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Pandemic spread of bla_{KPC-2} among *Klebsiella pneumoniae* ST11 in China is associated with horizontal transfer mediated by IncFII-like plasmids

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ABSTRACT

Objectives: The aim of this study was to investigate the spread of the bla_{KPC-2} gene among *Klebsiella pneumoniae* and to illustrate the mechanism of dissemination of *K. pneumoniae* carbapenemase-producing *K. pneumoniae* (KPC-*Kp*) ST11 in China.

Methods: A total of 354 *K. pneumoniae* isolates were collected from four hospitals in China and were characterized by multilocus sequence typing (MLST). Mobile genetic elements (MGEs) and pulsed-field gel electrophoresis (PFGE) analysis were used to identify the subtypes of *K. pneumoniae* ST11. Polymerase chain reaction (PCR)-based amplification and sequencing were performed to analyse Tn*1721* transposons and IncFII-like plasmids. Electroporation experiments and whole-genome sequencing (WGS) were used to reveal the genetic environment of the bla_{KPC-2} gene.

Results: As the primary type (87.1%) of KPC-*Kp*, *K. pneumoniae* ST11 was not predominant in non-KPC-*Kp* (3.1%). ST11 KPC-*Kp* was clonally heterogeneous and could be further classified into 11 MGE types and 14 PFGE subtypes. Five Tn*1721*-bla_{KPC-2} variants were identified on IncFII-like plasmids. The detection rate of IncFII-like plasmids was much higher in ST11 KPC-*Kp* (100%) compared with non-ST11 KPC-*Kp* (16.0%) and the non-KPC-*Kp* group (7.5%). Moreover, the IncFII plasmid (with II*a* replicon) was primarily detected on the MGE-F type (61.7%). The IncFII*k* plasmid (with II*k* replicon) was clustered into two subtypes: MGE-A (28.3%) and -F (41.5%). The detection of the IncFII and IncFII*k* plasmids on MGE-A was 57.1% (20/35) and 42.9% (15/35), respectively.

Conclusions: A close correlation was shown between ST11 KPC-*Kp* and IncFII-like plasmids. Horizontal transfer mediated by IncFII-like plasmids plays an important role in the pandemic expansion of bla_{KPC-2} among *K. pneumoniae* ST11 in China.

1. Introduction

Klebsiella pneumoniae carbapenemase (KPC) enzymes are globally spread β-lactamases of Ambler class A and are mainly found in *K. pneumoniae* [1]. KPC-producing *K. pneumoniae* (KPC-*Kp*) was first reported in 2001, and its incidence has greatly increased since then [2,3]. Although different KPC variants have been described, KPC-2 and -3 remain the most commonly identified variants [4]. However, controlling the dissemination of the KPC-encoding gene (bla_{KPC}) is problematic because bla_{KPC} is located on transferable plasmids and transposons, such as Tn*4401,* or Tn*4401*-like transposons that are associated with insertion sequence (IS) elements, thereby facilitating the inter- and intra-species dissemination of resistance [5-7]

Interestingly, although KPCs are present in >100 different *K. pneumoniae* sequence types (STs), the mass dissemination of KPC-*Kp* has been primarily restricted to clone complex 258 (CC258), namely, ST258, ST11, ST340, and ST512 [1,8,9]. The epidemic spread of *K. pneumoniae* ST11 is particularly significant in China [8]. Although previous studies have indicated that the KPC pandemic is primarily driven by the dissemination of clone CC258 [8,10], the bla_{KPC} gene can also spread between species by horizontal gene transfer (HGT) [11]. HGT has been reported to be mediated by various forms of mobile genetic elements (MGEs), such as plasmids, transposons and integrative conjugative elements (ICEs), which generate significant genotypic and phenotypic variation within bacterial populations [12]. MGEs carrying antimicrobial resistance (AMR) are commonly found in *K. pneumoniae* and can cause in-

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creased drug-resistant infections in hospitals [13]. Many studies have indicated that bla_{KPC} can be identified on plasmids of different incompatibility (Inc) replicon groups. Several different KPC-containing plasmids (i.e., IncF, IncI2, IncX, IncA/C, IncR, and ColE1) have also been identified in CC258 [14], but the predominant plasmid type is incompatibility group F (IncF) plasmids that harbor several FII replicons (IncFII-like plasmids) [15].

Although the first *K. pneumoniae* strain containing the bla_{KPC} gene was identified in a non-CC258 isolate in 1996 in the southern United States [3], CC258 has been considered the predominant clone of carbapenem-resistant *K. pneumoniae* (CR-*Kp*) in the United States since 2009 [16]. Epidemiological observations have demonstrated that non-CC258 KPC-*Kp* did not have the same global success as CC258 KPC-*Kp* [1]; however, the mechanism behind the spread of bla_{KPC} in *K. pneumoniae* CC258 warrants further exploration.

Although most previous reports have focused on KPC-*Kp* strains, statistical information on non-KPC-*Kp* strains is lacking. In China, it is unclear whether ST11 is a dominant type in clinical *K. pneumoniae* isolates (including KPC-*Kp* and non-KPC-*Kp*) or is dominant only in KPC-*Kp* strains through a mechanism that has yet to be understood. To resolve this issue, large-scale investigations into both KPC-*Kp* and non-KPC-*Kp* strains are warranted to elucidate the genetic background and prevalence of the bla_{KPC} gene in China.

The present analysis was conducted based on sequence comparisons of *K. pneumoniae* strains and plasmids obtained from the GenBank database. In addition, 354 clinical *K. pneumoniae* isolates were collected from four Chinese hospitals to clarify the genetic backgrounds surrounding bla_{KPC} and the potential functions of transferable IncFII plasmids. The aim of this study was to explore the association between ST11 KPC-*Kp*, Tn*1721* and IncFII plasmids to clarify the potential mechanism underlying the spread of ST11 KPC-*Kp* in China.

