Replicative transposition contributes to the evolution and dissemination of KPC-2-producing plasmid in Enterobacterales

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Abstract

Klebsiella pneumoniae carbapenemase (KPC)-producing Enterobacterales are prevalent worldwide and pose an alarming threat to public health. The incidence and transmission of bla\textsubscript{KPC-2} gene via horizontal gene transfer (e.g. transposition) have been well documented. However, the dynamics of transposon structure bearing bla\textsubscript{KPC-2} and their exact effects on the evolution and dissemination of KPC-2 gene are not well characterized. Here, we collected all 161 carbapenem-resistant Enterobacterales (CRE) isolates during the early stage of CRE pandemic. We observed that the prevalence of KPC-2-producing Enterobacterales was mediated by multiple species and sequence types (STs), and that bla\textsubscript{KPC-2} gene was located on three diverse variants of Tn\textsubscript{1721} in multi-drug resistance (MDR) region of plasmid. Notably, the outbreak of KPC-2-producing plasmid is correlated with the dynamics of transposon structure. Furthermore, we experimentally demonstrated that replicative transposition of Tn\textsubscript{1721} and IS\textsubscript{26} promotes horizontal transfer of bla\textsubscript{KPC-2} and the evolution of KPC-2-producing plasmid. The Tn\textsubscript{1721} variants appearing concurrently with the peak of an epidemic (A2- and B-type) showed higher transposition frequencies and a certain superior ability to propagation. Overall, our work suggests replicative transposition contributes to the evolution and transmission of KPC-2-producing plasmid and highlights its important role in the inter- and intra-species dissemination of bla\textsubscript{KPC-2} gene in Enterobacterales.

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Introduction

Klebsiella pneumoniae carbapenemase (KPC) is a class A serine \( \beta \)-lactamase that efficiently hydrolyzes most \( \beta \)-lactam antimicrobial agents, including carbapenems, limiting treatment options in infected patients seriously [1]. KPC-producing Enterobacterales, particularly \( K. \) pneumoniae, have spread worldwide over the last decade, becoming an urgent public health threat [2]. KPC-2, the most commonly identified variant, is a dominant factor leading to carbapenem resistance in Enterobacterales. The bla\textsubscript{KPC-2} gene is typically identified in mobile transposon, which is most often situated on conjugative plasmids [2–4]. Tn\textsubscript{4401} is the major vehicle of bla\textsubscript{KPC-2} in most countries and regions, such as Europe [5], the United States [6], and Brazil [7]. In Asia, bla\textsubscript{KPC-2} is mostly located on diverse variants of Tn\textsubscript{1721} and IS\textsubscript{26} [8–10].

Horizontal gene transfer (HGT) plays an important role in the evolution of bacteria and the dissemination of antibiotic resistance genes [11]. Tn\textsubscript{4401} and Tn\textsubscript{1721}, typical replicative transposons belonging to Tn3 family, have been proved to mobilize bla\textsubscript{KPC-2} at a high transposition frequency, and the latter is capable of transferring bla\textsubscript{KPC-2} both internal and external to this element [12,13]. However, few published studies provided comprehensively analysis of epidemic of KPC-2-producing Enterobacterales, the dynamics of genetic structure surrounding bla\textsubscript{KPC-2} and transposition mechanism of these elements. Notably, bla\textsubscript{KPC-2} is located on diverse variants of Tn\textsubscript{1721} that exhibited various transposition frequencies and movement patterns [13], and the mechanism of movement of A2-type (Tn\textsubscript{1721}-bla\textsubscript{KPC-2}-IS\textsubscript{26}) remains undetermined.

Here, we show that outbreak of KPC-2-producing plasmid is correlated with the dynamics of transposon structure in Enterobacterales. The A2- and B-type Tn\textsubscript{1721} appearing concurrently with the peak of the epidemic of bla\textsubscript{KPC-2}-carrying isolates demonstrated higher transposition frequency. Their specific replicative transposition (the IS\textsubscript{26}...
pattern for A2-type and the Tn1721-bla\textsubscript{KPC-2} gene pattern for B-type) had a certain superior ability to conjugate into another strain. Thus, replicative transposition contributes to the evolution and dissemination of KPC-2-producing plasmid in Enterobacteriales, facilitating the inter- and intra-species dissemination of bla\textsubscript{KPC-2}.

