Engineering 12 (2022) 90–100

Contents lists available at ScienceDirect

Engineering

journal homepage: www.elsevier.com/locate/eng

Research
Antimicrobial Resistance—Article

Genomic and Phenotypic Diversity of Carbapenemase-Producing Enterobacteriaceae Isolates from Bacteremia in China: A Multicenter Epidemiological, Microbiological, and Genetic Study

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ARTICLE INFO

Article history:
Received 30 April 2020
Revised 7 September 2020
Accepted 9 October 2020
Available online 25 December 2020

Keywords:
Carbapenemase
Carbapenemase-producing
Enterobacteriaceae
Plasmid-mediated
China
Extended-spectrum β-lactamase

ABSTRACT

Carbapenemase-producing Enterobacteriaceae (CPE) isolates are recognized as one of the most severe threats to public health. However, the population structure and genetic characteristics of CPE isolates among bloodstream infections (BSIs) are largely unknown. To address this knowledge gap, in this study, we included patients with clinically significant BSIs due to Enterobacterales isolates, recruited from 26 sentinel hospitals in China (2014–2015). CPE isolates were microbiologically and genomically characterized, including their susceptibility profiles, molecular typing, phylogenetic features, and genetic context analysis of carbapenemase-encoding genes. Of the 2569 BSI Enterobacterales isolates enrolled, 42 (1.6%) were carbapenemase-positive. Moreover, among the 2242 investigated isolates, 1111 (49.6%) extended-spectrum β-lactamase (ESBL)-producing isolates were identified in *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Proteus mirabilis* (*P. mirabilis*), and *Klebsiella oxytoca*. Whole genome sequencing analysis showed the clonal spread of *K. pneumoniae* carbapenemase (KPC)-2-producing *K. pneumoniae* sequence type (ST) 11 and New Delhi metallo-β-lactamase (NDM)-5-producing *E. coli* ST167 in our collection. Plasmid analysis revealed that carbapenemase-encoding genes were located on multiple plasmids. A high prevalence of biofilm-encoding type 3 fimbriae clusters and yesiniabactin-associated genes was observed in *K. pneumoniae* isolates. This work demonstrates the high prevalence of ESBLs and the wide dissemination of CPE among BSI isolates in China, which represent real clinical threats. Moreover, our findings first illustrate a more comprehensive genome scenario of CPE isolates among BSIs. The clonal spread of KPC-2-producing *K. pneumoniae* ST11 and NDM-5-producing *E. coli* ST167 needs to be closely monitored.

1. Introduction

The emergence and spread of carbapenem resistance, especially in extended-spectrum β-lactamase (ESBL)-producing bacteria, are of particular clinical relevance [1,2]. Recently, carbapenem-resistant Enterobacteriaceae (CRE) were listed as the most critical group of pathogens by the World Health Organization [3], indicating that they have emerged as a global threat to the antibiotic era [4]. Among these microorganisms, carbapenemase-producing Enterobacteriaceae (CPE) are recognized as the most worrying threat due to the limited therapeutic options against these pathogens and their mobility [5,6]. As per the literature, concomitant use of antimicrobial agents and proton pump inhibitors prolongs the duration of the gastrointestinal colonization by CPE [7]. Our recent work also revealed that antibiotic exposure, surgical history, and CPE positivity are correlated [8].

Bloodstream infections (BSIs) are major causes of infectious diseases worldwide and have become one of the leading causes of death in developed countries [9,10]. Approximately 9300 healthcare-associated infections are caused by CPE each year in the United

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https://doi.org/10.1016/j.eng.2020.10.015

States, and almost half of the hospitalized patients with BSIs due to CPE die from the infection [11]. Therefore, it is particularly important to understand how resistance elements spread between strains and the extent of transmission that goes undetected between cases [11]. However, only a few large-scale resistance surveillance programs have targeted the testing of BSI isolates [12].

In China, an increasing incidence of CPE-mediated infections has been observed over the past decade [13]. To date, only a few studies have reported the national surveillance of CPE isolates [13–15]. A high prevalence of ESBL-producing Enterobacteriaceae in BSIs in China was reported recently; however, the detection of CPE was not described in that study [16]. In our previous study, we also reported the preliminary data of BSIs caused by Enterobacteriaceae in China [17]. The vast majority of these studies were based on epidemiological data or Sanger sequencing data; thus, those studies were not able to perform comparative genomics analysis to track the dissemination and transmission of such strains or antimicrobial resistance genes (ARGs).

Thus far, the population structure and genetic characteristics of CPE isolates among BSIs remain largely unknown. To address this knowledge gap, we recruited patients with clinically significant BSIs caused by Enterobacteriaceae isolates from China. Moreover, a comprehensive phenotypic characterization was conducted on Enterobacteriaceae isolates causing BSIs. We further employed the whole genome sequencing (WGS) and plasmid analysis methods in an effort to obtain a snapshot of the epidemiology of CPE among and within hospitals. Our findings help in understanding the implications of the dissemination of resistance within and between species and geographical areas in China.

2. Materials and methods

2.1. Study design, setting, and bacterial isolates

The Consortium of Blood Bacterial Resistant Investigation Collaborative Systems (BRICS) is a prospective, multicenter, and observational consortium for tracking antimicrobial resistance among BSI-causing isolates in China since 2014 [17]. Patients with BSI judged to be clinically significant were identified by the responsible clinical microbiologist of the sentinel hospital. As part of this program, all nonduplicate BSI isolates were collected from 26 sentinel hospitals located in 21 cities (Fig. 1) between January 2014 and December 2015 in this study. Outpatients or patients with incomplete data were excluded. All isolates were sent to the central laboratory of the First Affiliated Hospital of Zhejiang University to be tested and analyzed. Clinical data were extracted using centralized queries from clinical and medical record systems used for patients with CPE infections. Ethical approval was granted by the Ethics Committee of the First Affiliated Hospital of Zhejiang University. Individual consent was obtained from all patients either face-to-face or by phone.

2.2. Identification, antimicrobial susceptibility testing, and molecular biology

Bacterial species were identified using matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) (Bruker Daltonics, Germany). Antimicrobial susceptibility testing (AST) was done using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) standards [18]. AST of colistin and tigecycline was performed using the microbroth dilution method as described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[†]. The

[†] http://www.eucast.org/.