2. Materials and methods

2.1. Identification and antimicrobial susceptibility testing of *K. pneumoniae* isolates

This study involved four hospitals and 354 *Kp* strains (Hua Shan Hospital, 235 strains; Jin Shan Hospital, 29 strains; Tai Zhou Municipal Hospital, 60 strains; and Shan Dong Provincial Hospital, 30 strains) covering three provinces in China, from January 2017 to February 2018. Bacteria were routinely cultured at 37°C on blood culture plates**.** Identification and antimicrobial susceptibility testing (AST) were performed using a VITEK 2 system with GNI and GN13 cards (bioMerieux, Marcy L'Etoile, France). A total of 223 CR-*Kp* isolates were continuously collected based on AST outcomes, meanwhile 131 carbapenem-sensitive *K. pneumoniae* (CS-*Kp*) isolates from similar clinical departments were enrolled in this study. The 354 *K. pneumoniae* strains were isolated from various specimens, including sputum, 61.9% (219 isolates); urine, 19.2% (68 isolates); blood, 5.4% (19 isolates); and others, 13.8% (39 isolates).

2.2. Total DNA extraction, PCR amplification and multilocus sequence typing (MLST)

Total DNA of the isolates was extracted using the QIAamp DNA mini kit (QIAGEN) according to the manufacturer's instructions. The bla_{KPC} subtypes were identified through the amplification and sequence analysis of a 750-bp polymerase chain reaction (PCR) product [17]. MLST was performed with seven housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB,* and *tonB*) on 354 *K. pneumoniae* isolates according to the protocol described on the *K. pneumoniae* MLST database website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). Our MLST findings were compared with those of the database, which was created in 2005 at the Pasteur Institute in Paris, France [18].

2.3. Analysis of the Tn*1721*-bla_{KPC-2} genetic structure of clinical samples

ST11 KPC-*Kp* was subjected to the amplification and sequencing of Tn*1721* by junction PCR, mapping PCR and crossing PCR, as described in our previous reports, with minor revisions [17] (primer sequences and target sites are shown in S1 and S2). Another pair of primers (primer 11) was added to detect a more comprehensive backbone of *tnpA*.

2.4. MGE analysis and pulsed-field gel electrophoresis analysis

Genetic relatedness among the ST11 KPC-*Kp* isolates was initially determined by MGE analysis based on five MGEs, including prophage residual elements and integrative and conjugative elements (ICEs). Deleo et al. (2014) previously reported ten putative prophages and two ICEs according to one ST258 *K. pneumoniae* NJST258_1 [10]. Following this report, we conducted a genomic DNA sequence comparison of 21 strains of *K. pneumoniae* ST11 that were obtained from GenBank by BLAST Ring Image Generator (brig) (http://sourceforge.net/projects/brig/). We cited phage 258.2 (*pcap*), ICE Kp258.2 (*ICE*), phage 258.4(*shock*), phage 258.5 (*repA*) and one ICE (*irp3*) as markers to identify different ST11 subtypes [10]. These 21 strains that represented a worldwide distribution of *K. pneumoniae* ST11 from 2008 to 2016 were further divided into eight MGE types based on different combination profiles of the presence of five genes (MGE-A, B, C, D, E, F, G, and H). The primer sequences, target sites and gene combination profiles of 21 *K. pneumoniae* ST11 strains are shown in S1, S3 and S4, respectively.

Genetic relatedness among the ST11 KPC-*Kp* strains was also determined and confirmed using pulsed-field gel electrophoresis (PFGE) of XbaI-digested total DNA with a CHEF Mapper electrophoresis system (Bio-Rad, Hercules, CA, USA) using conditions outlined in the PulseNet protocol for Enterobacteriaceae. Dendrograms were conducted using the Dice coefficient and the unweighted pair–group method using average linkage clustering [19].

2.5. Analysis of IncFII-like plasmids from the GenBank database and Clinical *K. pneumoniae* isolates

Based on various KPC-K*p* plasmid sequences in the GenBank database, we analysed the Inc typing of those plasmids and conducted a detailed comparison of plasmids isolated from ST11 KPC-*Kp*. The plasmid analysis was performed using the plasmid MLST website (https://pubmlst.org/plasmid/).

Identification of IncFII-like plasmids in the clinical *K. pneumoniae* strains was conducted by PCR-based replicon typing. A total of 200–400 ng of DNA per amplification reaction was used as a template. The PCR amplification and sequencing of IncFII replicons (II*a,* II*k* and II*s*) were performed using previously reported primers [20]. The IncFII-like plasmid was confirmed by the positive detection of any of the three IncFII replicons (II*a,* II*k* and II*s*).

2.6. Transformation experiments of IncFII plasmids

Thirty ST11 KPC-*Kp* strains were selected based on different MGE and PFGE types. Plasmids from wild-type isolates were extracted using a Qiagen Plasmid Midi kit (Qiagen, Germany). *E. coli* ElectroMAX DH5α competent cells were transformed with the extracted plasmids by electroporation (Micro-Pulser electroporator; Bio-Rad, USA). Transformants were selected on MacConkey agar containing imipenem (0.5 mg/L) for KPC-*Kp* and MacConkey agar containing ampicillin (0.5 mg/L) for non-KPC-*Kp*. Putative