Materials and methods

Media and growth of strains

Bacteria were routinely cultured at 37°C in Luria-Bertani (LB) medium or on LB agar. Plasmids were constructed in Escherichia coli DH5α (supE44 Δ lacU169 [φ 80 lacZ ΔM15] hsdR17 recA1 endA1 gyrA96 thi-1 relA1), HB101 (recA13 F- STR\textsuperscript{R}) and E. coli J53 (AZ\textsuperscript{R}) were used as recipient strains in transposition experiments and conjugation experiments, respectively. The following antibiotics were added at the indicated concentrations: imipenem (IPM), 1 mg/L; streptomycin (STR), 25 mg/L; trimethoprim (TMP), 25 mg/L and sodium azide (AZ), 100 mg/L.

Clinical isolates

A total of 161 non-duplicated, carbapenem-resistant Enterobacteriales (CRE) isolates were collected from August 2006 to December 2010 during routine identification and antimicrobial susceptibility testing by the Microbiology Laboratory, Huashan Hospital, Fudan University (Shanghai, China). This collection comprised all clinical isolates from the first occurrence of KPC-2-producing isolates (K. pneumoniae) to the prevalence of this carbapenemase (Dataset S1). For comparison, a susceptible collection of 112 carbapenem-sensitive K. pneumoniae (CS-KP) were also collected contemporaneously from similar departments (Dataset S2).

Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE)

MLST was performed according to the protocol described on the Pasteur Institute MLST website (https://bigdb.pasteur.fr/klebsiella/klebsiella.html for K. pneumoniae and https://bigdb.pasteur.fr/ecoli/ecoli.html for E.coli) and PubMLST website (https://pubmlst.org/organisms/citrobacter-spp for Citrobacter freundii and https://pubmlst.org/organisms/klebsiella-aerogenes for Klebsiella aerogenes). Genetic relatedness among the ST11 K. pneumoniae isolates was analyzed by XbaI-PFGE type as described previously [9]. Dendograms were conducted using the Dice coefficient and the unweighted pair-group method using average linkage clustering [14].

PCR screening of bla\textsubscript{KPC-2} and IncFII replicon and analysis of genetic environment of bla\textsubscript{KPC-2} gene among clinical isolates

The bla\textsubscript{KPC-2} were identified through the amplification and sequence analysis of a 750-bp polymerase chain reaction (PCR) product [15]. IncFII replicon screening was conducted by PCR-based replicon typing using previously reported primers [16]. The bla\textsubscript{KPC-2} bearing genetic structures were determined by a series of PCR assays as reported previously [15].

Transformation and conjugation experiments of bla\textsubscript{KPC-2}-bearing plasmids

Thirty-five bla\textsubscript{KPC-2} bearing plasmids were obtained by transformation or conjugation (Dataset S3). Plasmids of clinical isolates were extracted with a Qiagen Plasmid Mdi kit (Qiagen, Germany) and examined by agarose gel electrophoresis, and then transformed into E. coli DH5α Electrocompetent cells by electroporation (Micro-Pulsar electroporator; Bio-Rad, USA). Construction experiment was performed with E. coli J53 (AZ\textsuperscript{R}) as the recipient. Transformants and conjugants were selected on MacConkey agar containing IPM (AZ was also used in conjugants selection) and identified by VITEK 2 system (bioMérieux, France) and were further subjected to PCR amplification of IncFII replicons, bla\textsubscript{KPC-2} and genetic environment of bla\textsubscript{KPC-2} according to our previous operations. For plasmids originated from clinical isolates of E. coli, each transformant was also identified by detecting the deletion of lacY gene as E. coli DH5α lacks the lac operon. Primers are listed in Table S1.