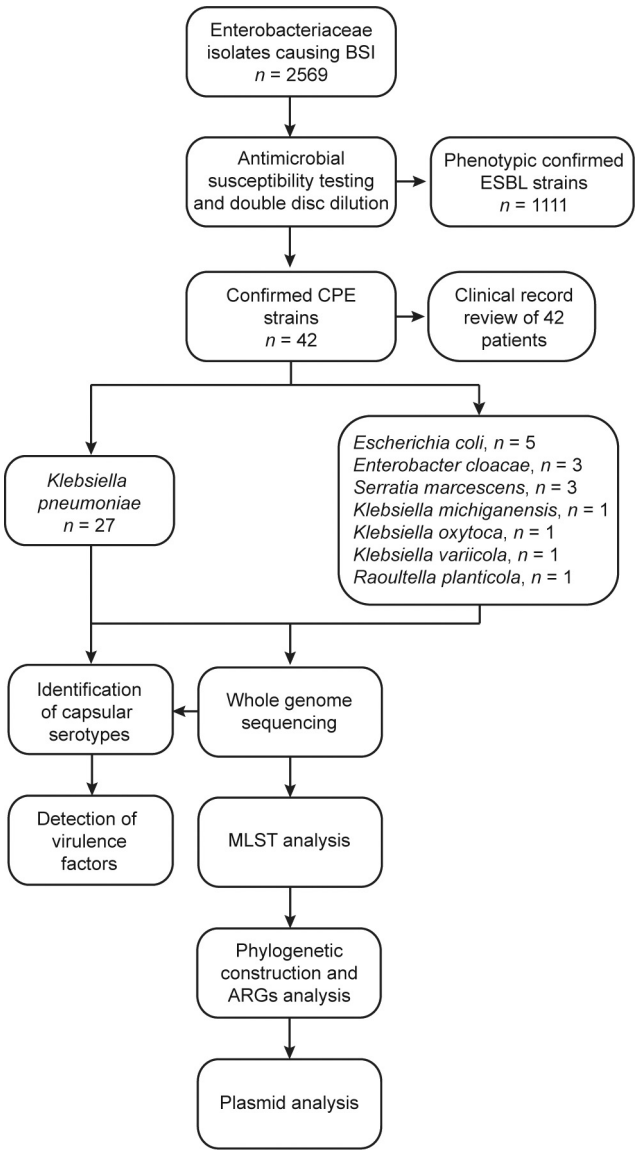

Fig. 1. Study flowchart. MLST: multilocus sequence typing.

ESBL production of *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Proteus mirabilis* (*P. mirabilis*), and *Klebsiella oxytoca* (*K. oxytoca*) isolates was phenotypically performed using the double-disk dilution method following CLSI recommendations. Carbapenem non-susceptible isolates were screened for the presence of carbapenemase-encoding genes of imipenemase (bla_{IMP}), Verona integrin-encoded metallo-β-lactamase (bla_{VIM}), *K. pneumoniae* carbapenemase (bla_{KPC}), oxacillinase (bla_{OXA-48}), and New Delhi metallo-β-lactamase (bla_{NDM}) as described previously [19].

2.3. WGS and genomics analysis

To characterize the genetic features of the CPE strains, WGS was performed on all CPE isolates. Total DNA was extracted using the Gentra Puregene Yeast/Bact. Kit (Qiagen, Germany) and then subjected to WGS using an Illumina Hiseq 2500 platform (Novogene Co., China). Paired reads were assembled into a number of scaffolds using Velvet 1.1 [20]. Multilocus sequence typing (MLST) analysis was performed on *E. coli*, *K. pneumoniae*, *Enterobacter cloacae* (*E. cloacae*), and *K. oxytoca* isolates as described previously [4]. ARGs were identified using the ResFinder 2.1 database [21]. Genome alignments of all CPE isolates were performed with progres-

siveMauve [22] based on an estimate of the shared gene content among each pair of input genomes with a multiple whole genome alignment algorithm. The putative coding sequences of the flanking region of carbapenemase-encoding genes and *rmpA*/*rmpA2* genes were manually inspected using open reading frame (ORF) finder programs[†].

2.4. Plasmid analysis

Nuclease digestion, pulsed-field gel electrophoresis (S1-PFGE), and Southern blot were performed to estimate the size of the bla_{NDM}/bla_{KPC}/bla_{IMP}/*rmpA*/*rmpA2*-carrying plasmids [23]. The sequence of the target plasmids was assembled using plasmidSPAdes [24]. To identify the targeted plasmids, a similarity search against the GenBank nucleotide (NT) database was performed by a basic local alignment search tool (BLAST) for each scaffold. Plasmid sequences were defined when > 70% of the scaffold length matched the plasmid sequences and < 30% matched either the mobile element or chromosomal sequences based on MUMmer alignment [25]. The best BLAST hit to each scaffold was manually inspected using the plasmid database (PLSDB), a resource of complete bacterial plasmids [26]. The estimated length of each plasmid was calculated as the sum of the length of all scaffolds associated with a specific reference plasmid. Reference plasmids having $\geq$ 99% identity with $\geq$ 70% overall coverage by scaffolds of one isolate were considered as present in that isolate. Plasmid Finder 1.3 was used to identify the incompatibility type of the plasmids [27]. The sequences of representative plasmids were compared against other plasmid sequences accessed from US National Center for Biotechnology Information (NCBI) using BLAST and plotted using the BLAST Ring Image Generator (BRIG)[‡].

2.5. Phylogenetic analysis of K. pneumoniae isolates

To further characterize the phylogenetic structure of *K. pneumoniae* carbapenemase (KPC)-2-producing *K. pneumoniae* isolates, we created a core single-nucleotide polymorphism (SNP)-based phylogenetic tree and identified SNPs via mapping of Illumina reads to a reference genome (*K. pneumoniae* strain HS11268, CP003200). Core genes were defined as previously described [28]. The maximum likelihood-based phylogenetic reconstruction was performed with RAxML version 8.2.10 using the generalized time reversible (GTR) evolutionary model after the removal of recombination sites [29]. Phylogenetic tree visualizations were produced using the Interactive Tree of Life[††].

2.6. Characterization of the known virulence and capsule genes of K. pneumoniae isolates

To identify the virulence and capsule genes in the 27 *K. pneumoniae* isolates, we further screened all short-read sets for the known alleles of virulence genes and *wzi* gene allele sequences using the *K. pneumoniae* Bacterial Isolate Genome Sequence database (BIGSdb) at Institute Pasteur[‡‡].

3. Results

3.1. High prevalence of ESBLs in BSI isolates

During the two-year study period, a total of 4801 unique cases of bacteremia were reported in 26 hospitals in 21 cities. Nonduplicate 2569 Enterobacterales isolates were prospectively collected, accounting for 53.5% (2569/4801) of all the Gram-negative bacteremia cases. Among the Enterobacteriaceae isolates, *E. coli* (n = 1617) was the predominant species, followed by *K. pneumoniae* (n = 570). Among the 2242 investigated isolates, 1111 (49.6%) isolates were confirmed as ESBL-producing isolates. The overall proportions of ESBL-producing *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *P. mirabilis* were 57.0% (922/1617), 30.0% (171/570), 33.3% (8/24), and 35.7% (10/28), respectively. Although most of the isolates were susceptible to trimethoprim/sulfamethoxazole (50.3%), polymyxin B (98.0%), meropenem (96.8%), and imipenem (95.9%), high-level resistances to amoxicillin (88.6%), cefazolin (60.6%), and cefuroxime (53.7%) were also observed.