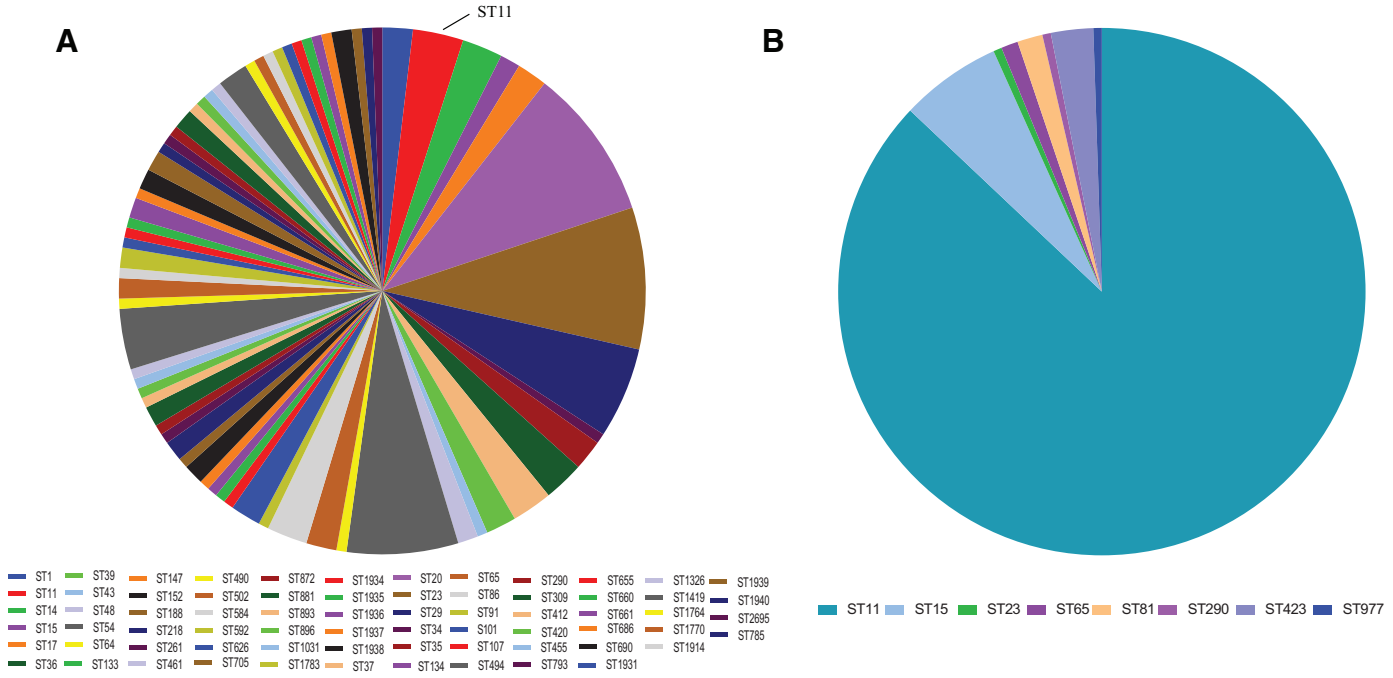

Fig. 1. Multilocus sequence typing (MLST) distribution of bla_{KPC-2}-positive (A.) and bla_{KPC-2}-negative (B.) *K. pneumoniae* isolates.

transformant colonies were selected and identified by the Vitek system and were further subjected to PCR amplification of the bla_{KPC-2}, Tn*1721* and IncFII replicons according to our previous operations.

2.7. Whole-genome sequencing and Nucleotide Blast of representative IncFII plasmids

Clinical *K. pneumoniae* strains no. 44 and no. 187 were recovered in 2016 and 2008, respectively, from Huashan Hospital (Shanghai, China). The genomic DNA of strains 44 and 187 were subjected to both short- and long-read massively parallel sequencing. Short-read sequencing was performed on the Illumina MiSeq system (Illumina), and long-read sequencing was performed using the Pacific Biosciences RS II platform (PacBio), reaching an average depth of more than 200 × in coverage (strain 44 reached 278 ×, and strain 187 reached 208 ×). The reads were assembled into contigs and scaffold sequences using the *de novo* assembler Newbler and CANU [21]. Furthermore, integration was needed for the assembled results of the Illumina short reads and PacBio long reads. The mummer software was employed to confirm the assembling results between the short- and long-read sequencing data, determine the location and arrangement of various contigs and fill the gaps among contigs [22]. The final whole-genome sequences were rectified using Pilon software [23] and trimmed into circular chromosome and plasmids. The whole-genome sequences identified on plasmids p44-2 and p187-2 have been released under the accession numbers CP025463.1 and CP025468.1, respectively.

A nucleotide Blast of the representative IncFII-like plasmids pHS062105-3, pKP048, pHS082416, pHS092753, and p44-2 (https://blast.ncbi.nlm.nih.gov/) was performed to determine the genetic structure of the Tn*1721* variants and bla_{KPC-2}. The genetic environment of Tn*1721* harboring bla_{KPC-2} was classified as either A- or B-type, based on those typical plasmids. pHS062105-3, pHS082416 and pHS092753 all originated from our laboratory and were chosen in this study to represent the genetic structures of A1-, B1- and B2-Tn*1721*-bla_{KPC-2}. The plasmid pKP048 (FJ628167.2) and the newly sequenced p44-2 were chosen to represent A2- and B3-Tn*1721*-bla_{KPC-2}.

2.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism 7. A *P*-value of less than 0.05 was considered statistically significant. Fisher's exact test was used to compare the presence of IncFII plasmids between different isolate groups.

3. Results

3.1. ST11 was the primary MLST type of KPC-Kp but was not predominant in non-KPC-Kp

Among the 354 *K. pneumoniae* isolates (223 CR-*Kp* and 131 CS-*Kp* strains) tested, 193 strains were found to harbor bla_{KPC-2}, and 161 strains were negative for bla_{KPC-2}. There was a significant difference in the MLST distribution between the KPC-*Kp* and non-KPC-*Kp* strains ($P<0.05$). In the bla_{KPC-2}-positive group, ST11 was the primary MLST type and accounted for 87.1% (168/193). ST15 was detected in 12 isolates (6.2%). Furthermore, six other types were detected, as follows: ST423 (5, 2.6%), ST81 (3, 1.6%), ST65 (2, 1.0%), ST23, ST290 and ST977 (1/193, 0.5% each). However, the MLST types were scattered, and there were no prevalent types in non-KPC-*Kp*. ST11 was detected at a rate of only 3.1% (5/161) in this group. The results of the MLST distribution are shown in Fig. 1.