Bioinformatics analysis

All complete genome sequences of Enterobacteriales publicly available (5152 in total) and all of the plasmid sequences harbored in these strains (10,507 in total) were downloaded from NCBI database in August 2021 (Chromosomes in Dataset S4 and plasmids in Dataset S5). The bla\textsubscript{KPC-2} gene was identified by using nucleotide BLAST. Plasmid incompatibility type was determined by comparing with information in the Plasmid MLST locus/sequence definitions database (https://pubmlst.org/bigdb?db=pubmlst_plasmid_seqdef& page = sequenceQuery).

Plasmids construction

The primers and plasmids used in this study are listed in Table S1 and Table S2, respectively. pHS10842 (GenBank accession no. KP125892), a vector favorable for use in the exploration of the transposition mechanism of Tn1721-like transposons, has been described previously [13].
For pHS10842-ΔtnpA_Tn1721, IRR and tnpR fragments were amplified with primers JP934/JP935 and JP936/JP937, respectively. The IRR fragment share 20-bp sequences with AhdI restriction sites of pHS10842 and the tnpR fragments, respectively. The tnpR fragments also share 20-bp sequences with the IRR fragment and AflIII restriction sites of pHS10842, respectively. The two fragments were subcloned into AhdI and AflIII restriction sites of pHS10842 by use of the NEBuilder HiFi DNA assembly master mix (New England BioLabs, USA), generating the derivative with the tnpA deletion of Tn1721, pHS10842-ΔtnpA_Tn1721.

The transposase deletion was introduced into IS26 by digesting plasmid pHS10842 with SwaI and XmnI to removal a 560-bp fragment (base 11,366 to base 11,925 in GenBank accession no. KP125892) and generate blunt ends. The DNA was ligated and transformed into competent cells of E. coli DH5α. pHS10842-ΔtnpA_Tn1721/Δtnp26 was constructed in a similar strategy with pHS10842-ΔtnpA_Tn1721.

Transposition assays and molecular characterization of transposition events

Transposition assays were performed as described previously [13,17]. Transposition frequency is calculated as the number of IPM<sup>T</sup> TMP<sup>T</sup> STR<sup>T</sup> transconjugants per TMP<sup>T</sup> STR<sup>T</sup> transconjugant. For each transposition event, the movement patterns were determined by agarose gel electrophoresis and Southern hybridization [13]. The exact insertion site and target site duplication of Tn1721 and IS26 were determined by using primers specific for regions internal to Tn1721 and IS26 and primers specific for R388 as described previously [13]. Primers are listed in Table S1.

Analysis of the target site consensus sequence

The relative frequencies of the AT and GC contents of the region extending from 50 bp upstream to 50 bp downstream of the duplicated target site for IS26 (8 bp) were calculated and plotted on a line graph. The pictures of the relative frequencies of the bases at each position were generated with the Pictogram program (http://genes.mit.edu/pictogram.html).

Statistics

Statistical significance was assessed by Fisher’s exact test or Chi-square test with Yates’ correction using GraphPad Prism8 software (https://www.graphpad.com/). P < 0.05 was considered statistically significant.

Results

The prevalence of KPC-2-producing Enterobacterales was mediated by multiple species and STs

All of the 161 CRE were identified as bla<sub>KPC-2</sub>-positive, including K. pneumoniae (112, 69.56%), E. coli (15, 9.32%), Citro. freundii (15, 9.32%), K. aerogenes (12, 7.45%) and other species (7, 4.35%) (Figure 1A). Among the 112 K. pneumoniae isolates, ST11 was the most prevalent ST, followed by ST423, ST65 and ST977, and PFGE of ST11 K. pneumoniae indicated five diverse subtypes with a criterion of 75% identity (Figure 1B). For comparison, we collected CS-KP as susceptible controls that were matched by time and ward. As expected, all CS-KP isolates were bla<sub>KPC-2</sub>-negative and the STs of the susceptible collection were scattered without any dominant STs (Figure S1). E. coli, Citro. freundii and K. aerogenes isolates were comprised of several STs. Together, these results suggested that bla<sub>KPC-2</sub> is the chief culprit leading to carbapenem resistance and that the prevalence of bla<sub>KPC-2</sub> in Enterobacterales was mediated by multiple species and STs, rather than clonal spread.

