

Research paper

The transferability and evolution of NDM-1 and KPC-2 co-producing *Klebsiella pneumoniae* from clinical settings

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ABSTRACT

Background: Emergence of KPC-2 and NDM-1-coproducing carbapenem-resistant *Klebsiella pneumoniae* (KPC-2-NDM-1-CRKP) has escalated threat of CRKP to healthcare. Currently, only 4 isolates had been reported sporadically. It remains unclear how KPC-2-NDM-1-CRKPs emerged, and whether they are stable and have transmission capacity.

Methods: PCR and Sanger sequencing was used to screen carbapenemase genes for 2057 CRKP isolates, to identify KPC-2-NDM-1-CRKPs. The clinical information of the patients was collected from medical records. Antimicrobial susceptibility, plasmid stability, plasmid conjugation and growth curve were measured. The whole genomes were sequenced on PacBio and Illumina platforms. Similar plasmids were searched against public database. Phylogenetic tree was built based on related genomes which carried similar plasmids, to infer the evolutionary history of KPC-2-NDM-1-CRKP.

Findings: We identified to date the largest cohort of 7 KPC-2-NDM-1-CRKPs. An increased incidence rate was observed. Plasmid transferability assay and phylogenetic analysis suggested an evolutionary pathway that KPC-2-NDM-1-CRKP emerged from a KPC-2-CRKP progenitor which acquired another highly transferable *bla*_{NDM-1} plasmid later. Further, demographics and stability test revealed that these isolates were stable and had the potential for transmission among patients.

Interpretation: Our study extends our concern on KPC-2-NDM-1-CRKP about its high stability and non-inferior fitness, sheds light on the evolution of KPC-2-NDM-1-CRKP, strengthens the importance of continued surveillance on these isolates, and would improve clinical treatment and management.

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1. Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) represents an enormous global public health threat due to the presence of mobile carbapenemase that confers resistance to carbapenem and multiple other antibiotic classes, greatly challenging clinical management [1]. In fact, CRKP have caused serious infections in debilitated and immunocompromised patients worldwide, leading to prolonged hospital stay and increased mortality rates [2]. Our recent nationwide survey demonstrated that the predominant carbapenemases were KPC-2 (76.5%), a class A serine β -lactamase, and NDM-1 (9.3%), a class B metallo β -lactamase, during 2012–2016 [3]. Both KPC-2 and NDM-1 can hydrolyte a wide spectrum of β -lactams, including penicillins,

cephalosporins and carbapenems. KPC-2 can also hydrolyte monobactams, but can be inhibited by a number of inhibitors, such as avibactam. On the contrary, NDM-1 could not hydrolyte monobactams, but is unresponsive to avibactam [4,5]. Thus, the synergistic effect of different carbapenemase classes such as KPC-2 and NDM-1 may confer even higher level of resistance to carbapenems as well as other antimicrobials.

Although KPC-producing CRKP and NDM-producing CRKP have been widely identified, CRKP with co-existence of KPC-2 and NDM-1 have been rarely reported. To our knowledge, only 4 KPC-2 and NDM-1 co-producing CRKP isolates (KPC-2-NDM-1-CRKPs) have been identified in different countries (India, Pakistan and China) [6–9]. However, each study only presented single KPC-2-NDM-1-CRKP or lacked any clinical or microbiological characteristics, which may be not representative enough to reflect the full picture of those double-positive CRKPs. For example, it remains unclear about the

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Research in Context

Evidence before this study

We searched PubMed with the terms “KPC-2 AND NDM-1 AND *Klebsiella pneumoniae*” and followed the references and citations for reports published up to May 29, 2019, with no language restrictions. We also blasted *bla*_{KPC-2} and *bla*_{NDM-1} against NCBI NT and RefSeq Assembly databases for complete genome sequences released up to May 29, 2019. After filtration of literatures without reporting any KPC-2 and NDM-1 coproducing carbapenem-resistant *Klebsiella pneumoniae* (KPC-2-NDM-1-CRKP), there were only 3 relevant papers describing the emerging of 4 KPC-2-NDM-1-CRKPs in India, Pakistan and China, and an undocumented genome sequence for an extra KPC-2-NDM-1-CRKP. All of them were case reports with routine description on the antimicrobial resistance profile and plasmid transferability in vitro, and only one had done genome sequencing. However, no one had provided long term surveillance information, or evidence for stability to maintain KPC-2 and NDM-1, or evidence for cross infection, i.e. transferability in vivo. Furthermore, how KPC-2-NDM-1-CRKP emerged and clinical treatment practice, were largely unknown.