3.2. Identification and distribution of CPE isolates

In total, 66 meropenem non-susceptible isolates and 73 imipenem non-susceptible isolates were identified, including 42 carbapenemase-positive isolates confirmed using polymerase chain reaction (PCR) and sequencing. Among these isolates, 27 were *K. pneumoniae*, followed by *E. coli* (n = 5), *Serratia marcescens* (*S. marcescens*) (n = 3), *E. cloacae* (n = 3), *Klebsiella michiganensis* (*K. michiganensis*) (n = 1), *Klebsiella variicola* (*K. variicola*) (n = 1), *K. oxytoca* (n = 1), and *Raoultella planticola* (*R. planticola*) (n = 1). All CPE isolates were non-susceptible to carbapenems and resistant to a broad array of antimicrobials. Among these isolates, total resistance to cefotaxime and piperacillin–tazobactam was observed (100.0%), followed by very high incidences of resistance to aztreonam (95.2%) and ceftazidime (92.9%). As a result, the most active antimicrobials against CPE isolates were tigecycline, polymyxin B, and trimethoprim/sulfamethoxazole. All isolates were susceptible to tigecycline, while only one *S. marcescens* isolate was resistant to polymyxin B.

Sanger sequencing further revealed that bla_{KPC-2} was the most prevalent variant found in 31 (91.3%) isolates, followed by bla_{NDM-5} (n = 5), bla_{NDM-1} (n = 4), bla_{IMP-4} (n = 1), and bla_{IMP-8} (n = 1). Interestingly, carbapenemase genes were present in 27 (4.7%) of 570 *K. pneumoniae* isolates and 5 (0.3%) of 1617 *E. coli* strains. Additionally, these CPE isolates were collected from 14 hospitals located in 12 cities, and the highest CPE ratio was observed in the First Affiliated Hospital of Wannan Medical College, with a rate of 17.4%.

3.3. Clinical description of CPE-positive patients

All patients with CPE infections were hospitalized. The mean age of the 42 patients was 61.1 years with a range of 1–90 years, and 66.7% (28/42) were males. Twenty-one (50.0%) patients were admitted to the intensive care unit (ICU) (including the emergency ICU (EICU) and neonatal ICU (NICU)). As a result, 15 of 42 (35.7%) patients died in this study. It is worth noting that 5 of 6 (83.3%) patients infected with *rmpA*/*rmpA2*-producing *K. pneumoniae* isolates died, suggesting the high risk of infection with such hypervirulent pathogens.

3.4. Clonal spread of K. pneumoniae ST11 and E. coli ST167 isolates

All genome assemblies of the 42 CPE isolates were deposited in GenBank and are registered under BioProject accession No. PRJNA393804. The results of the WGS data are summarized in Table 1. Through comparative genomic analyses within each species and sequence type (ST), we identified eight CPE clades (Fig. 2), which is in line with the species identification. It is worth noting that KPC-2-producing *K. pneumonia* ST11 and New Delhi metallo-β-lactamase (NDM)-5-producing *E. coli* ST167 isolates were detected in ten and four sentinel hospitals, respectively.

[†] https://www.ncbi.nlm.nih.gov/orffinder/.
[‡] http://brig.sourceforge.net/.
[††] https://itol.embl.de/.
[‡‡] http://bigsdb.web.pasteur.fr.

Table 1
Overview of CPE assembly statistics and genome coverage based on the assembly data.

Isolate ID	Contigs	N50 (bp)	Assembled bases (bp)	× Coverage	Accession No.
EN3287	56	153 355	4 797 988	385	NPGJ00000000
EN3600	139	132 334	5 391 491	234	NPGK00000000
E987	90	138 052	5 170 714	300	NPGF00000000
E4219	158	113 310	5 139 090	292	NPHP00000000
E834	138	100 008	5 095 123	257	NPHS00000000
E2619	182	119 182	5 158 320	222	NPHR00000000
E2239	146	136 971	5 024 459	314	NPHO00000000
E4903	107	136 972	4 982 100	285	NPHQ00000000
K1439	84	314 574	6 135 809	141	NPHU00000000
K3678	103	215 513	6 273 403	217	NPHT00000000
K3657	105	170 401	5 663 831	283	NPHN00000000
K2147	115	203 939	5 659 063	268	NPHH00000000
K604	65	340 693	5 669 428	242	NPHL00000000
K4803	108	164 600	5 829 038	228	NPHG00000000
K315	99	192 313	5 643 629	189	NPHF00000000
K5012	86	160 075	5 478 142	288	NPHE00000000
K1433	90	203 939	5 505 326	262	NPHI00000000
K3893	96	185 914	5 628 838	274	NPHA00000000
K3871	98	185 914	5 632 206	293	NPHB00000000
K5014	95	157 792	5 483 445	274	NPHD00000000
K3862	104	203 938	5 553 192	362	NPHC00000000
K85	95	176 166	5 810 616	217	NPHM00000000
K666	96	144 277	5 451 010	262	NPHK00000000
K669	96	160 072	5 447 971	189	NPHJ00000000
K3902	98	171 014	5 618 419	362	NPGZ00000000
K2646	100	192 313	5 545 597	271	NPGX00000000
K950	79	203 695	5 675 827	307	NPGM00000000
K1177	107	192 313	5 733 347	359	NPGU00000000
K2080	104	203 896	5 585 536	139	NPGS00000000
K3194	94	192 313	5 636 447	184	NPGW00000000
K4280	99	192 313	5 726 611	270	NPGV00000000
K2701	89	203 896	5 581 788	219	NPGR00000000
K5065	63	291 843	5 822 605	273	NPGT00000000
K4311	115	222 925	5 529 081	263	NPGP00000000
K4307	83	203 939	5 525 783	246	NPGQ00000000
K4324	93	203 939	5 530 708	192	NPGN00000000
K4314	84	203 939	5 524 464	242	NPGO00000000
K2631	208	241 238	6 293 921	187	NPGY00000000
R3467	63	357 979	6 138 099	229	NPGL00000000
SE768	55	227 823	5 499 067	334	NPGI00000000
SE3605	57	288 863	5 412 855	298	NPGH00000000
SE4145	73	202 460	5 427 821	242	NPGG00000000

bp: base pair.

These data suggest the clonal spread of KPC-2-producing *K. pneumoniae* ST11 and NDM-5-producing *E. coli* ST167 in our study.

3.5. Characterization of ARGs

Using the WGS data, we identified a broad array of ARGs associated with a broad array of antimicrobials: aminoglycosides, β-lactams, fosfomycin, carbapenems, quinolones, sulfonamide, trimethoprim, and phenicol (Fig. 2). Moreover, 33 of 42 (78.6%) isolates carrying carbapenemases also harbored a predicted ESBL. Interestingly, among these ESBLs, cefotaxime hydrolyzing β-lactamase (CTX-M)-65 was the most predominant cluster (47.6%, 20/42), followed by CTX-M-14 (23.8%, 10/42). Seven isolates produced two $bla_{\text{CTX-M}}$ alleles. As expected, at least one variant of the sulfhydryl reagent variable (SHV) enzyme was found in 30 *Klebsiella* isolates. Among these isolates, 22 harbored $bla_{\text{SHV-11}}$ and 11 harbored $bla_{\text{SHV-178}}$.