bla<sub>KPC-2</sub> genes were usually located on three diverse variants of Tn1721 in MDR region of plasmid

The bla<sub>KPC-2</sub> genes were usually reported to be located on MDR region of plasmid, and IncFII plasmids contributed significantly to the global prevalence of bla<sub>KPC</sub> among K. pneumoniae [4,9]. To determine the correlation between IncFII plasmids and bla<sub>KPC-2</sub> gene, we conducted a bioinformatic analysis of 5152 complete genome sequences of Enterobacterales publicly available (including 5152 chromosomes and 10,506 plasmids in total, Dataset S4 and Dataset S5). Among these, 311 strains were identified to be bla<sub>KPC-2</sub>-positive, and the overwhelming majority of bla<sub>KPC-2</sub> genes were found on plasmids (300/311, 96.46%). Remarkably, most of the bla<sub>KPC-2</sub>-bearing plasmids belonged to IncFII group, but this group was less common in bla<sub>KPC-2</sub>-negative plasmids [174/300 (58%) versus 2195/10206 (21.51%), P < 0.0001; Table 1]. Moreover, IncFII plasmids were significantly over-represented in bla<sub>KPC-2</sub>-positive K. pneumoniae compared with that in bla<sub>KPC-2</sub>-negative group [151/204 (74.02%) versus 657/2701 (24.32%), P < 0.0001; Table 1]. These findings suggest that bla<sub>KPC-2</sub> genes are mostly located on plasmids in Enterobacterales, and that IncFII is the most common Incompatibility group, especially in K. pneumoniae.

To evaluate this correlation in clinical isolates, we first performed IncFII replicon screening on all clinical isolates. Significantly, the detection rate of IncFII replicon was 84.82% in K. pneumoniae and the rate
was slightly lower for *E. coli* (73.33%) and *K. aerogenes* (66.67%) (Table S3). None of *Citro. freundii* isolates was detected to IncFII-positive. However, the rate was much lower in carbapenem-susceptible collection in comparison with either CR-KP or CRE collection (49.11% vs. 84.82% for CR-KP, \( P < 0.0001 \); 49.11% vs. 72.05% for CRE, \( P = 0.0001 \), Figure 2A). Next, the linkage between IncFII plasmids and *bla*\(_{KPC-2}\) gene was determined in thirty-five transformants and conjugants containing *bla*\(_{KPC-2}\)-positive plasmid by PCR amplification of IncFII replicons and genetic environment of *bla*\(_{KPC-2}\) (see Dataset S3 for details). Interestingly, 80% of plasmids carrying *bla*\(_{KPC-2}\) gene (28/35) belong to IncFII group. Finally, five plasmids in various sizes (two IncFII-negative and four IncFII-positive, Dataset S3) were selected for complete sequence analysis to furtherly confirm the correlation between IncFII plasmids and *bla*\(_{KPC-2}\) gene.

Moreover, our analysis of genetic environment of *bla*\(_{KPC-2}\) gene among 161 CRE identified three diverse variants of Tn1721 bearing *bla*\(_{KPC-2}\) (designated A1-, A2- and B-type) (Figure 2B). All of them possess a core element (Tn1721) that consists of two 38-bp terminal inverted repeats (IRs; the right inverted

