial agents and chemotherapy,* pp. AAC. 01127-16, 2016.

[30] D. Pomakova *et al.*, "Clinical and phenotypic differences between classic and hypervirulent Klebsiella pneumonia: an emerging and under-recognized pathogenic variant," *European journal of clinical microbiology & infectious diseases,* vol. 31, no. 6, pp. 981-989, 2012.

[31] C. Struve *et al.*, "Mapping the evolution of hypervirulent Klebsiella pneumoniae," *MBio,* vol. 6, no. 4, pp. e00630-15, 2015.

[32] X. Nassif, J. Fournier, J. Arondel, and P. Sansonetti, "Mucoid phenotype of Klebsiella pneumoniae is a plasmid-encoded virulence factor," *Infection and immunity,* vol. 57, no. 2, pp. 546-552, 1989.

[33] X. Nassif and P. J. Sansonetti, "Correlation of the virulence of Klebsiella pneumoniae K1 and K2 with the presence of a plasmid encoding aerobactin," *Infection and Immunity,* vol. 54, no. 3, pp. 603-608, 1986.

[34] Y.-T. Chen, H.-Y. Chang, Y.-C. Lai, C.-C. Pan, S.-F. Tsai, and H.-L. Peng, "Sequencing and analysis of the large virulence plasmid pLVPK of Klebsiella pneumoniae CG43," *Gene,* vol. 337, pp. 189-198, 2004.

[35] C.-R. Lee *et al.*, "Antimicrobial resistance of hypervirulent Klebsiella pneumoniae: epidemiology, hypervirulence-associated determinants, and resistance mechanisms," *Frontiers in cellular and infection microbiology,* vol. 7, p. 483, 2017.

[36] R. M. Martin and M. A. Bachman, "Colonization, infection, and the accessory genome of Klebsiella pneumoniae," *Frontiers in cellular and infection microbiology,* vol. 8, p. 4, 2018.

[37] L. Surgers, A. Boyd, P.-M. Girard, G. Arlet, and D. Decré, "ESBL-producing strain of hypervirulent Klebsiella pneumoniae K2, France," *Emerging infectious diseases,* vol. 22, no. 9, p. 1687, 2016.

[38] Y. M. Liu *et al.*, "Clinical and molecular characteristics of emerging hypervirulent Klebsiella pneumoniae bloodstream infections in mainland China," *Antimicrobial agents and chemotherapy*, pp. AAC. 02523-14, 2014.

[39] R. Zhang, D. Lin, E. W.-c. Chan, D. Gu, G.-X. Chen, and S. Chen, "Emergence of carbapenem-resistant Serotype K1 hypervirulent Klebsiella pneumoniae (hvKP) strains in China," *Antimicrobial agents and chemotherapy*, pp. AAC. 02173-15, 2015.

[40] Y. Zhang *et al.*, "Emergence of a hypervirulent carbapenem-resistant Klebsiella pneumoniae isolate from clinical infections in China," *Journal of Infection*, vol. 71, no. 5, pp. 553-560, 2015.

[41] Y. Davies *et al.*, "Virulence and antimicrobial resistance of Klebsiella pneumoniae isolated from passerine and psittacine birds," *Avian Pathology*, vol. 45, no. 2, pp. 194-201, 2016.

[42] A. Tomás *et al.*, "Functional genomic screen identifies Klebsiella pneumoniae factors implicated in blocking NF-κB signalling," *Journal of Biological Chemistry*, p. jbc. M114. 621292, 2015.

[43] G. Cortés, N. Borrell, B. de Astorza, C. Gómez, J. Sauleda, and S. Albertí, "Molecular analysis of the contribution of the capsular polysaccharide and the lipopolysaccharide O side chain to the virulence of Klebsiella pneumoniae in a murine model of pneumonia," *Infection and immunity*, vol. 70, no. 5, pp. 2583-2590, 2002.

[44] Y.-J. Pan *et al.*, "Genetic analysis of capsular polysaccharide synthesis gene clusters in 79 capsular types of Klebsiella spp," *Scientific reports*, vol. 5, p. 15573, 2015.

[45] K. L. Wyres *et al.*, "Identification of Klebsiella capsule synthesis loci from whole genome data," *Microbial genomics*, vol. 2, no. 12, 2016.

[46] S. Brisse *et al.*, "wzi gene sequencing, a rapid method to determine the capsular type of Klebsiella strains," *Journal of clinical microbiology*, pp. JCM. 01924-13, 2013.

[47] W.-L. Yu *et al.,* "Association between rmpA and magA genes and clinical syndromes caused by Klebsiella pneumoniae in Taiwan," *Clinical infectious diseases,* vol. 42, no. 10, pp. 1351-1358, 2006.

[48] K.-M. Yeh *et al.,* "Capsular serotype K1 or K2, rather than magA and rmpA, is a major virulence determinant for Klebsiella pneumoniae liver abscess in Singapore and Taiwan," *Journal of clinical microbiology,* vol. 45, no. 2, pp. 466-471, 2007.

[49] D. Witkowska *et al.,* "Major structural proteins of type 1 and type 3 Klebsiella fimbriae are effective protein carriers and immunogens in conjugates as revealed from their immunochemical characterization," *FEMS Immunology & Medical Microbiology,* vol. 45, no. 2, pp. 221-230, 2005.

[50] C. N. Murphy, M. S. Mortensen, K. A. Krogfelt, and S. Clegg, "Role of Klebsiella pneumoniae type 1 and type 3 fimbriae in colonizing silicone tubes implanted into the bladder of mice as a model of catheter-associated urinary tract infections," *Infection and immunity,* pp. IAI. 00348-13, 2013.

[51] C. A. Broberg, M. Palacios, and V. L. Miller, "Klebsiella: a long way to go towards understanding this enigmatic jet-setter," *F1000prime reports,* vol. 6, 2014.