3.6. Genetic context of carbapenemase-encoding genes

S1-PFGE, Southern hybridization, and replicon typing analysis revealed that all carbapenemase-encoding genes were plasmid-mediated (Table 2 and Fig. S1 in Appendix A). Plasmids harboring $bla_{\text{KPC-2}}$ (n = 31) were genetically divergent and could be categorized into the following groups: IncFII plasmid with a size of ~79 to ~260 kilobase (kb), IncR plasmid with a size of ~55 kb, and IncP-6 plasmid with a size of ~38 kb (Fig. S1). Although bla_{KPC} was mainly associated with the Tn3 transposon with three main structures, they were found to share a similar conserved structure, IS*Kpn27*–$bla_{\text{KPC-2}}$–IS*Kpn6*–*korC*-*hp*–*klcA*, implying that this mobile element played a key role in the transmission of the $bla_{\text{KPC-2}}$ gene in China (Fig. 3(a)).

In addition, $bla_{\text{IMP-4}}$ and $bla_{\text{IMP-8}}$ were located on ~55 kb IncN and ~73 kb non-typeable plasmids, respectively (Table 2 and Fig. S1(e)). *In silico* analysis revealed that $bla_{\text{IMP-4}}$ was carried by a class I integron in the $bla_{\text{IMP-4}}$–*ltrA*–*qacED1*–*sul1* cassette array on pEN987-imipenemase (IMP), which shows sequence similarity with plasmid p19501-IMP from *K. pneumoniae* (MF344565) (Fig. 3(b)). In contrast, $bla_{\text{IMP-8}}$ was harbored by a class I integron in the $bla_{\text{IMP-8}}$–*aac(6′)-Ib*–$bla_{\text{OXA-1}}$–*catB3*–*arr-3*–*qacEdelta1*-*sul1* cassette array on pEN3600-IMP. Interestingly, this structure exhibits high similarity with the sequence of the $bla_{\text{KPC-2}}$ plasmid p112298-KPC, although $bla_{\text{IMP-8}}$ is absent from that plasmid (Fig. 3(c)).

Plasmids harboring bla_{NDM} from *E. coli* (n = 5) and *E. cloacae* (n = 1) all belonged to IncX3 with a size of ~46 kb; however, two *Klebsiella* isolates carried bla_{NDM} genes encoded by the IncFI plasmid with a size of ~55 kb and a non-typeable plasmid with a size of ~220 kb (Table 2 and Fig. S1(d)). Although a fragment consisting of *dsbC*–*trpF*–ble_{MBL}–bla_{NDM} was observed in all the bla_{NDM}-bearing

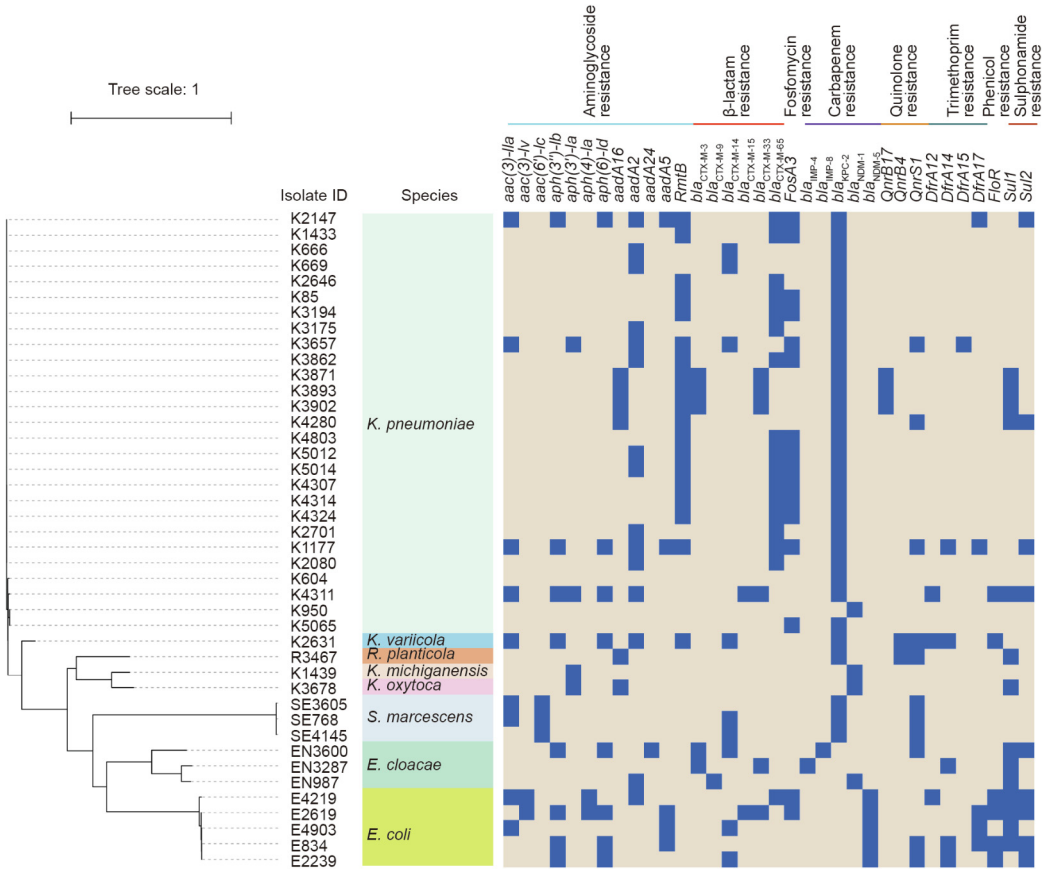

Fig. 2. Alignment of genome sequences of 42 CPE isolates and ARGs identified via WGS. The figure was generated using progressiveMauve based on an estimate of the shared gene content among each pair of input genomes with a multiple whole genome alignment algorithm. ARGs are shown on the right side (presence represented by blue shapes).

Table 2
Features of 42 carbapenemase-encoding plasmids identified from BSIs.