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**Figure 1.** The molecular epidemiological investigation of CRE isolates (A) Species distribution and sequence types (STs) of *K. pneumoniae, E. coli, Citro. freundii* and *K. aerogenes*. (B) Dendrogram of ST11 *K. pneumoniae* isolates based on pulsed-field gel electrophoresis (PFGE) of XbaI-digested DNA.
repeat [IRR] and the left inverted repeat [IRL]), transposase (TnpA), resolvase (TnpR) and resolution site (res). The B-type is composed of Tn1721, blaKPC-2, an additional inverted repeat (IRL2), as well as several unrelated elements (Tn3, ISKpn6, and ISKpn8). The sequence of A1-type (Tn1721-blaKPC-2) shares 100% identity with that of B-type, but it does not contain the additional IRL, IRL2. In the A2-type, Tn3 was disrupted by an IS26, forming the chimera, Tn1721-blaKPC-2-IS26. The A2-type Tn1721 accounted for more than half the samples (59.01%, 95/161), and A1- and B-type accounted for 19.25% (31/161) and 18.63% (30/161), respectively (Table 2 and Figure 2B).

**Outbreak of KPC-2-producing plasmids is correlated with the dynamics of transposon structure**

In order to clarify the progression of blaKPC-2 outbreak, we comprehensively analyzed all data obtained in molecular epidemiological investigation. Figure 3 provides an overview of the evolution of blaKPC-2 bearing structure in Enterobacterales. Samples are plotted by month of isolation, wards, species, STs of most common resistant specie (K. pneumoniae) and transposable elements carrying blaKPC-2 gene. As shown in Figure 3, blaKPC-2 genes that were located in three distinct Tn1721-like transposons on plasmid, originated from K. pneumoniae, then became increasingly prevalent in this specie and spread in Enterobacterales further. The epidemic of blaKPC-2-carrying plasmid reported

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**Table 1. Incompatibility group analysis of plasmids with or without blaKPC-2 obtained from NCBI database.**

| Plasmid host | blaKPC-2-positivea | blaKPC-2-negative | P-value |
|-------------|--------------------|-------------------|---------|
| K. pneumoniae | 151/204 (74.02%) | 657/2701 (24.32%) | < 0.0001 |
| E. coli | 5/24 (20.83%) | 780/3716 (20.99%) | 0.8161 |
| Citro. freundii | 3/20 (15%) | 42/274 (15.33%) | 1.0000 |
| K. aerogenes | 1/3 (33.33%) | 1/48 (2.08%) | 0.1153 |
| Others | 14/49 (28.57%) | 715/3467 (20.62%) | 0.2359 |
| In total | 174/300 (58%) | 2195/10206 (21.51%) | < 0.0001 |

aData are number of IncFII plasmids/total number (% of IncFII-positive rate). P-value for comparisons of the IncFII-positive rates of blaKPC-2-positive and blaKPC-2-negative groups.

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**Figure 2.** IncFII screen of clinical isolates and genetic structures surrounding blaKPC-2. (A) IncFII-positive rates of blaKPC-2-positive and blaKPC-2-negative groups. (B) Schematic representation of blaKPC-2-bearing genetic elements classified as A1-, A2- and B-type. Genes are depicted as arrows according to the direction of transcription. blaKPC-2 is shown as spotted arrows. Inverted repeats are indicated by rectangles in different colors: Tn1721 (black), Tn3 (dark gray), ISKpn6 (light gray), and IS26 (white). Regions sharing identical sequences across plasmids are indicated by gray shading between the different representations of the plasmids. The GenBank accession numbers for pHS10505, pHS062105, and pHS10842 are KF826292, KF623109, and KP125892, respectively.
here had three stages, designated as (i) distributed period (August 2006–April 2009, 19 isolates), (ii) epidemic period (May 2009–February 2010, 50 isolates), and (iii) mixed epidemic period (March–December 2010, 92 isolates). In the distributed period, \( \text{bla}\text{KPC-2} \) appeared sporadically in \( K.\text{pneumoniae} \) without any predominate STs, and all Tn1721-like transposons belonged to A1-type. At the second epidemic stage, KPC-2-producing plasmid were prevalent in \( K.\text{pneumoniae} \) ST11 that consisted of several different subtypes, and spread into other \( \text{Enterobacterales} \). A2-type Tn1721 was the dominant structure carrying \( \text{bla}\text{KPC-2} \). In the mixed epidemic period, KPC-2-producing isolates increased furtherly. This stage involved three distinct Tn1721 variants, and the number of emerging B-type was almost equal to that for A2-type during the same period.