[52] C. Struve, M. Bojer, and K. A. Krogfelt, "Characterization of Klebsiella pneumoniae type 1 fimbriae by detection of phase variation during colonization and infection and impact on virulence," *Infection and immunity,* vol. 76, no. 9, pp. 4055-4065, 2008.

[53] T. A. S. Sebghati, T. K. Korhonen, D. B. Hornick, and S. Clegg, "Characterization of the type 3 fimbrial adhesins ofKlebsiella strains," *Infection and immunity,* vol. 66, no. 6, pp. 2887-2894, 1998.

[54] M. D. Alcántar-Curiel *et al.*, "Multi-functional analysis of Klebsiella pneumoniae fimbrial types in adherence and biofilm formation," *Virulence,* vol. 4, no. 2, pp. 129-138, 2013.

[55] R. Pal and K. Gokarn, "Siderophores and pathogenecity of microorganisms," *J. Biosci. Tech,* vol. 1, no. 3, pp. 127-134, 2010.

[56] E. Panina, A. Mironov, and M. Gelfand, "Comparative analysis of FUR regulons in gamma-proteobacteria," *Nucleic acids research,* vol. 29, no. 24, pp. 5195-5206, 2001.

[57] M. S. Lawlor, C. O'Connor, and V. L. Miller, "Yersiniabactin is a virulence factor for Klebsiella pneumoniae during pulmonary infection," *Infection and immunity*, vol. 75, no. 3, pp. 1463-1472, 2007.

[58] M. A. Bachman, S. Lenio, L. Schmidt, J. E. Oyler, and J. N. Weiser, "Interaction of lipocalin 2, transferrin, and siderophores determines the replicative niche of Klebsiella pneumoniae during pneumonia," *MBio*, vol. 3, no. 6, pp. e00224-11, 2012.

[59] M. A. Bachman, V. L. Miller, and J. N. Weiser, "Mucosal lipocalin 2 has pro-inflammatory and iron-sequestering effects in response to bacterial enterobactin," *PLoS pathogens*, vol. 5, no. 10, p. e1000622, 2009.

[60] S. I. Müller, M. Valdebenito, and K. Hantke, "Salmochelin, the long-overlooked catecholate siderophore of Salmonella," *Biometals*, vol. 22, no. 4, pp. 691-695, 2009.

[61] T. A. Russo, R. Olson, U. MacDonald, J. Beanan, and B. A. Davidson, "Aerobactin, but not yersiniabactin, salmochelin and enterobactin, enables the growth/survival of hypervirulent (hypermucoviscous) Klebsiella pneumoniae ex vivo and in vivo," *Infection and immunity*, pp. IAI. 00430-15, 2015.

[62] C. R. Raetz and C. Whitfield, "Lipopolysaccharide endotoxins," *Annual review of biochemistry*, vol. 71, no. 1, pp. 635-700, 2002.

[63] J. Kubler-Kielb *et al.*, "The capsular polysaccharide and lipopolysaccharide structures of two carbapenem resistant Klebsiella pneumoniae outbreak isolates," *Carbohydrate research*, vol. 369, pp. 6-9, 2013.

[64] S. Kim *et al.*, "Bilayer properties of lipid A from various Gram-negative bacteria," *Biophysical journal*, vol. 111, no. 8, pp. 1750-1760, 2016.

[65] H. V. Ratajczak, "Klebsiella, infection and immunity," in *Encyclopedic Reference of Immunotoxicology*: Springer, 2005, pp. 365-368.

[66] S. Alberti *et al.*, "C1q binding and activation of the complement classical pathway by Klebsiella pneumoniae outer membrane proteins," *Infection and immunity*, vol. 61, no. 3, pp. 852-860, 1993.

[67] P.-F. Hsieh *et al.*, "Klebsiella pneumoniae peptidoglycan-associated lipoprotein and murein lipoprotein contribute to serum resistance, antiphagocytosis, and proinflammatory cytokine stimulation," *The Journal of infectious diseases,* vol. 208, no. 10, pp. 1580-1589, 2013.

[68] E. Sugawara, S. Kojima, and H. Nikaido, "Klebsiella pneumoniae major porins OmpK35 and OmpK36 allow more efficient diffusion of β-lactams than their Escherichia coli homologs OmpF and OmpC," *Journal of bacteriology,* pp. JB. 00590-16, 2016.

[69] E. Padilla, E. Llobet, A. Doménech-Sánchez, L. Martínez-Martínez, J. A. Bengoechea, and S. Albertí, "Klebsiella pneumoniae AcrAB efflux pump contributes to antimicrobial resistance and virulence," *Antimicrobial agents and chemotherapy,* vol. 54, no. 1, pp. 177-183, 2010.

[70] H.-C. Chou, C.-Z. Lee, L.-C. Ma, C.-T. Fang, S.-C. Chang, and J.-T. Wang, "Isolation of a chromosomal region of Klebsiella pneumoniae associated with allantoin metabolism and liver infection," *Infection and immunity,* vol. 72, no. 7, pp. 3783-3792, 2004.

[71] J. O'Neill, "Review on antimicrobial resistance," *Antimicrobial resistance: tackling a crisis for the health and wealth of nations,* 2014.

[72] S. Santajit and N. Indrawattana, "Mechanisms of antimicrobial resistance in ESKAPE pathogens," *BioMed research international,* vol. 2016, 2016.

[73] S. Navon-Venezia, K. Kondratyeva, and A. Carattoli, "Klebsiella pneumoniae: a major worldwide source and shuttle for antibiotic resistance," *FEMS microbiology reviews,* vol. 41, no. 3, pp. 252-275, 2017.

[74] C. J. von Wintersdorff *et al.*, "Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer," *Frontiers in microbiology,* vol. 7, p. 173, 2016.