Replicon type	No. of plasmids	Approximate size (kb)	Species	Carbapenemase genes	ST(s)[a] carrying plasmids
IncN	1	55	*E. cloacae*	bla_{IMP-4}	ST97
Non-typeable	1	73	*E. cloacae*	bla_{IMP-8}	ST25
IncFII	1	260	*K. pneumoniae*	bla_{KPC-2}	ST11
IncFII	6	170	*K. pneumoniae*	bla_{KPC-2}	ST11
IncFII	1	170	*K. variicola*	bla_{KPC-2}	ND
IncFII	8	146	*K. pneumoniae*	bla_{KPC-2}	ST11
IncFII	1	125	*K. pneumoniae*	bla_{KPC-2}	ST11
IncFII	5	106	*K. pneumoniae*	bla_{KPC-2}	ST11/ST268
IncFII	1	106	*R. ornithinolytica*	bla_{KPC-2}	ND
IncFII	2	106	*S. marcescens*	bla_{KPC-2}	ND
IncFII	2	95	*K. pneumoniae*	bla_{KPC-2}	ST11/ST23
IncFII	2	79	*K. pneumoniae*	bla_{KPC-2}	ST11/ST15
IncR	1	55	*S. marcescens*	bla_{KPC-2}	ND
IncP-6	1	38	*K. pneumoniae*	bla_{KPC-2}	ST11
IncX3	1	46	*E. cloacae*	bla_{NDM-1}	ST600
IncFI	1	55	*K. michiganensis*	bla_{NDM-1}	ND
Non-typeable	1	220	*K. oxytoca*	bla_{NDM-1}	ND
IncFI	1	55	*K. pneumoniae*	bla_{NDM-1}	ST23
IncX3	5	46	*E. coli*	bla_{NDM-5}	ST167/new ST

ND: not determined; kb: kilobase.
[a] STs were determined for *K. pneumoniae*, *E. coli*, and *E. cloacae* complex isolates.

plasmids, eight plasmids exhibited three different types of bla_{NDM-1} gene contexts (Figs. 3(d) and (e)). The absence of insertion sequence *Aba125* (IS*Aba125*) was identified in three plasmids (pK950-NDM, pK3287-NDM, and E4219-NDM), and pK3287-NDM showed low similarity with other plasmids, except for the bla_{NDM-1}-containing region.

3.7. The genetic diversity of K. pneumoniae ST11 isolates

Phylogenetic analysis of *K. pneumoniae* isolates was further investigated given that *K. pneumoniae* was the most prevalent organism identified in the 42 carbapenemase-positive isolates. We identified a total of 21 711 core genome SNPs among 27 *K.*

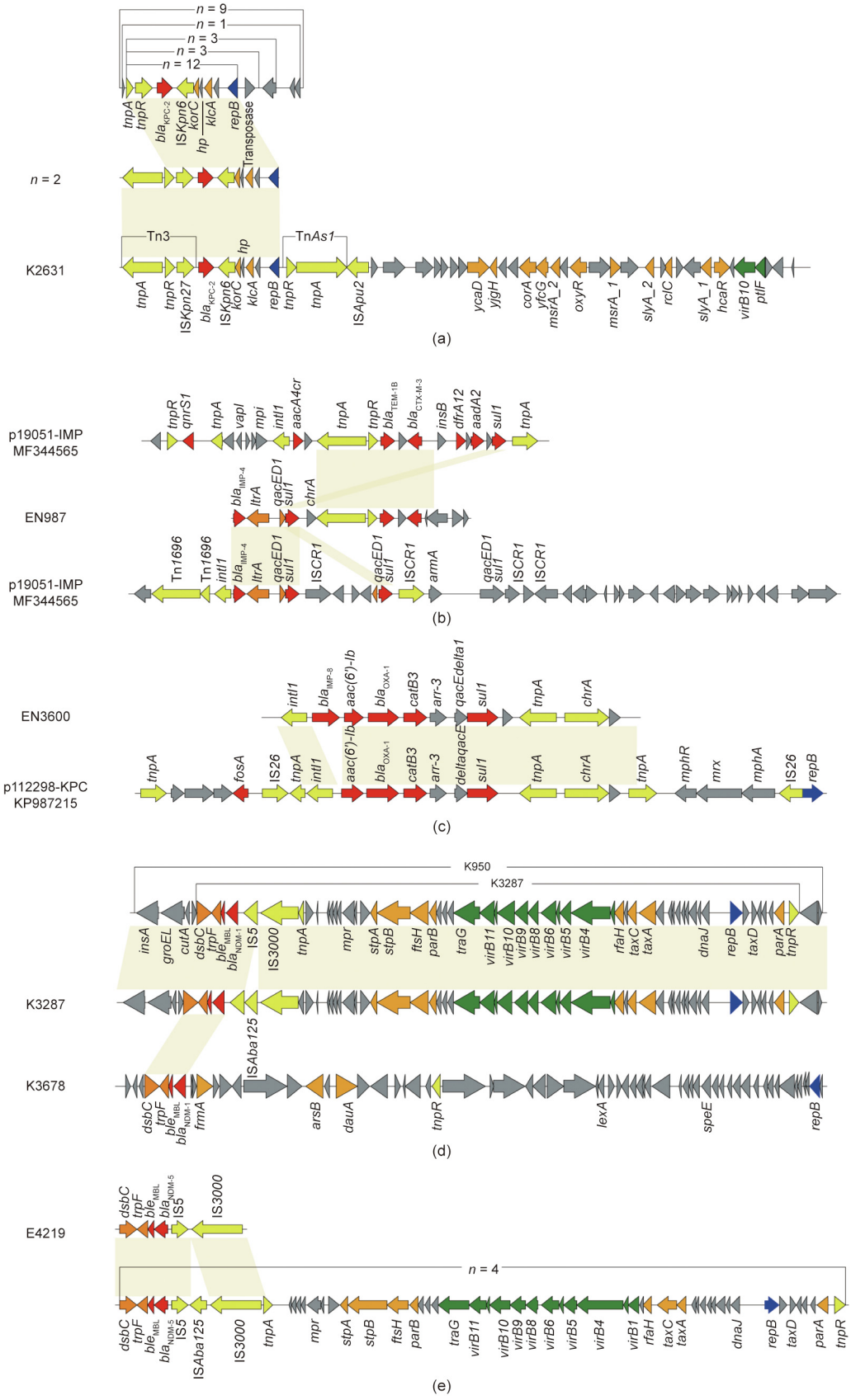

Fig. 3. Genetic environment of carbapenemase-encoding genes in 42 CPE isolates. (a) Colinear genome alignment among 31 bla_{KCP-2}-harboring plasmids. (b) Genetic environment of the bla_{IMP-4} gene in isolate EN987. (c) Genetic environment of the bla_{IMP-8} gene in isolate EN3600. (d) Genomic map of the bla_{NDM-1}-carrying plasmids. (e) Comparative genomics of five bla_{NDM-5}-harboring plasmids. The Easyfig program was applied in comparative genomics. Colored arrows indicate ORFs, and the shaded region reflects sequence similarity. Arrows indicate the directions of transcription of the genes, and different genes are shown in different colors. The ARGs are indicated in red. Isolates with different sizes of the core region of carbapenemase-encoding genes are indicated by vertical lines as well as numbers. IS: insertion sequence.