Added value of this study

This study is based on the largest Carbapenem-resistant Enterobacteriaceae (CRE) Network in China, covering the population of 65 hospitals in 25 provinces and municipalities across China for 5 years. Our surveillance data allows us to monitor the incidence of KPC-2-NDM-1-CRKP. We observed an increased incidence rate. We had the complete genomes sequenced for all the 7 KPC-2-NDM-1-CRKPs, and based on phylogenetic analysis we proposed that KPC-2-NDM-1-CRKP emerged from a KPC-2-CRKP progenitor which acquired another highly transferable *bla*_{NDM-1} plasmid later. Through serial passage for 10 days, we found 5 KPC-2-NDM-1-CRKPs were quite stable to maintain the resistant plasmids. Importantly, we observed 2 clusters of isolates which were the evidences of clonal transmission among patients. We collected the clinical information of the patients who had been cured for the infection. Based on antimicrobial susceptibility tests we found the compound ceftazidime/avibactam which had just been introduced in China to fight against CRKP had little effect on KPC-2-NDM-1-CRKP, while the novel compound aztreonam/avibactam was of great value for future treatment.

Implications of all the available evidence

The present study revealed an increased incidence, high stability, non-inferior fitness and transferability among patients of KPC-2-NDM-1-CRKPs. These evidences extend our concern on KPC-2-NDM-1-CRKP, and highlight KPC-2-NDM-1-CRKP as an emerging and growing threat in the hospital and the community. Thus, the surveillance should be continued and clinical management should be put into practice to prevent cross infection or even outbreak. Additionally, this work suggested a mechanism how KPC-2-NDM-1-CRKP emerged: a KPC-2-CRKP progenitor acquired a highly transferable *bla*_{NDM-1} plasmid from broad hosts. Therefore, it's necessary to monitor the prevalence of *bla*_{NDM-1} plasmid and warn the high probability of *bla*_{NDM-1} incorporating with native Enterobacteriaceae to extend their resistant profile, not merely with KPC-2-CRKP.

the genetic background of those isolates remain largely unknown. These are critical questions required to be elucidated as the combination of “super-antimicrobial resistance” may greatly escalate the public-health threat of the “superbug”.

The Chinese Carbapenem-resistant Enterobacteriaceae (CRE) Network has been ongoing since its establishment in 2014 [3,10]. It is the largest CRE surveillance program in China that monitors CRE epidemiology and antimicrobial resistance profile. During the ongoing CRE surveillance, we identified 7 cases of infection caused by KPC-2-NDM-1-CRKPs. To the best of our knowledge, our study represents the largest number of KPC-2-NDM-1-CRKP isolates, thus will provide us a unique opportunity to address the questions mentioned above.

2. Materials and methods

2.1. CRE network and bacterial isolates

The CRE Network was described previously [3,10]. Specifically, a total of 2057 non-repetitive clinical CRKP isolates from 65 hospitals in 25 provinces and municipalities across China were collected from January 1, 2012 to December 31, 2017. CRKP isolates resistant to any carbapenem (imipenem, meropenem, or ertapenem), as determined by standard methods, were obtained from patients at participating hospitals. All isolates were reconfirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker Daltonik, Bremen, Germany) at Peking University People's Hospital and were stored at −80 °C for further usage.

2.2. Identification of KPC-2-NDM-1-CRKP and clinical data collection

All CRKP isolates collected from the CRE Network were subjected to polymerase chain reaction (PCR) and Sanger sequencing on the genes encoding carbapenemases, including *bla*_{KPC}, *bla*_{NDM}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{SIM} and *bla*_{OXA-48} [3]. Seven cases with KPC-2-NDM-1-CRKP infection were identified and the clinical information on these patients was collected by a standard and predefined case report form. The form included demographics, underlying medical conditions, clinical presentations, antimicrobial therapy and clinical evaluation and outcomes. The study was reviewed and approved by the research ethics committee of Peking University People's Hospital. A waiver of written informed consent was granted.