3.2. Circulating ST11 KPC-Kp was clonally heterogeneous and divided into different subtypes

MGEs have been reported to generate significant genotypic and phenotypic variation within bacterial populations [13]. Comparative sequence analysis of worldwide, public *K. pneumoniae* ST11 isolated from 2008 to 2017 confirmed that the five MGEs underwent significant divergence over a long timescale. We

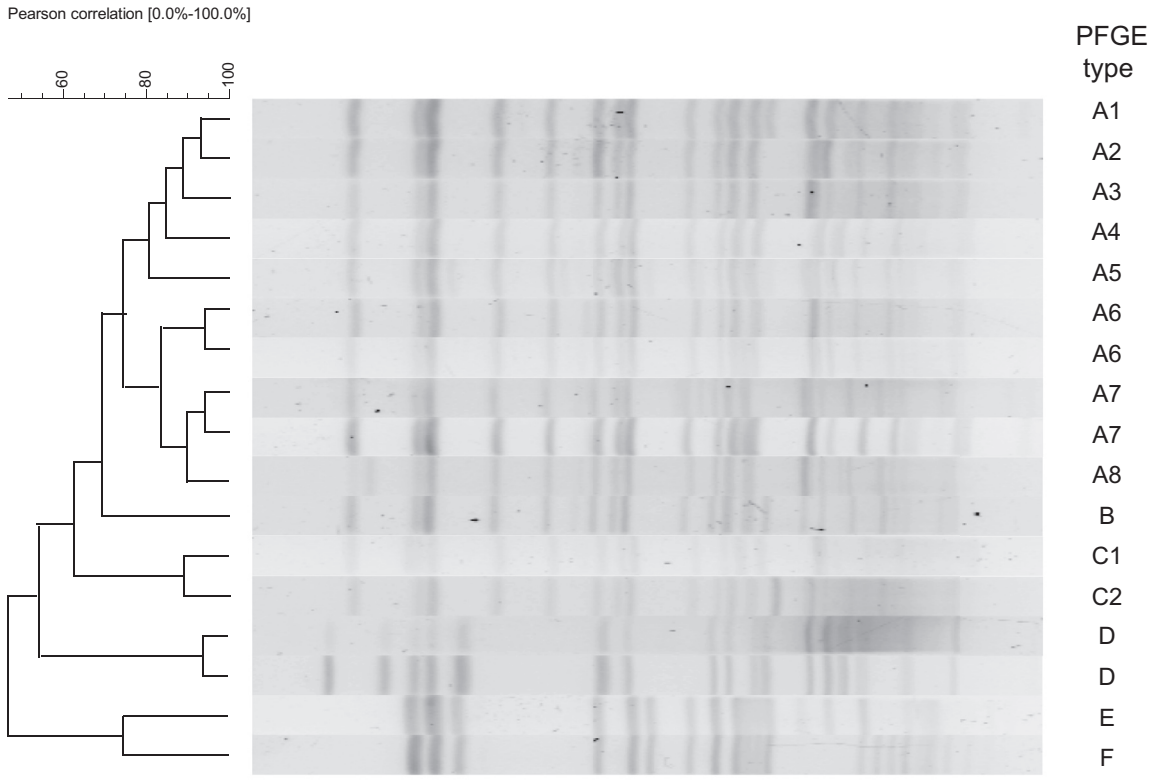

Fig. 2. Dendrogram of ST11 KPC-*Kp* strains based on pulsed-field gel electrophoresis (PFGE) of XbaI-digested total DNA with a CHEF Mapper electrophoresis system.

Table 1
MGE types among 168 ST11 KPC-*Kp* strains.

MGE type	*pcap*	*ICE*	*irp3*	*shock*	*prepA*	Number (%)
A	+	-	+	+	+	35 (20.83)
B	+	-	-	-	+	2 (1.19)
C	-	+	-	+	+	8 (4.76)
D	-	-	+	+	-	1 (0.60)
E	-	-	-	+	+	13 (7.74)
F	+	-	-	+	+	93 (54.76)
G	-	+	+	+	-	2 (1.19)
H	-	-	+	+	+	8 (5.36)
I	+	+	+	+	-	2 (1.19)
J	+	+	-	+	+	2 (1.19)
K	-	-	-	-	-	2 (1.19)

hypothesized that these MGEs would be appropriate for subtype definition. To test this hypothesis, we analysed public *K. pneumoniae* ST11 sequences and successfully divided them into different MGE subtypes. Then, the 168 clinical strains collected in this study were also shown to represent different subtypes according to MGE analysis. Concurrently, partial clinical strains were selected for PFGE verification, which also revealed different ST11 subtypes. Therefore, MGE analysis combined with PFGE could effectively demonstrate that the ST11 strains were clonally heterogeneous.

Among 168 ST11 KPC-*Kp* strains, 11 subtypes were detected: MGE-A, B, C, D, E, F, G, H, I, J and -K. MGE-F was the most common type (54.8%, 93/168), and MGE-A (20.8%, 35/168) was the second most common type. The other types accounted for 23.8% in total (40/168). PFGE analysis showed that the ST11 KPC-*Kp* isolates could be divided into 6 clusters and 14 subtypes (A1, A2, A3, A4, A5, A6, A7, A8, B, C1, C2, D, E, and F). These data indicate that the prevalence of ST11 KPC-*Kp* was not clonally homogeneous but consisted of several different subtypes (Table 1 and Fig. 2).

3.3. There was a very close linkage among IncFII plasmids, bla_{KPC} gene and K. pneumoniae ST11

First, a comparative sequence analysis was conducted of 86 plasmids carrying the bla_{KPC} gene that were deposited in GenBank. Remarkably, most of the plasmids (60.5%, 52/86) were identified as IncFII plasmids, and other Inc types were identified as follows: IncFI (8.1%, 7/86), IncA/C (5.8%, 5/86), IncN (16.3%, 14/86) IncX (8.1%,7/86) and IncI (1.2%, 1/86). The accession numbers and information of the 86 plasmids can be found in S5.