[75] R. Li, "Molecular characterization of intrinsic and transferable genetic elements harbored by B-lactams and colistin resistant gram-negative bacterial strains," The Hong Kong Polytechnic University, 2017.

[76] J. M. Blair, M. A. Webber, A. J. Baylay, D. O. Ogbolu, and L. J. Piddock, "Molecular mechanisms of antibiotic resistance," *Nature Reviews Microbiology*, vol. 13, no. 1, p. 42, 2015.

[77] J. A. Olivares Pacheco, A. Bernardini, G. Garcia-Leon, F. Corona, M. B. Sanchez, and J. L. Martinez, "The intrinsic resistome of bacterial pathogens," *Frontiers in microbiology*, vol. 4, p. 103, 2013.

[78] E. Bouza and E. Cercenado, "Klebsiella and enterobacter: antibiotic resistance and treatment implications," in *Seminars in respiratory infections*, 2002, vol. 17, no. 3, pp. 215-230.

[79] E. L. Fonseca, N. da Veiga Ramos, B. G. N. Andrade, L. L. Morais, M. F. A. Marin, and A. C. P. Vicente, "A one-step multiplex PCR to identify Klebsiella pneumoniae, Klebsiella variicola, and Klebsiella quasipneumoniae in the clinical routine," *Diagnostic microbiology and infectious disease*, vol. 87, no. 4, pp. 315-317, 2017.

[80] M. Barlow, "What antimicrobial resistance has taught us about horizontal gene transfer," in *Horizontal Gene Transfer*: Springer, 2009, pp. 397-411.

[81] S. M. Soucy, J. Huang, and J. P. Gogarten, "Horizontal gene transfer: building the web of life," *Nature Reviews Genetics*, vol. 16, no. 8, p. 472, 2015.

[82] A. H. Holmes *et al.*, "Understanding the mechanisms and drivers of antimicrobial resistance," *The Lancet*, vol. 387, no. 10014, pp. 176-187, 2016.

[83] A. P. Magiorakos *et al.*, "Multidrug‐resistant, extensively drug‐resistant and pandrug‐resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance," *Clinical microbiology and infection*, vol. 18, no. 3, pp. 268-281, 2012.

[84] J. D. Pitout, P. Nordmann, and L. Poirel, "Carbapenemase-producing Klebsiella pneumoniae: a key pathogen set for global nosocomial dominance," *Antimicrobial agents and chemotherapy*, pp. AAC. 01019-15, 2015.

[85] H. Yigit *et al.*, "Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of Klebsiella pneumoniae," *Antimicrobial agents and chemotherapy*, vol. 45, no. 4, pp. 1151-1161, 2001.

[86] T. Naas, G. Cuzon, M.-V. Villegas, M.-F. Lartigue, J. P. Quinn, and P. Nordmann, "Genetic structures at the origin of acquisition of the β-lactamase blaKPC gene," *Antimicrobial agents and chemotherapy,* vol. 52, no. 4, pp. 1257-1263, 2008.

[87] C.-R. Lee, J. H. Lee, K. S. Park, Y. B. Kim, B. C. Jeong, and S. H. Lee, "Global dissemination of carbapenemase-producing Klebsiella pneumoniae: epidemiology, genetic context, treatment options, and detection methods," *Frontiers in microbiology,* vol. 7, p. 895, 2016.

[88] P. Shen *et al.*, "Novel genetic environment of the carbapenem-hydrolyzing β-lactamase KPC-2 among Enterobacteriaceae in China," *Antimicrobial agents and chemotherapy,* vol. 53, no. 10, pp. 4333-4338, 2009.

[89] R. Zhang *et al.*, "Nationwide surveillance of clinical carbapenem-resistant Enterobacteriaceae (CRE) strains in China," *EBioMedicine,* vol. 19, pp. 98-106, 2017.

[90] J. Rodríguez-Baño, B. Gutiérrez-Gutiérrez, I. Machuca, and A. Pascual, "Treatment of infections caused by extended-spectrum-beta-lactamase-, AmpC-, and carbapenemase-producing Enterobacteriaceae," *Clinical microbiology reviews,* vol. 31, no. 2, pp. e00079-17, 2018.

[91] L. Villa, C. Feudi, D. Fortini, A. García-Fernández, and A. Carattoli, "Genomics of KPC-producing Klebsiella pneumoniae sequence type 512 clone highlights the role of RamR and ribosomal S10 protein mutations in conferring tigecycline resistance," *Antimicrobial agents and chemotherapy,* vol. 58, no. 3, pp. 1707-1712, 2014.

[92] M. Hentschke, M. Wolters, I. Sobottka, H. Rohde, and M. Aepfelbacher, "ramR mutations in clinical isolates of Klebsiella pneumoniae with reduced susceptibility to tigecycline," *Antimicrobial agents and chemotherapy,* vol. 54, no. 6, pp. 2720-2723, 2010.

[93] S. Roy, S. Datta, R. Viswanathan, A. K. Singh, and S. Basu, "Tigecycline susceptibility in Klebsiella pneumoniae and Escherichia coli causing neonatal septicaemia (2007–10) and role of an efflux pump in tigecycline non-susceptibility," *Journal of Antimicrobial Chemotherapy,* vol. 68, no. 5, pp. 1036-1042, 2013.

[94] A. Ruzin, M. A. Visalli, D. Keeney, and P. A. Bradford, "Influence of transcriptional activator RamA on expression of multidrug efflux pump AcrAB and tigecycline susceptibility in Klebsiella pneumoniae," *Antimicrobial agents and chemotherapy,* vol. 49, no. 3, pp. 1017-1022, 2005.

[95] M. E. Pitt *et al.*, "Multifactorial chromosomal variants regulate polymyxin resistance in extensively drug-resistant Klebsiella pneumoniae," *Microbial genomics,* vol. 4, no. 3, 2018.