2.3. Microbiological characteristics, antimicrobial susceptibility testing, plasmid conjugation assay, and growth curve assay

Capsular genotyping, multilocus sequence typing and hypermucoviscosity were performed. Antimicrobial susceptibility was evaluated according to Clinical and Laboratory Standards Institute guidelines (M07-A9, 2017) and US Food and Drug Administration standard. Plasmid transferability was tested using *E. coli* EC600 (rifampicin resistance) as the recipient strain. Growth curve for each strain was also measured and compared using a two-way ANOVA with Tukey's honest significant difference (HSD) correction. Detailed procedures were documented in the supplementary file.

2.4. Plasmids stability testing

Plasmid stability was assessed as previously described with minor modifications [11]. Briefly, cultures grew at 37 °C in a shaking bath (200 rpm) and went on serial passage for 10 days (approximately 200 generations) with 1:1000 dilution in antibiotic-free LB broth. Samples were taken and stored in glycerol in −20 °C each day. After 10 days, cultures were serially diluted and plated on antibiotic-free LB agar, imipenem (3 mg/L) LB agar and imipenem (3 mg/L) + EDTA (0.05 mM) LB agar. The retention rate of KPC-2 and NDM-1 activities was calculated by dividing the number of colonies on imipenem-

stability of those isolates, fitness cost on bearing an extra carbapenemase and how those isolates emerged. In addition, the clinical characteristics and outcomes of KPC-2-NDM-1-CRKP infections as well as

containing LB agar by the number of colonies on antibiotic-free LB agar. And the retention rate of KPC-2 activity was calculated by dividing the number of colonies on imipenem and EDTA containing LB agar by the number of colonies on antibiotic-free LB agar. In addition, on day 10, 40 colonies were randomly selected and subjected to PCR validation of *bla*_{KPC-2} and *bla*_{NDM-1} genes.

2.5. Plasmid incompatibility assay

Plasmid incompatibility assay was performed as previously described with slight modifications [12]. Briefly, a transconjugant bearing both *bla*_{KPC-2} and *bla*_{NDM-1} plasmids obtained from plasmid conjugation assay was cultured in antibiotic-free LB broth at 37 °C in a shaking bath (200 rpm) overnight. Cultures were diluted and plated on antibiotic-free LB agar next day. Single colony was selected for *bla*_{KPC-2} and *bla*_{NDM-1} detection by PCR. Plasmids were considered incompatible when more than 80% colonies lost either or both of *bla*_{KPC-2} and *bla*_{NDM-1} plasmids.

2.6. Whole genome sequencing, assembly and annotation

All seven KPC-2-NDM-1-CRKPs were subjected to the whole genome sequencing on PacBio Sequel platform and Illumina NextSeq 550 platform. The hybrid assembling was performed using Unicycler v0.4.6 [13], and the assembly was annotated by Prokka v1.12 [14] and several databases. Detailed procedures were documented in the supplementary file. All draft genome sequences were deposited into NCBI Genome database, and organized under BioProject PRJNA534178.

2.7. Plasmid comparison

Plasmid sequences were compared using blastn, and illustrated by R ggplot2 package. Homology was defined as $\geq 80\%$ identity. Secondary alignments and alignments smaller than 1 Kbp were removed. Relevant plasmids were searched in NCBI nt and plasmid database until March 18, 2019. Redundant hits were removed.

2.8. Phylogenetic analysis

Multiple sequence alignments from relevant plasmids were generated by Mugsy v1.2.3 [15], and multiple sequence alignments from genomes were generated by Roary v3.12.0 [16]. All gaps were removed before detecting possible recombination events using ClonalFrameML v1.11 [17]. After recombination region removal, RAXML v8.2.12 [18] was used to build phylogenetic tree, under GTRGAMMA model and 'autoFC' bootstrapping criteria. Final tree was demonstrated using R ggtree [19] and ggplot2 package.

3. Results

3.1. Incidence of KPC-2-NDM-1-CRKP in China

In this study, a total of 2057 non-repetitive clinical CRKP isolates from 2012 to 2017 were analyzed for carbapenem resistant genes. None of KPC-2-NDM-1-CRKP was found during 2012–2015. KPC-2-NDM-1-CRKP emerged in 2016 with an annual incidence of 0.28% (2/705). In 2017, 5 more KPC-2-NDM-1-CRKPs were identified. The annual incidence of KPC-2-NDM-1-CRKP was increased from 0.28% in 2016 to 0.58% (5/856) in 2017 in China.