Next, we focused on IncFII plasmids and performed IncFII screening on all clinical *K. pneumoniae* isolates (including non-KPC-*Kp* and KPC-*Kp* strains). Significantly, the detection rate of the IncFII plasmids in ST11 KPC-*Kp* was 100%; the rate was much lower for non-ST11 KPC-*Kp* (16.0%) and the non-KPC-*Kp* group (7.45%). Seemingly, there is a very close linkage among IncFII plasmids, the bla_{KPC} gene and *K. pneumoniae* ST11.

To confirm the association between the IncFII plasmids and ST11 KPC-*Kp*, a sequence analysis was conducted on 11 public plasmids isolated from ST11 KPC-*Kp*. Interestingly, 90.9% of plasmids (10/11) carrying the bla_{KPC} gene were classified as IncFII plasmids (Table 2). From further analysis of different IncFII replicons, two IncFII-like plasmids were characterized among the clinical ST11 strains; the detection of IncFII plasmids (with the II*a* replicon) reached 68.5% (115/168), and that of IncFII*k* plasmids (with the II*k* replicon) reached 31.5% (53/168).

3.4. Different IncFII-like plasmids participated in the spread of the bla_{KPC-2} gene among K. pneumoniae ST11 strains

The presence of different IncFII-like plasmids was analysed in clinical *K. pneumoniae* ST11 strains and the IncFII plasmid was primarily detected in the ST11 subtype MGE-F (61.7%) and could also be found in MGE-A (17.4%), -B (1.7%), -C (3.5%), -D (0.9%), -E (7.0%),

Table 2
Incompatibility group analysis of plasmids separated from eleven ST11 KPC-K*p* obtained from Genebank.

Strains	MGE type	Location and date	*bla* $_{KPC}$	Plasmids analysis		
				Location of *bla* $_{KPC}$	Accession number	Inc types
F44	A	China (2016)	+	p44-2	CP025463.1	IncFII
SWU01	A	China (2015)	+	SWU01 plasmid	CP018455.1	IncFII
FDAARGOS_443	A	Canada (2012)	+	FDAARGOS_443 plasmid	CP023938.1	IncFII
FDAARGOS_444	A	Canada (2013)	+	FDAARGOS_444 plasmid	CP023942.1	IncFII
GD4	A	China (2015)	+	pKPGD4	CP025952.1	IncFII
WCHKP649	A	China (2015)	+	pKPC2_095649	CP026584.1	IncFII
WCHKP8F4	A	China (2015)	+	pKPC2_095084	CP027067.1	IncFII
JM45	B	China (2010)	+	JM45 plasmid p1	CP006657.1	IncFIIk
CAV1392	C	UK (2011)	+	pKPC_CAV1392	CP011575.1	No-IncFII
HS11286	G	China (2011)	+	pKPHS2	CP003224.1	IncFIIk
JS187	G	China (2008)	+	p187-2	CP025468.1	IncFIIk

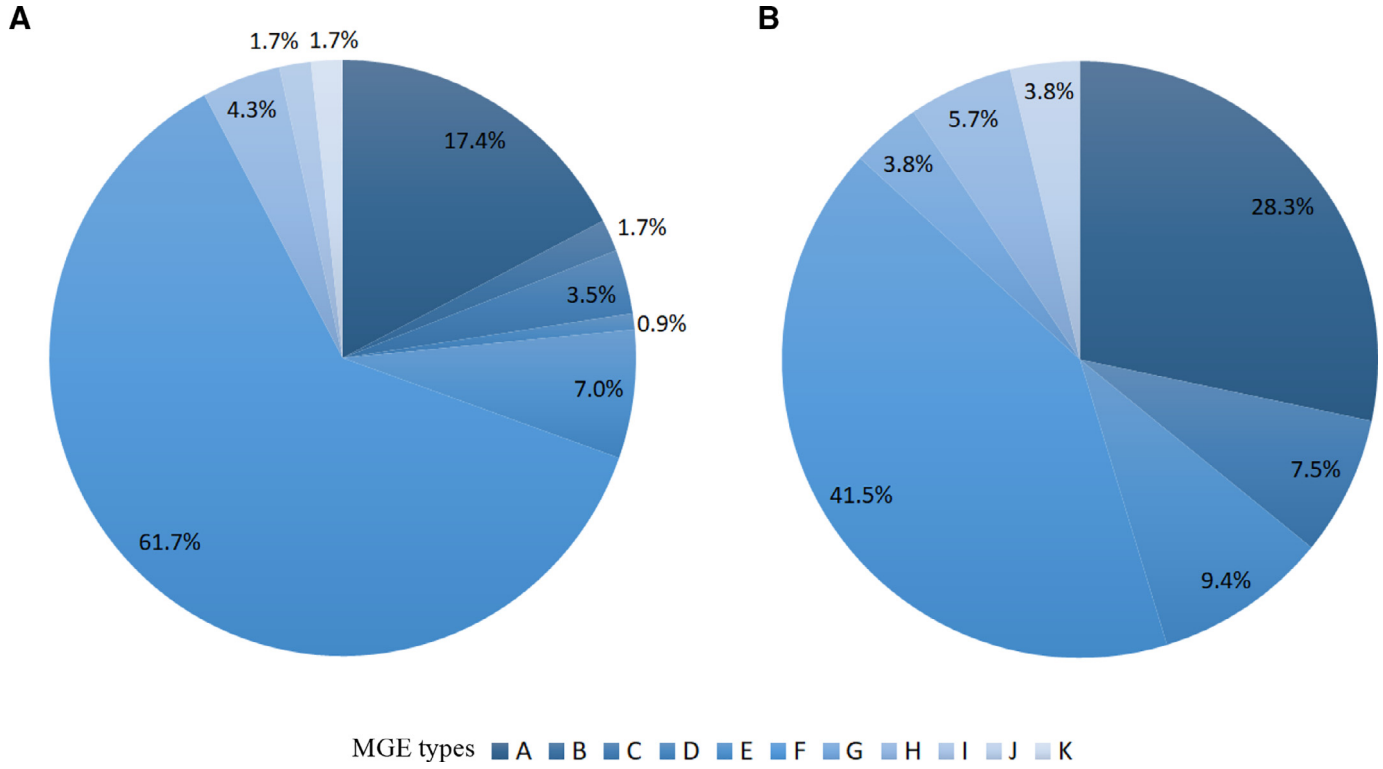

Fig. 3. The detection of different *K. pneumoniae* ST11 sublineages carrying IncFII plasmids (A.) and IncFII*k* plasmids (B.).