[96] M. S. Wright *et al.*, "Genomic and transcriptomic analyses of colistin-resistant clinical isolates of Klebsiella pneumoniae reveal multiple pathways of resistance," *Antimicrobial agents and chemotherapy,* vol. 59, no. 1, pp. 536-543, 2015.

[97] Y.-H. Cheng, T.-L. Lin, Y.-T. Lin, and J.-T. Wang, "Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in Klebsiella pneumoniae," *Antimicrobial agents and chemotherapy,* pp. AAC. 00009-16, 2016.

[98] L. Poirel *et al.*, "The mgrB gene as a key target for acquired resistance to colistin in Klebsiella pneumoniae," *Journal of Antimicrobial Chemotherapy,* vol. 70, no. 1, pp. 75-80, 2014.

[99] A. Cannatelli *et al.*, "MgrB inactivation is a common mechanism of colistin resistance in KPC carbapenemase-producing Klebsiella pneumoniae of clinical origin," *Antimicrobial agents and chemotherapy,* pp. AAC. 03110-14, 2014.

[100] J. Kluytmans, "Plasmid-encoded colistin resistance: mcr-one, two, three and counting," *Eurosurveillance,* vol. 22, no. 31, 2017.

[101] J. Hawkey *et al.*, "ISMapper: identifying transposase insertion sites in bacterial genomes from short read sequence data," *BMC genomics,* vol. 16, no. 1, p. 667, 2015.

[102] J. M. Heather and B. Chain, "The sequence of sequencers: the history of sequencing DNA," *Genomics,* vol. 107, no. 1, pp. 1-8, 2016.

[103] S. Goodwin, J. D. McPherson, and W. R. McCombie, "Coming of age: ten years of next-generation sequencing technologies," *Nature Reviews Genetics,* vol. 17, no. 6, p. 333, 2016.

[104] C. Børsting and N. Morling, "Next generation sequencing and its applications in forensic genetics," *Forensic Science International: Genetics,* vol. 18, pp. 78-89, 2015.

[105] B. E. Slatko, A. F. Gardner, and F. M. Ausubel, "Overview of Next‐Generation Sequencing Technologies," *Current protocols in molecular biology,* vol. 122, no. 1, p. e59, 2018.

[106] M. Kchouk, J.-F. Gibrat, and M. Elloumi, "Generations of sequencing technologies: From first to next generation," *Biology and Medicine,* vol. 9, no. 3, 2017.

[107] S. Bialek-Davenet *et al.,* "Genomic definition of hypervirulent and multidrug-resistant Klebsiella pneumoniae clonal groups," *Emerging infectious diseases,* vol. 20, no. 11, p. 1812, 2014.

[108] M. M. Lam *et al.,* "Genetic diversity, mobilisation and spread of the yersiniabactin-encoding mobile element ICEKp in Klebsiella pneumoniae populations," *Microbial genomics,* 2018.

[109] M. M. Lam *et al.,* "Tracking key virulence loci encoding aerobactin and salmochelin siderophore synthesis in Klebsiella pneumoniae," *bioRxiv,* p. 376236, 2018.

[110] R. R. Wick, E. Heinz, K. E. Holt, and K. L. Wyres, "Kaptive Web: user-friendly capsule and lipopolysaccharide serotype prediction for Klebsiella genomes," *Journal of clinical microbiology,* pp. JCM. 00197-18, 2018.

[111] I. Letunic and P. Bork, "Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees," *Nucleic acids research,* vol. 44, no. W1, pp. W242-W245, 2016.

[112] M. V. Larsen *et al.,* "Multilocus sequence typing of total genome sequenced bacteria," *Journal of clinical microbiology,* pp. JCM. 06094-11, 2012.

[113] K. G. Joensen *et al.,* "Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli," *Journal of Clinical Microbiology,* vol. 52, no. 5, pp. 1501-1510, 2014.

[114] E. Zankari *et al.,* "Identification of acquired antimicrobial resistance genes," *Journal of antimicrobial chemotherapy,* vol. 67, no. 11, pp. 2640-2644, 2012.

[115] A. Carattoli *et al.*, "PlasmidFinder and pMLST: in silico detection and typing of plasmids," *Antimicrobial Agents and Chemotherapy*, pp. AAC. 02412-14, 2014.

[116] M. Inouye *et al.*, "SRST2: Rapid genomic surveillance for public health and hospital microbiology labs," *Genome medicine*, vol. 6, no. 11, p. 90, 2014.

[117] P. Siguier, J. Pérochon, L. Lestrade, J. Mahillon, and M. Chandler, "ISfinder: the reference centre for bacterial insertion sequences," *Nucleic acids research*, vol. 34, no. suppl_1, pp. D32-D36, 2006.

[118] R. D. Fleischmann *et al.*, "Whole-genome random sequencing and assembly of Haemophilus influenzae Rd," *Science*, vol. 269, no. 5223, pp. 496-512, 1995.

[119] S. J. Salipante, D. J. SenGupta, L. A. Cummings, T. A. Land, D. R. Hoogestraat, and B. T. Cookson, "Application of whole genome sequencing for bacterial strain typing in molecular epidemiology," *Journal of clinical microbiology*, pp. JCM. 03385-14, 2015.

[120] K.-M. Wu *et al.*, "Genome sequencing and comparative analysis of Klebsiella pneumoniae NTUH-K2044, a strain causing liver abscess and meningitis," *Journal of bacteriology*, vol. 191, no. 14, pp. 4492-4501, 2009.

[121] D. E. Fouts *et al.*, "Complete genome sequence of the N2-fixing broad host range endophyte Klebsiella pneumoniae 342 and virulence predictions verified in mice," *PLoS genetics*, vol. 4, no. 7, p. e1000141, 2008.