3.2. Clinical characteristics of patients with KPC-2-NDM-1-CRKP

Clinical characteristics of the 7 patients with KPC-2-NDM-1-CRKPs were collected (Table 1). Notably, patients P2315 and P2343 were hospitalized at the same department two months apart.

Patients P2972 and P2974 were hospitalized at different departments from the same hospital, while patient P2601 was hospitalized at another hospital of the same city three months apart.

Patient P2660 was an outpatient with no detailed information obtained. However, this case underlined the threat of KPC-2-NDM-1-CRKP not only for in-patient, but also for community. Among the rest patients, 5 out of 6 had cardiovascular diseases and 3 out of 6 had carbapenem exposure history within 30 days before therapeutic antimicrobial usage.

3.3. Phenotypes and microbiological characteristics of KPC-2-NDM-1-CRKP

Molecular typing showed that the 7 KPC-2-NDM-1-CRKPs belonged to 3 MLSTs and 4 capsule antigen serotypes (Table 2). These isolates from the same geography displayed the same types, except that C2601 (isolate from patient P2601; the same nomenclature for the others) evolved to different capsule antigen serotype from C2972 and C2974.

All isolates displayed resistance to penicillins, cephalosporins and carbapenems, but were susceptible to colistin and tigecycline. Although all isolates displayed high resistance to aztreonam (MIC > 64 mg/L), the addition of avibactam to aztreonam greatly reduced the MICs (ranging from 0.125 to 0.5 mg/L except C2414, which exhibited a MIC of 2 mg/L). The two patients (P2315 and P2343) recovered from amikacin treatment, and one patient (P2601) recovered from tigecycline treatment, indeed infected with the isolates susceptible to these drugs.

To evaluate the transferability of *bla*_{KPC-2} and *bla*_{NDM-1}, conjugation assays were performed by co-culturing the KPC-2-NDM-1-CRKPs with *E. coli* EC600. C2315 and C2343 were excluded due to their resistance to rifampicin. All remaining isolates were able to transfer their *bla*_{NDM-1} plasmids to the recipient strain, while only C2601, C2972 and C2974 were able to transfer their *bla*_{KPC-2} plasmids (Table 3). Notably, only C2974 was able to simultaneously transfer both *bla*_{KPC-2} and *bla*_{NDM-1} plasmids to EC600. The KPC-bearing transconjugants were sensitive to carbapenems, for which MICs of meropenem were below 1 mg/L. While the MICs range of meropenem for NDM-bearing transconjugants were between 2 and 8 mg/L. That also reached 8 mg/L with both *bla*_{NDM-1} and *bla*_{KPC-2} genes. But interaction between species and plasmids might influence the resistance phenotype.

Plasmid incompatibility was further assessed in the transconjugant C2974T-3 which bears both *bla*_{KPC-2} and *bla*_{NDM-1} plasmids. A total of 94 single colonies were selected for *bla*_{KPC-2} and *bla*_{NDM-1} detection. Of interest, both *bla*_{KPC-2} and *bla*_{NDM-1} plasmids were present in 58 colonies (61.7%, 58/94), suggesting that those two plasmids were compatible.

As highlighted above, C2315 was isolated two months later after C2343, and C2972 11 days later and C2601 three months later after C2974. These KPC-2-NDM-1-CRKPs seemed quite stable in the real world. Thus, the stability of all KPC-2-NDM-1-CRKPs was re-evaluated during serial passage in laboratory last for ten days (Fig. 1(b)). C2414, C2660, C2972 and C2974 displayed high stability, as the retention rates were still over 80% at the end of the experiment, for both KPC-2 and NDM-1 which were confirmed by PCR. C2601 gradually lost its KPC-2 activity, and less than half of the cells retained KPC-2 at the end. On the contrary, C2315 and C2343 lost KPC-2 activity in the first day, and both KPC-2 and NDM-1 within 2 days. This discrepancy may be resulted from the antibiotic pressure presented in real hospital scenario.