pneumoniae genomes (Fig. 4(a)). Then, these genomes were divided into four distinct phylogenetic clades based on analysis of the core gene maximum likelihood tree using RAxML. Additionally, SNP analysis identified three pairs of closely related isolates (K4314 & K4324, K666 & K559, and K3871 & K3902) shared by two patients, which indicates likely hospital transmission of these isolates. Analysis of the capsular polysaccharide (cps) locus showed that 27 *K. pneumoniae* isolates contained six different *wzi* alleles (Fig. 4(b)). According to the Klebsiella PasteurMLST database, these *wzi* alleles corresponded to different K types: KL47 (n = 16), KL64 (n = 6), K1 (n = 2), K20 (n = 1), K19 (n = 1), and KL13 (n = 1). Interestingly, five K types were detected among ST11 isolates (n = 23), and the

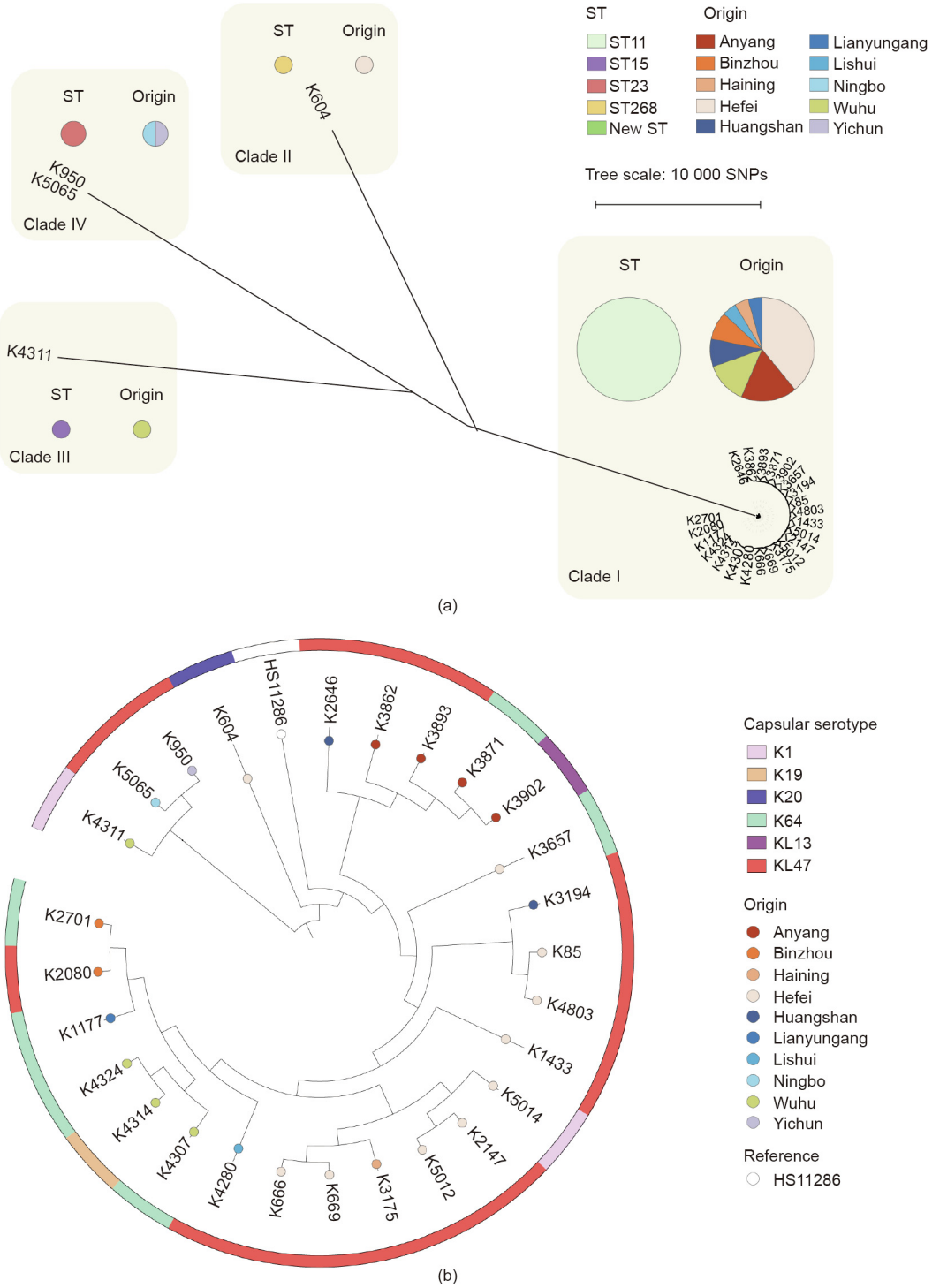

Fig. 4. Comparative genomics of 27 KPC-2-producing *K. pneumoniae* isolates. (a) Maximum likelihood-based phylogenetic tree built from 21 711 core genome SNPs of 27 BSI isolates mapped to the reference genome of *K. pneumoniae* strain HS11268 (CP003200). Isolates were grouped with a threshold of the maximum 1000 SNPs to the nearest group member, and the four resulting groups are shown. The origins of the isolates containing different STs are indicated in pie charts, and the different origins are shown in different colors. (b) Distribution of capsular types among 27 KPC-2-producing *K. pneumoniae* isolates. The origins of the isolates containing different capsular types are indicated in circles, and the different origins are shown in different colors.

analysis of the *K. pneumoniae* ST11 clone also indicated that the median pairwise SNP distance was 15 (range: 0–42) (Fig. 4(b)). These data suggest a genetic diversity among the ST11 lineage.

3.8. Distribution of virulence factors among K. pneumoniae isolates

A total of 76 known virulence factors were detected in *K. pneumoniae* isolates (Fig. 5). A high prevalence of biofilm-encoding *mrk* gene clusters and *ybt* genes was observed in these isolates. All 27 *K. pneumoniae* isolates encoded *mrk* genes, and 26 isolates harbored *ybt* genes. Moreover, aerobactin, allantoinase, colibactin, the ferric uptake operon, microcin, *KP1_1364* and *KP1_1371*, and salmochelin-associated genes were detected in seven, two, three, three, two, two, and two isolates, respectively. Surprisingly, the isolate K950 possessed the highest number of virulence genes and encoded 74 virulence factors, all except for the *ybbW* and *mceJ* genes.