[122] M. McClelland *et al.*, "Complete genome sequence of Salmonella enterica serovar Typhimurium LT2," *Nature*, vol. 413, no. 6858, p. 852, 2001.

[123] N. Stoesser *et al.*, "Genome sequencing of an extended series of NDM-Klebsiella pneumoniae neonatal infections in a Nepali hospital characterizes the extent of community versus hospital-associated transmission in an endemic setting," *Antimicrobial agents and chemotherapy*, pp. AAC. 03900-14, 2014.

[124] A. J. Mathers *et al.*, "Klebsiella pneumoniae carbapenemase (KPC) producing K. pneumoniae at a Single Institution: Insights into Endemicity from Whole Genome Sequencing," *Antimicrobial agents and chemotherapy*, pp. AAC. 04292-14, 2015.

[125] B. A. Espedido *et al.*, "Whole genome sequence analysis of the first Australian OXA-48-producing outbreak-associated Klebsiella pneumoniae isolates: the resistome and in vivo evolution," *PLoS One*, vol. 8, no. 3, p. e59920, 2013.

[126] X. Wang *et al.*, "Whole-Genome-Sequencing characterization of bloodstream infection-causing hypervirulent Klebsiella pneumoniae of capsular serotype K2 and ST374," *Virulence*, vol. 9, no. 1, pp. 510-521, 2018.

[127] K. Zhou *et al.*, "Use of whole-genome sequencing to trace, control and characterize the regional expansion of extended-spectrum β-lactamase producing ST15 Klebsiella pneumoniae," *Scientific reports*, vol. 6, p. 20840, 2016.

[128] M. M. Lam *et al.*, "Population genomics of hypervirulent Klebsiella pneumoniae clonal-group 23 reveals early emergence and rapid global dissemination," *Nature communications*, vol. 9, no. 1, p. 2703, 2018.

[129] J. R. Bowers *et al.*, "Genomic analysis of the emergence and rapid global dissemination of the clonal group 258 Klebsiella pneumoniae pandemic," *PLoS One*, vol. 10, no. 7, p. e0133727, 2015.

[130] L. S. Munoz-Price and J. P. Quinn, "The spread of Klebsiella pneumoniae carbapenemases: a tale of strains, plasmids, and transposons," ed: The University of Chicago Press, 2009.

[131] G. Cuzon *et al.*, "Worldwide diversity of Klebsiella pneumoniae that produce β-lactamase blaKPC-2 gene," *Emerging infectious diseases*, vol. 16, no. 9, p. 1349, 2010.

[132] L. S. Munoz-Price *et al.*, "Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases," *The Lancet infectious diseases*, vol. 13, no. 9, pp. 785-796, 2013.

[133] Y. Qi, Z. Wei, S. Ji, X. Du, P. Shen, and Y. Yu, "ST11, the dominant clone of KPC-producing Klebsiella pneumoniae in China," *Journal of Antimicrobial Chemotherapy*, vol. 66, no. 2, pp. 307-312, 2010.

[134] S. Gaiarsa *et al.*, "Genomic epidemiology of Klebsiella pneumoniae in Italy and novel insights into the origin and global evolution of its resistance to carbapenem antibiotics," *Antimicrobial agents and chemotherapy*, vol. 59, no. 1, pp. 389-396, 2015.

[135] L. Chen, B. Mathema, J. D. Pitout, F. R. DeLeo, and B. N. Kreiswirth, "Epidemic Klebsiella pneumoniae ST258 is a hybrid strain," *MBio*, vol. 5, no. 3, pp. e01355-14, 2014.

[136] P. Liu *et al.*, "Complete genome sequence of Klebsiella pneumoniae subsp. pneumoniae HS11286, a multidrug-resistant strain isolated from human sputum," *Journal of bacteriology,* vol. 194, no. 7, pp. 1841-1842, 2012.

[137] D. Bi *et al.*, "Mapping the resistance-associated mobilome of a carbapenem-resistant Klebsiella pneumoniae strain reveals insights into factors shaping these regions and facilitates generation of a 'resistance-disarmed'model organism," *Journal of Antimicrobial Chemotherapy,* vol. 70, no. 10, pp. 2770-2774, 2015.

[138] Y. Jiang, Z. Wei, Y. Wang, X. Hua, Y. Feng, and Y. Yu, "Tracking a hospital outbreak of KPC-producing ST11 Klebsiella pneumoniae with whole genome sequencing," *Clinical Microbiology and Infection,* vol. 21, no. 11, pp. 1001-1007, 2015.

[139] Clinical and L. S. Institute, "Performance Standards for Antimicrobial Testing: Twenty-Sixth Informational Supplement M100-S26," ed: CLSI Wayne, PA, USA, 2016.

[140] D. Antipov, A. Korobeynikov, J. S. McLean, and P. A. Pevzner, "hybridSPAdes: an algorithm for hybrid assembly of short and long reads," *Bioinformatics,* vol. 32, no. 7, pp. 1009-1015, 2015.

[141] A. Bankevich *et al.*, "SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing," *Journal of computational biology,* vol. 19, no. 5, pp. 455-477, 2012.

[142] R. Overbeek *et al.*, "The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST)," *Nucleic acids research,* vol. 42, no. D1, pp. D206-D214, 2013.

[143] T. Seemann, "Prokka: rapid prokaryotic genome annotation," *Bioinformatics,* vol. 30, no. 14, pp. 2068-2069, 2014.

[144] Y. Zhou, Y. Liang, K. H. Lynch, J. J. Dennis, and D. S. Wishart, "PHAST: a fast phage search tool," *Nucleic acids research,* vol. 39, no. suppl_2, pp. W347-W352, 2011.