Further, the effect of acquiring *bla*_{KPC-2} and *bla*_{NDM-1} plasmids on biological fitness cost was evaluated. Of interest, no significant differences in the growth rate were observed among the recipient strain EC600 and the transconjugants harboring either *bla*_{KPC-2} plasmid or/and *bla*_{NDM-1} plasmids (Fig. 1(a) and Supplementary Fig. 1(a)). To further gain insight into the intrinsic influence of *bla*_{KPC-2} and *bla*_{NDM-1} plasmids on biological

Table 1
Clinical characteristics of patients with KPC-2-NDM-1-CRKP.

Variables/patients	P2315	P2343	P2414	P2601	P2660	P2972	P2974
Clinical characteristics							
Age	51 years	74 years	81 years	5 months	28 years	3 months	3 months
Gender	Male	Male	Male	Female	Male	Female	Male
City	Qinhuangdao	Qinhuangdao	Wuhan	Jinan	Beijing	Jinan	Jinan
Ward	Surgery	Surgery	Neurology	Pediatric ICU	TCM	Pediatric ICU	Surgery
Underlying conditions	Emphysema, ischemic bowel disease, mesenteric artery stenosis, hypertension	Rectum perforation	Pneumonia history, coronary heart disease, chronic renal disease, hypertension	Pneumonia history, liver function damage, congenital heart disease, endophthalmitis	Unknown	Congenital heart disease	Ventricular septal defect
Invasive procedures							
Mechanical ventilation	No	No	No	Yes	No	Yes	Yes
Drainage catheters	Yes	Yes	Yes	No	No	Yes	Yes
Surgery	No	Yes	No	No	No	Yes	Yes
Date of specimen collection	10/09/2017	08/07/2017	10/18/2017	01/15/2017	08/01/2017	10/29/2016	10/18/2016
Infection type	Intra-abdominal infection	Intra-abdominal infection	Urinary tract infection	Severe pneumonia	Urinary tract infection	Bronchopneumonia	Pulmonary infection
Specimen type	Intra-abdominal fluid	Intra-abdominal fluid	Urine	Sputum	Pus	Sputum	Sputum
Prior antibiotic usage within 30 days	Yes	Yes	No	Yes	Unknown	Yes	No
Hospitalization within 90 days	Yes	No	No	No	Unknown	Yes	Yes
Empirical antimicrobial usage	MEM+ONZ	TCC+ONZ	CAZ	CFM+RXM, CMZ+ERY+MEM	Unknown	MEM+CSL, IPM/CS	CTM
Clinical presentations							
Temperature (T _{max}) (°C)	38.6	37.4	38.5	39.4	Unknown	39.8	37.8
WBC ($\times 10^9/L$)	15.52	7.93	19.68	7.3	Unknown	11.13	7.14
Therapeutic antimicrobial usage	AMK	AMK+CIP	CAZ	TGC	Unknown	CSL+LZD	CAZ, IPM/CS
Clinical outcomes							
Length of stay (days)	54	81	22	30	N/A	46	26
Days of mechanical ventilation	N/A	N/A	N/A	3	N/A	7	7
Duration of ICU stay (days)	N/A	N/A	5	21	N/A	11	11
Outcome	Recovered	Recovered	Recovered	Recovered	Unknown	Recovered	Recovered

No further information was obtained from P2660 because he came to the out-patient clinics. ICU: intensive care unit; TCM: traditional Chinese medicine; AMK: amikacin; CAZ: ceftazidime; CFM: cefixime; CIP: ciprofloxacin; CMZ: cefmetazole; CSL: cefoperazone/sulbactam; CTM: cefotiam; ERY: erythromycin; IPM/CS: imipenem/cilastatin; LZD: linezolid; MEM: meropenem; ONZ: ornidazole; RXM: roxithromycin; TCC: ticarcillin/clavulanate; TGC: tigecycline. N/A: not applicable.

Table 2
Microbiological characteristics of KPC-2-NDM-1-CRKP.