-H (4.3%), -I (1.7%) and -K (1.7%). The IncFII*k* plasmid was mainly identified in two ST11 subtypes, MGE-A (28.3%) and -F (41.5%); the other subtypes, such as MGE-C, -E, -G, -H, and -J, collectively accounted for 30.2% (16/53).

Similarly, the same ST11 subtype could harbor hetero-IncFII plasmids. Among the MGE-A type, the detection of the IncFII plasmid and IncFII*k* plasmid was 57.1% (20/35) and 42.9% (15/35), respectively. In the MGE-F group, the IncFII plasmid and IncFII*k* plasmid accounted for 76.3% (71/93) and 23.7% (22/93), respectively, as shown in Fig. 3.

3.5. Five different Tn*1721 elements carrying the* bla_{KPC-2} *gene were shown to be carried on IncFII-like plasmids*

Our molecular epidemiological data identified five different variants of Tn*1721* transposons among 168 ST11 KPC-*Kp* strains (Tn*1721*-A1, -A2, -B1, -B2, and -B3). A1-type (Tn*1721*-bla_{KPC-2}-Tn*3*) shared the same genetic environment as that found on pHS062105-3. A2-type (△IS*26*-Tn*1721*-bla_{KPC-2}-Tn*3*), with an IS*26* insertion before Tn*1721*-IR_R, was the same as pKP048. B1-type (△IS*26*-Tn*1721*-bla_{KPC-2}-△Tn*3*-IS*26*) and B2-type (Tn*1721*-bla_{KPC-2}-△Tn*3*-IS*26*), which were inverted by IS*26* at Tn*3* *tnpR* (△Tn*3*-IS*26*), shared the same region of bla_{KPC-2} as pHS082416 and pHS092753. With an IS*26* inserted within *tnpA*, B3-type (Tn*1721*-IS*26*- bla_{KPC-2}-△Tn*3*-IS*26*) exhibited the same structure as our whole-genome sequenced plasmids (p44-2 and p187-2). B1-Tn*1721* accounted for nearly half the samples (44.6%, 75/168), and A1-, A2-, B2- and B3-Tn*1721* accounted for 3.6% (6/168), 23.8% (40/168), 21.4% (36/193) and 6.7% (11/168), respectively (Fig. 4 and Table 3).

The nucleotide Blast analysis for representative IncFII plasmids (pHS062105-3, pKP048, pHS082416, pHS092753, p44-2 and p187-2) confirmed that Tn*1721* variants harboring the bla_{KPC-2} gene were located on IncFII-like plasmids. The sequence locations of bla_{KPC-2} on two PacBio-sequenced IncFII-like plasmids (p44-2 and p187-2) were between 8266 bp and 9147 bp and between 103 900 bp and 104 781 bp, respectively. Together, the PCR amplification and sequencing that were conducted on the 30 transformant plasmids generated results that were consistent with the genomic findings that Tn*1721* and bla_{KPC-2} are located on IncFII-like plasmids.