[145] M. M. Lam *et al.*, "Frequent emergence of pathogenic lineages of Klebsiella pneumoniae via mobilisation of yersiniabactin and colibactin," *bioRxiv,* p. 098178, 2017.

[146] J. Liu *et al.*, "Emergence and establishment of KPC-2-producing ST11 Klebsiella pneumoniae in a general hospital in Shanghai, China," *European Journal of Clinical Microbiology & Infectious Diseases*, vol. 37, no. 2, pp. 293-299, 2018.

[147] L. Zhang, Y. Li, W. Shen, S. M. Wang, G. Wang, and Y. Zhou, "Whole-genome sequence of a carbapenem-resistant hypermucoviscous Klebsiella pneumoniae isolate SWU01 with capsular serotype K47 belonging to ST11 from a patient in China," *Journal of global antimicrobial resistance*, vol. 11, pp. 87-89, 2017.

[148] Z. Yao, Y. Feng, L. Wei, and Z. Zong, "Draft Genome Sequence of a Sequence Type 11 Klebsiella pneumoniae Clinical Strain Carrying a blaKPC-2 Carbapenemase Gene and an rmtB 16S rRNA Methylase Gene," *Genome announcements*, vol. 5, no. 6, pp. e01549-16, 2017.

[149] M. Ye *et al.*, "Clinical and genomic analysis of liver abscess-causing Klebsiella pneumoniae identifies new liver abscess-associated virulence genes," *Frontiers in cellular and infection microbiology*, vol. 6, p. 165, 2016.

[150] J. Zhu, R. Jiang, X. Weng, W. Kangle, and H. Kong, "Whole-genome sequence-based analysis of Klebsiella pneumoniae JM45," *Chinese Journal of Clinical Infectious Diseases*, vol. 7, no. 1, pp. 27-33, 2014.

[151] T. J. Treangen, B. D. Ondov, S. Koren, and A. M. Phillippy, "The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes," *Genome biology*, vol. 15, no. 11, p. 524, 2014.

[152] A. J. Page *et al.*, "Roary: rapid large-scale prokaryote pan genome analysis," *Bioinformatics*, vol. 31, no. 22, pp. 3691-3693, 2015.

[153] N.-F. Alikhan, N. K. Petty, N. L. B. Zakour, and S. A. Beatson, "BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons," *BMC genomics*, vol. 12, no. 1, p. 1, 2011.

[154] M. J. Sullivan, N. K. Petty, and S. A. Beatson, "Easyfig: a genome comparison visualizer," *Bioinformatics*, vol. 27, no. 7, pp. 1009-1010, 2011.

[155] Y.-J. Pan *et al.*, "Identification of capsular types in carbapenem-resistant Klebsiella pneumoniae strains by wzc sequencing and implications for capsule depolymerase treatment," *Antimicrobial agents and chemotherapy*, vol. 59, no. 2, pp. 1038-1047, 2015.

[156] M. M. D'Andrea *et al.*, "Diversity of capsular polysaccharide gene clusters in Kpc-producing Klebsiella pneumoniae clinical isolates of sequence type 258 involved in the Italian epidemic," *PLoS One,* vol. 9, no. 5, p. e96827, 2014.

[157] C. M. Hudson, Z. W. Bent, R. J. Meagher, and K. P. Williams, "Resistance determinants and mobile genetic elements of an NDM-1-encoding Klebsiella pneumoniae strain," *PloS one,* vol. 9, no. 6, p. e99209, 2014.

[158] P. I. P. Ramos *et al.*, "Pyrosequencing-based analysis reveals a novel capsular gene cluster in a KPC-producing Klebsiella pneumoniae clinical isolate identified in Brazil," *BMC microbiology,* vol. 12, no. 1, p. 173, 2012.

[159] C. Whitfield, "Biosynthesis and assembly of capsular polysaccharides in Escherichia coli," *Annu. Rev. Biochem.,* vol. 75, pp. 39-68, 2006.

[160] L. N. Andrade, L. Vitali, G. G. Gaspar, F. Bellissimo-Rodrigues, R. Martinez, and A. L. C. Darini, "Expansion and Evolution of a Virulent, Extensively Drug-Resistant (Polymyxin B-Resistant), QnrS1-, CTX-M-2-and KPC-2-producing Klebsiella pneumoniae ST11 International High-risk Clone," *Journal of clinical microbiology,* pp. JCM. 00088-14, 2014.

[161] Y. Doi *et al.*, "Whole genome assembly of Klebsiella pneumoniae co-producing NDM-1 and OXA-232 carbapenemases using single-molecule, real-time sequencing," *Antimicrobial agents and chemotherapy,* pp. AAC. 03180-14, 2014.

[162] P. I. P. Ramos *et al.*, "Comparative analysis of the complete genome of KPC-2-producing Klebsiella pneumoniae Kp13 reveals remarkable genome plasticity and a wide repertoire of virulence and resistance mechanisms," *BMC genomics,* vol. 15, no. 1, p. 54, 2014.

[163] L. Naparstek, Y. Carmeli, S. Navon-Venezia, and E. Banin, "Biofilm formation and susceptibility to gentamicin and colistin of extremely drug-resistant KPC-producing Klebsiella pneumoniae," *Journal of Antimicrobial Chemotherapy,* vol. 69, no. 4, pp. 1027-1034, 2014.

[164] R. El Fertas-Aissani, Y. Messai, S. Alouache, and R. Bakour, "Virulence profiles and antibiotic susceptibility patterns of Klebsiella pneumoniae strains isolated from different clinical specimens," *Pathologie Biologie,* vol. 61, no. 5, pp. 209-216, 2013.

[165] X. Wang *et al.*, "Increased prevalence of carbapenem resistant Enterobacteriaceae in hospital setting due to cross-species transmission of the blaNDM-1 element and clonal spread of progenitor resistant strains," *Frontiers in microbiology,* vol. 6, p. 595, 2015.