Variables/isolates	C2315	C2343	C2414	C2601	C2660	C2972	C2974
Typing							
Capsule antigen serotype	K140	K140	K47	K19	K64	K47	K47
MLST	ST3493	ST3493	ST11	ST15	ST11	ST15	ST15
MIC (mg/L)							
Piperacillin/tazobactam	>256	>256	>256	>256	>256	>256	>256
Cefoperazone/sulbactam	>256	>256	>256	>256	>256	>256	>256
Ceftazidime/avibactam	>128	>128	>128	>128	>128	>128	>128
Cefepime	64	32	>256	256	>256	>256	>256
Imipenem	8	4	>32	>32	>32	>32	>32
Meropenem	16	8	>32	>32	>32	>32	>32
Amikacin	1	2	>256	4	>256	4	4
Aztreonam	>64	>64	>64	>64	>64	>64	>64
Aztreonam/avibactam	0.25	0.5	2	0.25	0.5	0.125	0.5
Colistin	0.125	0.125	0.5	0.125	0.125	0.125	0.125
Fosfomycin	32	32	>256	256	128	>256	>256
Levofloxacin	0.064	32	>64	64	>64	>64	>64
Tigecycline	0.25	0.5	1	1	0.5	1	1

Bold flags susceptible, and single underscore intermediate.

Table 3
Transferability of *bla*_{KPC-2} and *bla*_{NDM-1} plasmids.

Strains and transconjugants	Carbapenemase	AMK	ATM	FEP	CAZ	CSL	CRO	COL	FOX	IPM	LVX	MEM	TGC	TZP
C2414	KPC-2+NDM-1	>256	>64	>256	>64	>256	>256	0.5	>256	>32	>64	>32	1	>256
C2414T-1	NDM-1	2	0.125	32	>256	256	256	0.5	>128	4	4	2	0.125	128
C2601	KPC-2+NDM-1	4	>64	256	>64	>256	>256	0.125	>256	>32	64	>32	1	>256
C2601T-1	NDM-1	2	0.064	32	>256	256	256	0.5	>128	8	0.25	4	0.125	256
C2601T-2	KPC-2	1	128	8	2	32	128	0.5	128	1	0.25	0.25	0.125	128
C2660	KPC-2+NDM-1	>256	>64	>256	>64	>256	>256	0.125	>256	>32	>64	>32	0.5	>256
C2660T-1	NDM-1	2	128	32	>256	256	256	0.5	>128	4	0.25	4	0.125	128
C2972	KPC-2+NDM-1	4	>64	>256	>64	>256	>256	0.125	>256	>32	>64	>32	1	>256
C2972T-1	NDM-1	2	0.064	32	>256	256	256	0.5	>128	16	0.25	8	0.125	256
C2972T-2	KPC-2	2	128	16	64	32	128	0.5	16	1	0.25	0.25	0.125	128
C2974	KPC-2+NDM-1	4	>64	>256	>64	>256	>256	0.125	>256	>32	>64	>32	1	>256
C2974T-1	NDM-1	2	0.064	32	>256	256	256	0.5	>128	16	0.25	8	0.125	256
C2974T-2	KPC-2	2	32	4	16	16	64	0.5	16	1	0.25	0.125	0.125	64
C2974T-3	KPC-2+NDM-1	2	64	32	>256	256	256	0.5	>128	16	0.25	8	0.125	256

AMK: amikacin; ATM: aztreonam; FEP: cefepime; CAZ: ceftazidime; CSL: cefoperazone/Sulbactam; CRO: ceftriaxone; COL: colistin; FOX: cefoxitin; IPM: imipenem; LVX: levofloxacin; MEM: meropenem; TGC: tigecycline; TZP: piperacillin/tazobactam. Bold flags susceptible and single underscore intermediate.

fitness cost in KPC-2-NDM-1-CRKP, we took advantage of the strains obtained from plasmid stability assay, where we obtained strains that lost either *bla*_{KPC-2} or *bla*_{NDM-1} plasmids. Specifically, we obtained C2343ΔpC2343-NDM and C2414ΔpC2414-NDM, which lost *bla*_{NDM-1} plasmids from their parental strains, and we obtained C2601ΔpC2601-KPC, which lost *bla*_{KPC-2} plasmid from its parental strain. Consistent with the findings from transconjugants, no significant differences were observed in the growth rates between KPC-2-NDM-1-CRKP and KPC-2-CRKP, or between KPC-2-NDM-1-CRKP and NDM-1-CRKP in the presence/absence of nutrients (Supplemental Fig. 1(b)). Taken together, these microbiological characteristics indicated that KPC-2-NDM-1-CRKP could carry both *bla*_{KPC-2} and *bla*_{NDM-1} without sacrifice of fitness, and bear them for a long time. This extended our concern about KPC-2-NDM-1-CRKP, because it's not only intractable, but also free of punishment for formation and hard to regress. To further understand genetic background for these characteristics, and uncover the formation pathway of these isolates, we had their genome sequenced and assembled.