Altogether, outbreak of KPC-2-producing plasmid was correlated with the dynamics of \( \text{bla}\text{KPC-2} \)-bearing transposon structure, and A2- and B-type Tn1721 appeared concurrently with the peak of \( \text{bla}\text{KPC-2} \) epidemic.

**Table 2. Detection rate of Tn1721-like transposons bearing \( \text{bla}\text{KPC-2} \) on 161 CRE.**

| Tn1721 types | Genetic structure surrounding \( \text{bla}\text{KPC-2} \) | Detection of \( K.\text{pneumoniae} \) | Detection of other \( \text{Enterobacterales} \) | Detection of \( \text{Enterobacterales} \) (%)
|--------------|---------------------------------|-------------------------------|---------------------------------|---------------------------------
| A1           | Tn1721-\( \text{bla}\text{KPC-2} \) | 10                            | 21                              | 31 (19.25%)                     |
| A2           | Tn1721-\( \text{bla}\text{KPC-2}-\text{IS26} \) | 73                            | 22                              | 95 (59.01%)                     |
| B            | Tn1721-\( \text{bla}\text{KPC-2}-\text{IRL2} \) | 28                            | 2                               | 30 (18.63%)                     |
| Others       | Undefined                        | 1                             | 4                               | 5 (3.11%)                       |

**Figure 3.** Overview of 161 CRE clinical isolates collected from Huashan Hospital. \( K.\text{pneumoniae} \) isolates are shown as solid signs: squares (ST423), circles (ST977), triangles (ST65), and asterisks (ST11). Other \( \text{Enterobacterales} \) isolates are shown as empty signs: circles (\( E.\text{coli} \)) and triangles (others). Isolates are plotted based on ward (vertical axis) and time of collection (horizontal axis). Color indicates distinct Tn1721 variants carrying \( \text{bla}\text{KPC-2} \): A1-type (green), A2-type (red), B-type (blue) and undefined (black).

**Replicative transposition promotes horizontal transfer of \( \text{bla}\text{KPC-2} \)**

A critical step in the dissemination process of \( \text{bla}\text{KPC-2} \) is the HGT of mobile genetic elements (MGEs) surrounding this determinant. According to our comprehensive analysis of epidemiological data, it was presumed that replicative transposons, Tn1721 and IS26, can mobilize \( \text{bla}\text{KPC-2} \) through transposition, promoting the dissemination of \( \text{bla}\text{KPC-2} \) in \( \text{Enterobacterales} \). In our previous study, A1- and B-type Tn1721 have been shown to transfer \( \text{bla}\text{KPC-2} \) both internal and external to this element, and target transposition into 5-bp region that gradually exhibits a degenerated degree of AT-rich regions from both sides to the middle and that is immediately flanked by GC-rich regions [13]. Here, we characterized the movement and target site of A2-type Tn1721 (Tn1721-\( \text{bla}\text{KPC-2}-\text{IS26} \)).

Two distinct patterns of movement mediated by Tn1721 and IS26 existed in this chimera. Tn1721 pattern was the same as the one detected in A1- and B-type Tn1721 previously, including cointegrate
forming and resolving steps [13]. In addition to having the Tn1721 pattern, a different IS26 pattern via replicative transposition was also detected in several cases of A2-type Tn1721 (Figure 4B). Only one plasmid (P3) was obtained from the transconjugant. The donor (pHS10842) and target (R388) plasmids generated this cointegrate (P3) in which both plasmids fused together by directly repeated copies of IS26. This result was supported by a series of data (Figure 4A), as follows. (i) the size of P3 was larger than that of R388 and P2. (ii) Southern blot analysis showed that P3 contained blaKPC-2, Tn1721, sul1, IS26. (iii) A series of PCRs confirmed that P3 had the same blaKPC-2-bearing genetic structure (Tn1721-blaKPC-2-IS26) as that of pHS10842, and the junctions between donor and target in each case were amplified and sequenced with primers specific for regions internal to IS26 and primers specific for R388.