3.9. Plasmid analysis of rmpA/rmpA2-harboring plasmids

We additionally identified the "regulators of mucoid phenotype" *rmpA* and *rmpA2* genes in three and six isolates, respectively

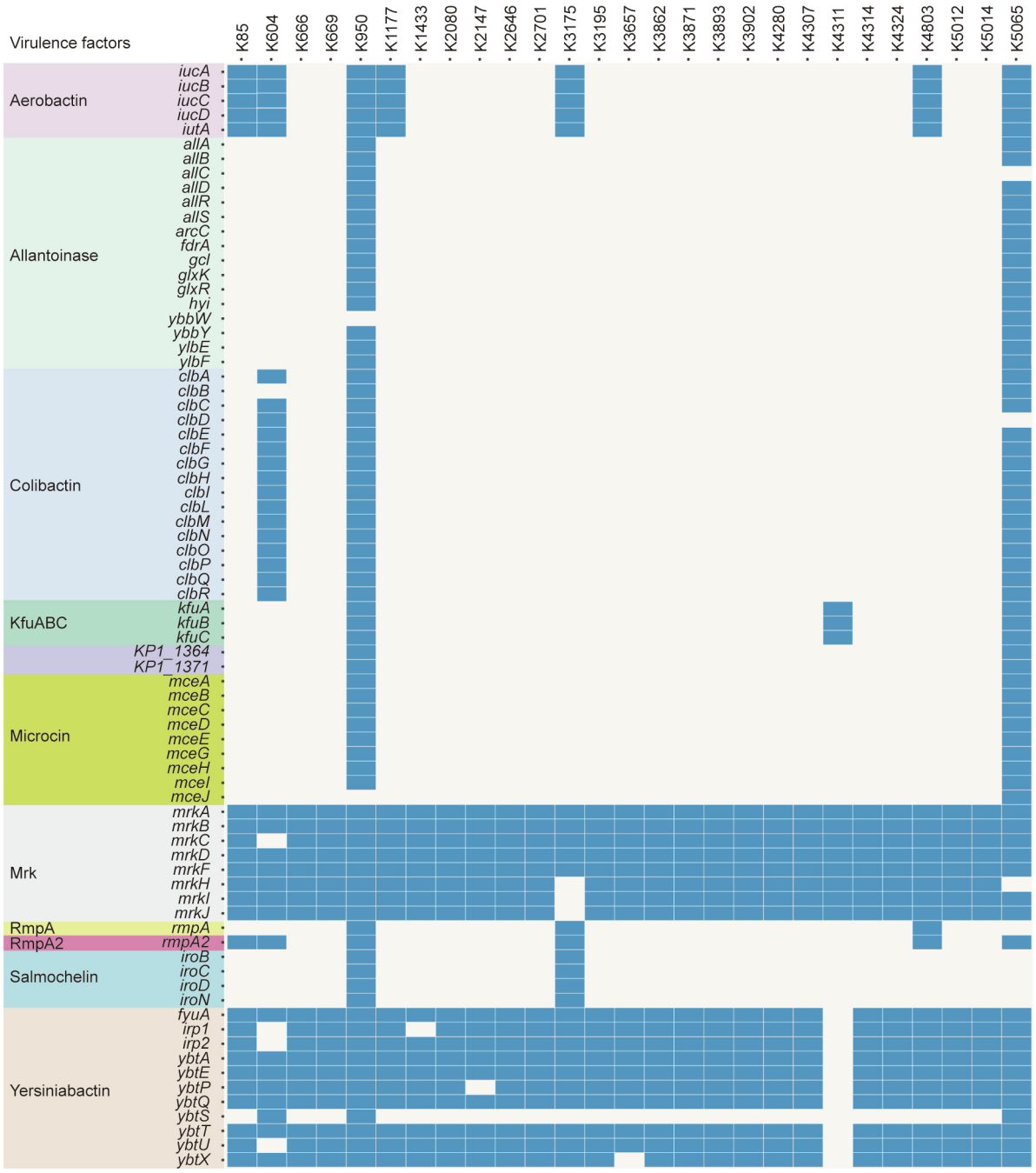

Fig. 5. Distribution of virulence-associated genes in *K. pneumoniae* strains. Heatmaps were generated by aligning the draft genome sequence of each isolate to the BIGSdb-Kp database. The presence of virulence genes in a specific genome is represented by a blue box, and the absence of virulence genes is represented by a cream-colored box. Virulence factors are shown on the left side. *iucABCD*: aerobactin-related genes; *iutA*: ferric aerobactin receptor gene; *allABCDRS/arcC/fdrA/gcl/ylbEF/ybbWY/hyi/glxKR*: allantoniase-related genes; *kfuABC*: ferric uptake operon-associated genes; *clbABCDEFGHILMNOPQR*: colibactin-associated genes; *mceABCDEGHIJ*: microcin-associated genes; *mrkABCDFHIJ*: type 3 fimbriae-associated genes; *rmpA/rmpA2*: cps transcriptional activator genes; *iroBCDN*: salmochelin-associated genes; *ybtAEPQSTUX/fyuA/irp1/irp2*: yersiniabactin-associated genes.

(Fig. S2 in Appendix A). S1-PFGE and Southern hybridization revealed that *rmpA* was carried by ~200 kb IncHIB and ~260 kb IncHIB plasmids (Fig. S2(a)). Compared to *rmpA*, the *rmpA2*-bearing plasmids were structurally more divergent, as the *rmpA2* gene was located on IncHIB plasmids with sizes of ~370, ~260, ~218, ~200, and ~150 kb (Fig. S2(b)). Notably, *rmpA* and *rmpA2* coexisted on the plasmids from isolates K950, K3175, and K4803 (Fig. S2 and Table 3). Comparative sequence analysis showed that K950 and K3175 shared the conserved genetic context of the *rmpA* gene, with a difference from that of K4803 (Fig. S2(c)). Further analysis identified chromosome-encoded salmochelin-associated genes *iroBCDN* in K950 and K3175, but it was absent in K4803 (Fig. 5). *In silico* analysis also found a similar core structure of the *rmpA*2 gene (Fig. S2(d)). These data indicated that *rmpA2*-bearing plasmids were genetically divergent, although these plasmids shared a similar structure surrounding *rmpA2*, indicating that this structure played a key role in the transmission of the *rmpA2* gene.

4. Discussion

Over the past decade, China has witnessed the emergence and rapid increase of CRE in some regions [13,15]. In light of the increasing burden of CRE infections, there is an urgent need to update our knowledge on the genomic characteristics of CRE isolates in China, which is pivotal for monitoring carbapenem resistance as well as for formulating relevant policies for the control and management of CRE infections. Moreover, bacteremia due to CRE is usually associated with increased rates of treatment failure, high mortality, and high hospitalization costs, and is gradually becoming a real clinical challenge [30,31]. Previous investigations of BSIs caused by CPE in China were mainly based on the epidemiological results of retrospective studies [14] and case series using small sample sizes [32,33]. Thus far, the genomic characteristics of CPE isolates from individuals with bacteremia are largely unknown. Here, we provide the first high-resolution genomic analysis of CPE isolates from a multicenter BSI surveillance in China.

This report documents that CPE are widespread in BSIs in China, accounting for 1.6% of the Enterobacteriaceae isolates, and were identified in 54.0% of the hospitals screened in this study. The combined results of this work and those of previous studies suggest that carbapenemase producers have disseminated throughout China. The dissemination of CPE among BSI isolates, as observed in our collection, raises concerns for the treatment of patients with Gram-negative sepsis and suggests a need to reduce selective pressure and control the spread of resistant organisms [34].