[166] D. Gu *et al.*, "A fatal outbreak of ST11 carbapenem-resistant hypervirulent Klebsiella pneumoniae in a Chinese hospital: a molecular epidemiological study," *The Lancet Infectious Diseases,* vol. 18, no. 1, pp. 37-46, 2018.

[167] B. L. Meatherall, D. Gregson, T. Ross, J. D. Pitout, and K. B. Laupland, "Incidence, risk factors, and outcomes of Klebsiella pneumoniae bacteremia," *The American journal of medicine,* vol. 122, no. 9, pp. 866-873, 2009.

[168] Y.-T. Lin *et al.*, "Pyogenic liver abscess as the initial manifestation of underlying hepatocellular carcinoma," *The American journal of medicine,* vol. 124, no. 12, pp. 1158-1164, 2011.

[169] M. D. Alcántar-Curiel and J. A. Girón, "Klebsiella pneumoniae and the pyogenic liver abscess: implications and association of the presence of rpmA genes and expression of hypermucoviscosity," *Virulence,* vol. 6, no. 5, pp. 407-409, 2015.

[170] H. Grundmann *et al.*, "Occurrence of carbapenemase-producing Klebsiella pneumoniae and Escherichia coli in the European survey of carbapenemase-producing Enterobacteriaceae (EuSCAPE): a prospective, multinational study," *The Lancet Infectious Diseases,* vol. 17, no. 2, pp. 153-163, 2017.

[171] J. F. Turton, C. Perry, S. Elgohari, and C. V. Hampton, "PCR characterization and typing of Klebsiella pneumoniae using capsular type-specific, variable number tandem repeat and virulence gene targets," *Journal of medical microbiology,* vol. 59, no. 5, pp. 541-547, 2010.

[172] Y. Huang *et al.*, "Widespread dissemination of carbapenem-resistant Escherichia coli ST167 strains harboring the blaNDM-5 in clinical settings in China," *Antimicrobial agents and chemotherapy,* pp. AAC. 00859-16, 2016.

[173] M. El-Mansi, K. J. Anderson, C. A. Inche, L. K. Knowles, and D. J. Platt, "Isolation and curing of the Klebsiella pneumoniae large indigenous plasmid using sodium dodecyl sulphate," *Research in microbiology,* vol. 151, no. 3, pp. 201-208, 2000.

[174] E. Diago-Navarro *et al.*, "Carbapenem-resistant Klebsiella pneumoniae exhibit variability in capsular polysaccharide and capsule associated virulence traits," *The Journal of infectious diseases,* vol. 210, no. 5, pp. 803-813, 2014.

[175] M. M. McLaughlin, M. R. Advincula, M. Malczynski, G. Barajas, C. Qi, and M. H. Scheetz, "Quantifying the clinical virulence of Klebsiella pneumoniae producing carbapenemase Klebsiella pneumoniae with a *Galleria mellonella* model and a pilot study to translate to patient outcomes," *BMC infectious diseases,* vol. 14, no. 1, p. 31, 2014.

[176] R. Li *et al.*, "Efficient generation of complete sequences of MDR-encoding plasmids by rapid assembly of MinION barcoding sequencing data," *GigaScience,* vol. 7, no. 3, p. gix132, 2018.

[177] R. R. Wick, L. M. Judd, C. L. Gorrie, and K. E. Holt, "Unicycler: resolving bacterial genome assemblies from short and long sequencing reads," *PLoS computational biology,* vol. 13, no. 6, p. e1005595, 2017.

[178] A. Sturn, J. Quackenbush, and Z. Trajanoski, "Genesis: cluster analysis of microarray data," *Bioinformatics,* vol. 18, no. 1, pp. 207-208, 2002.

[179] W.-L. Yu, M.-F. Lee, H.-J. Tang, M.-C. Chang, and Y.-C. Chuang, "Low prevalence of rmpA and high tendency of rmpA mutation correspond to low virulence of extended spectrum β-lactamase-producing Klebsiella pneumoniae isolates," *Virulence,* vol. 6, no. 2, pp. 162-172, 2015.

[180] I. R. Lee *et al.*, "Differential host susceptibility and bacterial virulence factors driving Klebsiella liver abscess in an ethnically diverse population," *Scientific reports,* vol. 6, p. 29316, 2016.

[181] R. Li *et al.*, "Genetic characterization of mcr-1-bearing plasmids to depict molecular mechanisms underlying dissemination of the colistin resistance determinant," *Journal of Antimicrobial Chemotherapy,* vol. 72, no. 2, pp. 393-401, 2016.

[182] A. A. J. Aljanaby and A. H. A. Alhasani, "Virulence factors and antibiotic susceptibility patterns of multidrug resistance Klebsiella pneumoniae isolated from different clinical infections," *African Journal of Microbiology Research,* vol. 10, no. 22, pp. 829-843, 2016.

[183] H.-L. Tang *et al.*, "Correlation between Klebsiella pneumoniae carrying pLVPK-derived loci and abscess formation," *European journal of clinical microbiology & infectious diseases,* vol. 29, no. 6, pp. 689-698, 2010.

[184] B. J. Walker *et al.*, "Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement," *PloS one,* vol. 9, no. 11, p. e112963, 2014.

[185] L. Chen *et al.*, "Complete sequence of a KPC-producing IncN multidrug-resistant plasmid from an epidemic Escherichia coli ST131 strain in China," *Antimicrobial agents and chemotherapy,* pp. AAC. 02587-13, 2014.

[186] S. He *et al.*, "Insertion sequence IS26 reorganizes plasmids in clinically isolated multidrug-resistant bacteria by replicative transposition," *MBio,* vol. 6, no. 3, pp. e00762-15, 2015.