Consistent with previous reports [18], 8-bp target site duplication was evidenced for each transposition events (Figure 5). At the target sites, the AT content for regions from t2 to t7 stabilize at 60-70%, while the AT content at t1 and t8 was slightly lower (46%). Nucleotide composition analysis revealed the t2 and t3 positions were found to be predominantly T residues (54% and 38%, respectively), while t6 and t7 positions were mostly A residues with the same percentage as that for t2 and t3, respectively. It is noteworthy that the insertion sites mostly carried one or even more AA or/and TT nucleotide tandems. These data suggested that IS26 preferentially targets AT-rich regions with AA and TT nucleotide tandems.

The transposition frequency of A2-type Tn1721 was measured to be $3.8 \times 10^{-6}$ (Table 3). This represented a 4-fold increase in efficiency compared to that of A1-type, a blaKPC-2-bearing structure lacking IS26, and 30% of that for B-type Tn1721 containing an additional left inverted repeat (IRL2). The inPA deletion of either Tn1721 or IS26 decreased half of the frequency, and both inPA deletions led to a functional inability to mobilize blaKPC-2. Hence, blaKPC-2 embedded between Tn1721 and IS26 was transferred at an apparently higher frequency owing to the existence of both elements.

Taken together, our findings indicate that all three transposons were capable of transferring blaKPC-2 through replicative transposition, and that A2- and B-type Tn1721 showed higher transposition frequencies and had a certain superior ability to propagation.

**Replicative transposition of Tn1721 contributes to the evolution and dissemination of KPC-2-producing plasmid**

Given that Tn1721 variants have been shown various capacities of transferring blaKPC-2 via replicative
transposition, an attractive hypothesis is that the A2- and B-type Tn1721 occasionally appearing in the late-stage stood out from the rest through selection for their ability to facilitate dissemination of blaKPC-2. As discussed above, the outbreak of KPC-2-producing plasmid was correlated with the dynamics of blaKPC-2-bearing Tn1721 structure, and A2- and B-type Tn1721 arising concurrently with the peak of blaKPC-2 epidemic showed apparently higher transposition frequencies. Such an association and different movement capabilities imply that replicative transposition of Tn1721 variants contributes to the evolution and dissemination of KPC-2-producing plasmid in Enterobacterales. This mechanism could enable blaKPC-2 gene to search for suitable host spontaneously, dealing with the antibiotic pressure from the environment.

Discussions

KPC-producing Enterobacterales that have spread extensively throughout the world, are an important cause of nosocomial infections, especially urinary tract infections, respiratory tract infections, and bloodstream-associated infections [1]. During the last decade, several studies have described of diverse transposable elements surrounding blaKPC-2 gene [9,10,12]. However, little is known about the exact impacts of these genetic environment change and its...
Transposable elements play an important role in the genetic variation and evolution of bacteria. The \textit{bla}_{KPC-2} gene is mostly located on transposable elements, such as \textit{Tn}1721, and \textit{Is26} [8,13]. To date, eight variants of \textit{Tn}4401 (\textit{Tn}4401a to \textit{Tn}4401h) have been identified, with \textit{Tn}4401a and \textit{Tn}4401b being the most widespread [1,12,19,20]. Notably, these isoforms demonstrate lacking \textit{tnpA} or/and \textit{tnpR}, or have deletions in the noncoding region upstream of \textit{bla}_{KPC} leading to enhanced or reduced expression of this carbapenemase [21]. It is widely reported that \textit{Tn}1721 variants are the dominant structures in Asia, particularly in China [8–10,15,22]. Given that the structure of \textit{Tn}1721 variants in current \textit{bla}_{KPC-2} epidemic is too complicated and diverse to track the dissemination process of this gene, we focus on the early stage of \textit{bla}_{KPC-2} epidemic. In addition to the dynamics of \textit{bla}_{KPC-2}-bearing transposon structure raised by this study, host and plasmid factors as well as antibiotic pressure from environment were also worth further study.