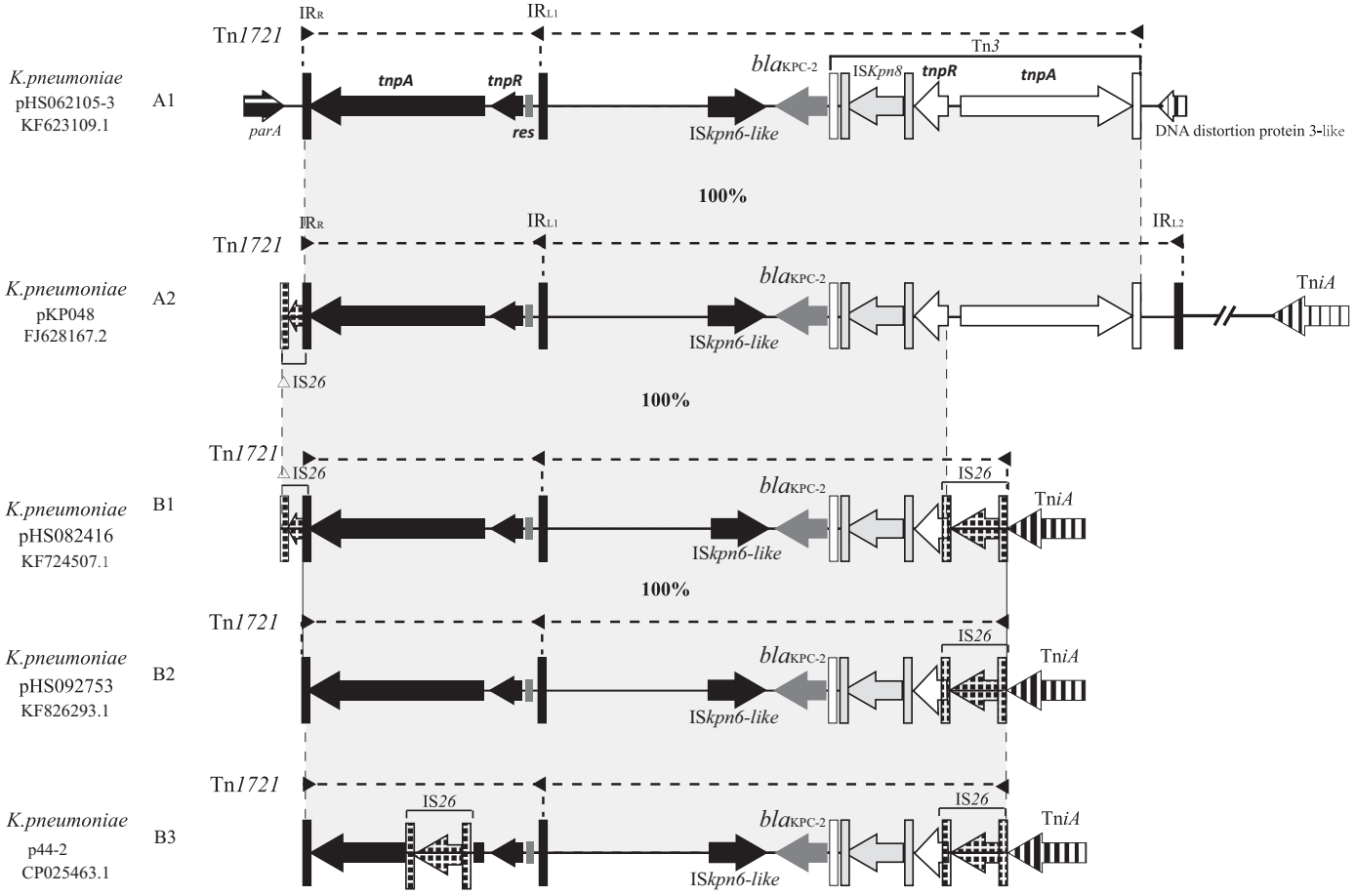

Fig. 4. Schematic representations of bla_{KPC-2}-bearing genetic elements classified as either A- or B-type and the sequence bearing bla_{KPC-2} in pHS062105-3 (GenBank accession no. KF623109.1), pKP048 (no. FJ628167.2), pHS082416 (KF724507.1), pHS092753 (KF826293.1) and p44-2(CP025463.1). Genes are depicted as arrows according to the direction of transcription. bla_{KPC-2} is depicted in dark gray. Inverted repeats are indicated by color-variable, vertical bars shown in the following corresponding matching colors: Tn*1721* (black), Tn*3* (white) and IS*26* (gray). Regions sharing identical or near-identical sequences across plasmids are indicated by the gray shading between the representations of different plasmids.

Table 3
Detection rate and location of five Tn*1721* -*bla* $_{KPC-2}$ variants on 168 ST11 KPC-*Kp*.

Tn*1721* types	Genetic environment surrounding *bla* $_{KPC-2}$	Location	Detection n (%)
A1	Tn*1721* -*bla* $_{KPC-2}$ -Tn*3*	incFII-like plasmid	6 (3.57)
A2	△IS*26* -Tn*1721* -*bla* $_{KPC-2}$-Tn*3*	incFII-like plasmid	40 (23.81)
B1	△IS*26* -Tn*1721* -*bla* $_{KPC-2}$-△Tn*3* -IS*26*	incFII-like plasmid	75 (44.64)
B2	Tn*1721* -*bla* $_{KPC-2}$-△Tn*3* -IS*26*	incFII-like plasmid	36 (21.43)
B3	Tn*1721* -IS*26* -*bla* $_{KPC-2}$-△Tn*3* -IS*26*	incFII-like plasmid	11 (6.55)

4. Discussion

K. pneumoniae is a common nosocomial pathogen that causes difficult-to-treat infections worldwide. The prevalence of KPC-*Kp* is increasing in China. Many studies have demonstrated that CC258 is responsible for the pandemic spread of KPC-*Kp* throughout the world [24]. ST11 is the dominant clone of KPC-*Kp* and has been widely reported in different regions in China [9,14,15,25,26]. This study provides key insights into the horizontal transfer of the Tn*1721* and IncFII plasmids, which appears to be a potential element driving the molecular diversification in *K. pneumoniae* ST11 strains. HGT mediated by IncFII-like plasmids plays an important role in the pandemic dissemination of bla_{KPC-2} among *K. pneumoniae* ST11 in China.

The results of our study confirm that *K. pneumoniae* ST11 is the primary type of bla_{KPC-2}-bearing *K. pneumoniae* (87.1%) but is not prevalent in non-KPC-*Kp* strains (3.1%). A previous report on KPC-*Kp* in China by Shen et al. (2016) also showed that ST11 KPC-*Kp* was not the first strain to emerge; rather, this strain gradually became dominant [25]. Twenty-one public *K. pneumoniae* ST11 strains isolated from 2008 to 2017 consisted of different sublineages; the 168 clinical isolates in this study exhibited a similar pattern. Acquisition of the bla_{KPC-2} gene of *K. pneumoniae* ST11 appears to follow a gradual evolutionary history rather than a one-step process. We hypothesized that the pandemic of *K. pneumoniae* ST11 occurred because ST11 had a better ability to capture or accumulate bla_{KPC} compared with the other types.

In the current study, approximately half the ST11 KPC-*Kp* samples (53.8%) shared the same MGE type-F, implicating the potential spread of this clone among clinical strains. However, the detection of 10 different MGE types and a total of 14 PFGE types of *K. pneumoniae* ST11 reveals that ST11 KPC-*Kp* strains in China constitute a generally non-clonal population. In a tertiary care hospital in Beijing, two major clusters (A and C) of ST11 KPC-*Kp* were

shown by PFGE to have been involved in a nosocomial outbreak [27]. A recent study in China also found that ST11 genomes are highly heterogeneous and cluster into at least three major lineages based on single nucleotide polymorphism (SNP) analysis [28]. Evidently, the molecular diversification in *K. pneumoniae* ST11 strains cannot be fully explained by simple clonal spread. Therefore, we focused on HGT transmission to elucidate the function and interaction of transfer elements in the pandemic of the bla_{KPC-2} gene among *K. pneumoniae* ST11 in China.