[187] A. M. Queenan and K. Bush, "Carbapenemases: the versatile β-lactamases," *Clinical microbiology reviews,* vol. 20, no. 3, pp. 440-458, 2007.

[188] Y. Matsumura *et al.*, "Genomic epidemiology of global VIM-producing Enterobacteriaceae," *Journal of Antimicrobial Chemotherapy,* vol. 72, no. 8, pp. 2249-2258, 2017.

[189] X. Zhang, D. Chen, G. Xu, W. Huang, and X. Wang, "Molecular epidemiology and drug resistant mechanism in carbapenem-resistant Klebsiella pneumoniae isolated from pediatric patients in Shanghai, China," *PloS one,* vol. 13, no. 3, p. e0194000, 2018.

[190] L. Chen and B. N. Kreiswirth, "Convergence of carbapenem-resistance and hypervirulence in Klebsiella pneumoniae," *The Lancet Infectious Diseases,* vol. 18, no. 1, pp. 2-3, 2018.

[191] B. J. Wiskur, J. J. Hunt, and M. C. Callegan, "Hypermucoviscosity as a virulence factor in experimental Klebsiella pneumoniae endophthalmitis," *Investigative ophthalmology & visual science,* vol. 49, no. 11, pp. 4931-4938, 2008.

[192] M. E. Wand, C. M. Müller, R. W. Titball, and S. L. Michell, "Macrophage and *Galleria mellonella* infection models reflect the virulence of naturally occurring isolates of B. pseudomallei, B. thailandensis and B. oklahomensis," *BMC microbiology,* vol. 11, no. 1, p. 1, 2011.

[193] R. Li, D. Lin, K. Chen, M. H. Y. Wong, and S. Chen, "First detection of AmpC β-lactamase blaCMY-2 on a conjugative IncA/C plasmid in Vibrio parahaemolyticus of food origin," *Antimicrobial agents and chemotherapy*, pp. AAC. 05008-14, 2015.

[194] B. Yao, X. Xiao, F. Wang, L. Zhou, X. Zhang, and J. Zhang, "Clinical and molecular characteristics of multi-clone carbapenem-resistant hypervirulent (hypermucoviscous) Klebsiella pneumoniae isolates in a tertiary hospital in Beijing, China," *International Journal of Infectious Diseases*, vol. 37, pp. 107-112, 2015.

[195] J. L. Insua *et al.*, "Modelling Klebsiella pneumoniae pathogenesis by infecting the wax moth *Galleria mellonella*," *Infection and immunity*, pp. IAI. 00391-13, 2013.

[196] N. Dong *et al.*, "Genome analysis of clinical multilocus sequence Type 11 Klebsiella pneumoniae from China," *Microbial genomics*, vol. 4, no. 2, 2018.

[197] P. Du, Y. Zhang, and C. Chen, "Emergence of carbapenem-resistant hypervirulent Klebsiella pneumoniae," *The Lancet Infectious Diseases*, vol. 18, no. 1, pp. 23-24, 2018.

[198] C. J. Harmer, R. A. Moran, and R. M. Hall, "Movement of IS26-associated antibiotic resistance genes occurs via a translocatable unit that includes a single IS26 and preferentially inserts adjacent to another IS26," *MBio*, vol. 5, no. 5, pp. e01801-14, 2014.

[199] C. J. Harmer and R. M. Hall, "IS26-mediated formation of transposons carrying antibiotic resistance genes," *mSphere*, vol. 1, no. 2, pp. e00038-16, 2016.

[200] M. Rozwandowicz *et al.*, "Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae," *Journal of Antimicrobial Chemotherapy*, vol. 73, no. 5, pp. 1121-1137, 2018.

[201] Y.-T. Chen *et al.*, "KPC-2-encoding plasmids from Escherichia coli and Klebsiella pneumoniae in Taiwan," *Journal of Antimicrobial Chemotherapy*, vol. 69, no. 3, pp. 628-631, 2013.

[202] D. Fortini *et al.*, "Double copies of blaKPC-3:: Tn4401a on an IncX3 plasmid in Klebsiella pneumoniae successful clone ST512 from Italy," *Antimicrobial agents and chemotherapy*, vol. 60, no. 1, pp. 646-649, 2016.

[203] B. Kitchel *et al.*, "Genetic factors associated with elevated carbapenem resistance in KPC-producing Klebsiella pneumoniae," *Antimicrobial agents and chemotherapy*, vol. 54, no. 10, pp. 4201-4207, 2010.

[204] T. D. Gootz *et al.*, "Genetic organization of transposase regions surrounding blaKPC carbapenemase genes on plasmids from Klebsiella strains isolated in a New York City hospital," *Antimicrobial agents and chemotherapy*, vol. 53, no. 5, pp. 1998-2004, 2009.

[205] A. E. Sheppard *et al.*, "Nested Russian doll-like genetic mobility drives rapid dissemination of the carbapenem resistance gene blaKPC," *Antimicrobial agents and chemotherapy*, pp. AAC. 00464-16, 2016.

[206] A. J. Mathers *et al.*, "Chromosomal integration of the Klebsiella pneumoniae carbapenemase gene, blaKPC, in Klebsiella species is elusive but not rare," *Antimicrobial agents and chemotherapy*, vol. 61, no. 3, pp. e01823-16, 2017.

[207] R. A. Weingarten *et al.*, "Genomic analysis of hospital plumbing reveals diverse reservoir of bacterial plasmids conferring carbapenem resistance," *MBio*, vol. 9, no. 1, pp. e02011-17, 2018.

[208] S. Conlan *et al.*, "Single-molecule sequencing to track plasmid diversity of hospital-associated carbapenemase-producing Enterobacteriaceae," *Science translational medicine*, vol. 6, no. 254, pp. 254ra126-254ra126, 2014.