Our findings indicated that all three \textit{Tn}1721 variants were capable of mobilizing \textit{bla}_{KPC-2} via replicative transposition, and \textit{A2}- and \textit{B-type} \textit{Tn}1721 exhibited higher transposition frequencies than \textit{A1-type}. Such various capacities are presumably associated with cointegrate resolution of \textit{Tn}1721 in transposition assay. For the transconjugants with the \textit{Tn}1721 pattern, only the cointegrate could be conjugated into the recipient (\textit{E. coli} HB101, STR resistant [STR\textsuperscript{R}]) and screened on LB agar containing IPM, TMP, and STR in the transposition assay. Once the cointegrate resolved in donor strain, neither plasmid generated here could survive. Because P1 lacked the TMP resistance (TMP\textsuperscript{R}) and the ability to conjugate into recipient (STR\textsuperscript{R}), even though it possessed IPM resistance (IPM\textsuperscript{R}), while P2 was an opposite case. In contrast, both the cointegrate and plasmid resolved from the cointegrate with \textit{Tn}1721-\textit{bla}_{KPC-2}-IRL2 pattern possessed IPM\textsuperscript{R} and TMP\textsuperscript{R}, as well as had the ability to conjugate to recipient (STR\textsuperscript{R}). For the transposition via \textit{Is26}, there is no resolution process. It means that the cointegrate (P3) with \textit{Is26} pattern possessed IPM\textsuperscript{R} and TMP\textsuperscript{R}, as well as had the ability of conjugal transfer. Consequently, it survived on LB agar containing IPM, TMP, and STR. In other words, the plasmid with \textit{Tn}1721-\textit{bla}_{KPC-2}-IRL2 pattern (in \textit{B-type}) or \textit{Is26} pattern (in \textit{A2-type}) had a certain superior ability of conjugal transfer and became more widespread. These results strongly supported our molecular epidemiological findings that the two peaks of \textit{bla}_{KPC-2} epidemic followed the appearance of \textit{A2-} and \textit{B-type} \textit{Tn}1721, reflecting an important role for replicative transposition of \textit{Tn}1721 and \textit{Is26} in the evolution and transmission of \textit{bla}_{KPC-2}-carrying plasmid in \textit{Enterobacterales}.

In conclusion, our work demonstrates replicative transposition facilitates the evolution and transmission of \textit{KPC-2}-producing plasmid in \textit{Enterobacterales}, and highlights its important role in the dissemination of antibiotic resistance genes between pathogenic bacterial species.

**Disclosure statement**

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**Table 3. Transposition frequency of \textit{Tn}1721-like transposons and derivatives\textsuperscript{a}***

| Donor plasmid | No. of independent determinations | Mean \(10^{-8}\) | Range\(\text{IPM}^{-\text{R}}\) |
|---------------|----------------------------------|----------------|----------------|
| pHS10842-\textit{Tn}1721B | 5 | 1.13 | 8.15–16 |
| pHS10842-\textit{Tn}1721A1 | 4 | 0.95 | 0.4–1.57 |
| pHS10842 (A2) | 4 | 3.8 | 3.4–4.2 |
| pHS10842-\textit{\textDelta}tnpA\textsubscript{tnpA1721} | 4 | 1.7 | 1.1–1.9 |
| pHS10842-\textit{\textDelta}tnp26 | 4 | 1.3 | 1.0–1.9 |
| pHS10842-\textit{\textDelta}tnpA\textsubscript{tnpA1721}\textit{\textDelta}tnp26 | 4 | <9 | 8.3–1.0 |

\textsuperscript{a}In all cases, the target was R388.

\textsuperscript{b}Transposition frequency is expressed as the number of IPM\textsuperscript{R} TMP\textsuperscript{R} STR\textsuperscript{R} transconjugants per TMP\textsuperscript{R} STR\textsuperscript{R} transconjugant.
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