In this work, bla_{KPC-2} and bla_{NDM-5} were found to be the major carbapenemase genes responsible for mediating the carbapenem resistance phenotypes in CPE. In China, the majority of CRE infections are due to *K. pneumoniae* ST11 that harbor the bla_{KPC-2} carbapenemase, which is closely related to the international epidemic clone ST258 [35]. A previous national survey of CRE identified KPC-2-producing *K. pneumoniae* strains as the predominant CRE isolates [13], which is consistent with our identified 80%-KPC-2-producing rate among CPE isolates. Moreover, the rapid spread of bla_{KPC-2} is mainly due to the clonal dissemination of *K. pneumoniae* ST11 strains. Previous investigations have described the high prevalence and mortality rates of ST11-KL47 and ST11-KL64 in China [36,37]. We detected that ST11 was partitioned into five clades, with ST11-KL47 being the predominant subclone. We also noted that *rmpA* and *rmpA2* coexisted on ST11-KL64 isolates, which is consistent with recent observations [8,37].

In contrast, a previous national survey revealed that CRE infections due to *E. coli* harboring carbapenemase are associated with the globally distributed ST131 lineage, which accounted for 33.0% of the isolates in that study, thus being the most dominant in China [13]. However, in recent years, there have been increasing reports of the detection of the NDM-5-producing *E. coli* ST167 lineage in clinical infections in China [13,38,39]. The observation of ST167 being the most common type of *E. coli* in our collection is in line with this trend. In addition to the wide spread of *E. coli* ST167 in China, some reports from European countries hint toward an emergence of a bla_{NDM} carrying *E. coli* ST167 [40–43], with epidemic potential. Appropriate measures to effectively control the spread of KPC-2-producing *K. pneumoniae* ST11 and NDM-5-producing *E. coli* ST167 are needed to prevent further spread in China as well as serious public health consequences.

Most of the bla_{KPC-2}/bla_{NDM}/bla_{IMP} genes detected in Enterobacteriaceae were located on transmissible plasmids [13]. Consistent with these data, characterization of the carbapenemase-harboring plasmids recovered in this study indicated that all carbapenemase-encoding genes among clinical CPE strains were plasmid-mediated. Here, we found KPC genes in four bacterial species and in $\geq$ 33 different STs, carried by 11 plasmid replicon types, suggesting that both plasmid spread and the mobility between plasmids play important roles in the dissemination of KPC enzymes in China. Even within ST11, we found at least eight different KPC plasmid replicon types, indicative of the success of this clone as a host of KPC plasmids. In contrast to bla_{KPC}, the bla_{NDM} genes (bla_{NDM-1} and bla_{NDM-5}) have frequently been reported to be located on IncX3 plasmids among multiple enterobacterial species, implying that IncX3 plasmids may provide an efficient vehicle for bla_{NDM} dissemination within bacterial strains and interspecies from humans [13,44]. Our observations further illustrate the clinical significance of IncX plasmids in the transmission of carbapenem resistance.

Notably, most *K. pneumoniae* isolates were found to be significantly prevalent with siderophore yersiniabactin, which is the most common *K. pneumoniae* high-virulence determinant and closely associated with the virulence of Enterobacteriaceae strains that cause invasive human infections [45,46]. Furthermore, it is well documented that the *rmpA* and *rmpA2* genes are significantly associated with invasive human infection compared with non-invasive or carriage isolates [28]. Therefore, the coexistence of these genes in *K. pneumoniae* ST11 KPC producers in BSIs is worrisome because they are simultaneously multidrug resistant, highly transmissible, and hypervirulent [47]. In addition, the combination of virulence factor genes such as aerobactin, allantoinase, colibactin, ferric uptake operon, microcin, salmochelin, *rmpA2*, and yersiniabactin was detected in the same isolate in this work. This finding may explain the high mortality rate associated with *K. pneumoniae* infections in this study and suggests that these isolates

Table 3
An overview of *rmpA*/*rmpA2*-encoding plasmids.

Plasmid	Status	Inc type	*rmpA*/*rmpA2* gene type	Plasmid size (kb)
pK85-*rmpA2*	Contig	IncHI1B	*rmpA2*	~218
pK604-*rmpA2*	Contig	IncHI1B	*rmpA2*	~370
pK950-*rmpA*	Contig	IncHI1B	*rmpA*/*rmpA2*	~260
pK3175-*rmpA*	Contig	IncHI1B	*rmpA*/*rmpA2*	~200
pK4803-*rmpA*	Contig	IncHI1B	*rmpA*/*rmpA2*	~200
pK5065-*rmpA2*	Contig	IncHI1B	*rmpA2*	~150

may potentially be hypervirulent in BSIs, although future experimental studies are needed for confirmation.

This study had several limitations. First, although hospitals in a broad geographic distribution of China were enrolled in this study, only a limited number of CPE strains were obtained and investigated. Therefore, these data may not be truly representative of the prevalence and characterization of CPE for the entirety of China. Second, our findings of carbapenemase-harboring plasmids should be explained with caution; thus, further studies are required to increase confidence in the findings of this work since only BSI-causing isolates were included in the collection.

5. Conclusions

In conclusion, our study reported the wide dissemination of ESBL and CPE isolates from individuals with bacteremia in China. Importantly, this work indicated that *K. pneumoniae* ST11 and *E. coli* ST167 have become serious clinical problems in China. The study also reveals a genomic picture of invasive CPE infections in China, which highlights the significance of country-level surveillance of BSIs. Plasmid analysis revealed that plasmids played important roles in the process of carbapenemase-encoding gene dissemination. The prevalence of CPE in BSIs should be continuously and closely monitored in China. Furthermore, large-scale detailed microbiological and genomic investigations into the epidemic clone *K. pneumoniae* ST11 with potential hypervirulence are also particularly needed to understand the transmission of the bla_{KPC-2} gene and virulence genes so that effective strategies for national control of CPE infections can be developed.

Acknowledgments

We gratefully acknowledge the financial support of the National Key Research and Development Program of China (2017YFC1200203 and 2016YFD0501105), the Mega-projects of Science Research of China (2018ZX10733402-004 and 2018ZX10712001-005), the National Natural Science Foundation of China (81741098 and 81711530049), the Zhejiang Provincial Key Research and Development Program (2015C03032), and the Zhejiang Provincial Natural Science Foundation of China (LY17H190003).

Compliance with ethics guidelines

Beiwen Zheng, Hao Xu, Lihua Guo, Xiao Yu, Jinru Ji, Chaoqun Ying, Yunbo Chen, Ping Shen, Huiming Han, Chen Huang, Shuntian Zhang, Tao Lv, and Yonghong Xiao declare that they have no conflict of interest or financial conflicts to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2020.10.015.

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