Certain mobile structures, such as transferable plasmids and transposons harboring the bla_{KPC} gene, play an important role in the dissemination of KPC-*Kp* [5,29]. Genetic structures surrounding the bla_{KPC} gene are mostly associated with two Tn*3*-based transposons [19]; one such structure is Tn*4401*, which has many variants [30], and another is Tn*1721,* which contains an integration structure of the Tn*3*-based transposon and the partial Tn*4401* segment [26]. Both of these Tn*3*-based transposons transposed the bla_{KPC} gene when integrated into a new target using a "paste-and-copy" mechanism [31]. While the Tn*1721* transposon was primarily shown to be associated with bla_{KPC-2} in China, distinct Tn*1721* elements carrying the bla_{KPC-2} gene were found to be carried on IncFII-like plasmids [31]. Conjugative plasmids play an important role in the horizontal transfer of bla_{KPC-2}, particularly the IncFII-like plasmid, which was postulated to contribute significantly to the global success of CC258 KPC-*Kp* [32]. Certain IncFII-like plasmids, such as pKP48 and pKPHS2, have already been described in strains from China [33,34]. Therefore, we chose those two mobile elements (Tn*1721* transposons and IncFII-like plasmids) to elaborate on their genetic functions in the evolutionary process of ST11 KPC-*Kp* in China.

The Inc analysis of 86 worldwide public plasmids indicated that IncFII-like plasmids mainly (60.5%) participated in the spread of bla_{KPC-2}, particularly in ST11 KPC-*Kp* strains (90.9%). Molecular detection analyses of clinical *K. pneumoniae* isolates showed that 100% of the ST11 KPC-*Kp* isolates harbored the IncFII-like plasmid, which is a much higher rate than that of the non-ST11 KPC-*Kp* and bla_{KPC-2}-negative groups. Molecular biological detection, electroporation experiments and WGS ascertained that five Tn*1721*-bla_{KPC-2} variants were carried by IncFII-like plasmids. These findings directly revealed an explicit correlation among bla_{KPC-2}, Tn*1721*, the IncFII-like plasmid and *K. pneumoniae* ST11. The IncFII-like plasmid appears to function as a good carrier of Tn*1721*-bla_{KPC} and promotes the spread of the bla_{KPC} gene among *K. pneumoniae* ST11.

Different IncFII-like replicons among the clinical ST11 KPC-*Kp* isolates were investigated. The IncFII plasmid was primarily detected in MGE-F (61.7%) and was also detected in eight other ST11 types (MGE-A, -B, -C, -D, -E, -H, -I, and -K). IncFII*k* plasmids can be grouped mainly in the MGE-A (28.3%) and -F (41.5%) types and are also detected in MGE-C, -E, -G, -H, and -J. Meanwhile, the same ST11 subtype was shown to harbor hetero-IncFII-like plasmids. For example, the IncFII plasmid (57.1%) and the IncFII*k* plasmid (42.9%) were detected among the MGE-A type isolates. The diversity of IncFII-like plasmids and ST11 subtypes cannot be fully explained by clonal transmission. Different IncFII-like plasmids carrying Tn*1721*-bla_{KPC} appear to interexchange and intertransfer among diverse *K. pneumoniae* ST11 subtypes. We reasoned that HGT mediated by hetero-IncFII-like plasmids contributed to the spread of bla_{KPC} among *K. pneumoniae* ST11. The horizontal transfer of Tn*1721* and IncFII plasmids appears to be an important element driving molecular diversification among *K. pneumoniae* ST11 strains.

A previous report showed that the transposition of Tn*4401* is not very efficient, and the frequency is very low [35]. Some plasmids that have acquired Tn*4401* may in turn enhance the spreading efficiency of the bla_{KPC} genes, particularly IncFII-like plasmids, in the evolution of CC258. Chmelnitsky et al. (2013) proposed that the successful global dissemination and survival of ST258 were, in part, dependent on the combination of bla_{KPC} on IncF plasmids with factors inherently present on the chromosome of this high-risk clone [36]. Other selection mechanisms also potentially participate in the evolution of CC258. Our epidemic and laboratory findings revealed a clear relationship among IncFII-like plasmids, Tn*1721*-bla_{KPC-2} and *K. pneumoniae* ST11. Based on our findings, we propose that the HGT of Tn*1721*-bla_{KPC-2} is mainly and effectively driven by IncFII-like plasmids among *K. pneumoniae* ST11 strains.

IncFII-like plasmids harboring Tn*1721*-bla_{KPC-2} contributed significantly to the evolutionary dominance of ST11 KPC-*Kp* in China. There was a close correlation between ST11 KPC-*Kp* and IncFII-like plasmids, whereby ST11 KPC-*Kp* was a seemingly good colonizer to capture IncFII-like plasmids, which were broadly transmitted in clinical environments. As the interaction of ST11 KPC-*Kp* and IncFII-like plasmids may be strengthened by a mechanism that has yet to be identified, it is feasible that IncFII-like plasmids were either more easily acquired or more easily retained after entering *K. pneumoniae* ST11.

5. Conclusions

Our study provides comprehensive research on the distribution of STs and the genetic environment of the bla_{KPC-2} gene in KPC-*Kp* and non-KPC-*Kp* isolates from China. ST11 KPC-*Kp* was shown to be clonally heterogeneous and to consist of a number of sublineages that have acquired bla_{KPC-2} genes (in varying Tn*1721*-like transposons). HGT mediated by IncFII-like plasmids contributes significantly to the pandemic dissemination of bla_{KPC-2} among *K. pneumoniae* ST11 in China, and further research is warranted to clarify the underlying mechanism.

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Competing Interests

None

Ethical Approval

Not required

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.03.014.

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