3.4. Genomic architecture

Each strain had one *bla*_{KPC-2} plasmid and one *bla*_{NDM-1} plasmid (Supplementary Table 1), except that C2315 had an extra copy of *bla*_{KPC-2} on its genome (Fig. 2). Consistent with previous reported cases, *bla*_{KPC-2} preferred to IncFII plasmid, while *bla*_{NDM-1} located on diverse background, such as IncN, IncX and IncHI1B plasmids. Most *bla*_{NDM-1} plasmids had multiple copies per cell, except pC2660-NDM. On the contrary, most *bla*_{KPC-2} plasmids were single copy, except pC2414-KPC and pC2660-KPC. Except for *bla*_{KPC-2} and *bla*_{NDM-1}, each strain also carried other antimicrobial resistance (AMR) genes (Fig. 2 and Supplementary Table 1). Especially, C2315 and C2343 carried the broadest spectrum of AMR genes, which mainly co-located with *bla*_{NDM-1} on the IncHI1B plasmids, and the IncFIA(HI1)+IncFII plasmids only contained orphan *bla*_{KPC-2}. C2414 and C2660 also carried large number of AMR genes, but main part of these AMR genes co-located with *bla*_{KPC-2} on the IncR+IncFII(pHN7A8) plasmids. pC2414-NDM, an IncN plasmid, also was a multi-class resistance plasmid.

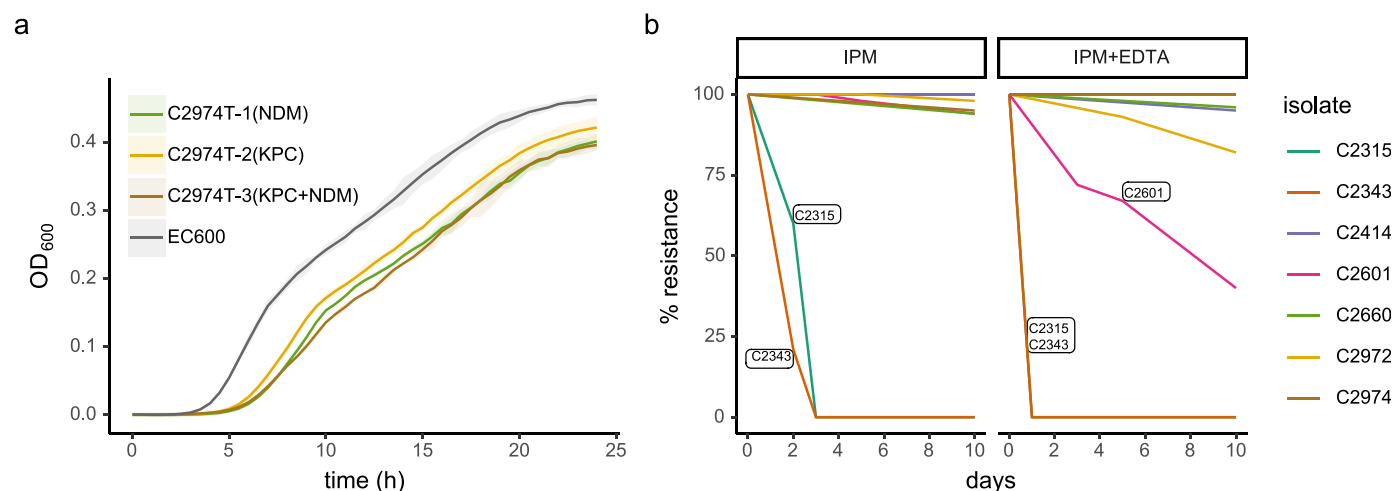


Fig. 1. Growth curves of transconjugants and stability of *bla*_{KPC-2} or *bla*_{NDM-1} plasmids. (a) Growth curves of three kinds of C2974 transconjugants and the recipient strain EC600. The shadow area below the line represents the 95% confident interval (3 replicates). (b) Stability of *bla*_{KPC-2} and *bla*_{NDM-1} plasmids along 10-day serial passage. IPM panel reflects the stability of *bla*_{KPC-2} and *bla*_{NDM-1} plasmids, while IPM+EDTA panel reflects the stability of *bla*_{KPC-2} plasmid.

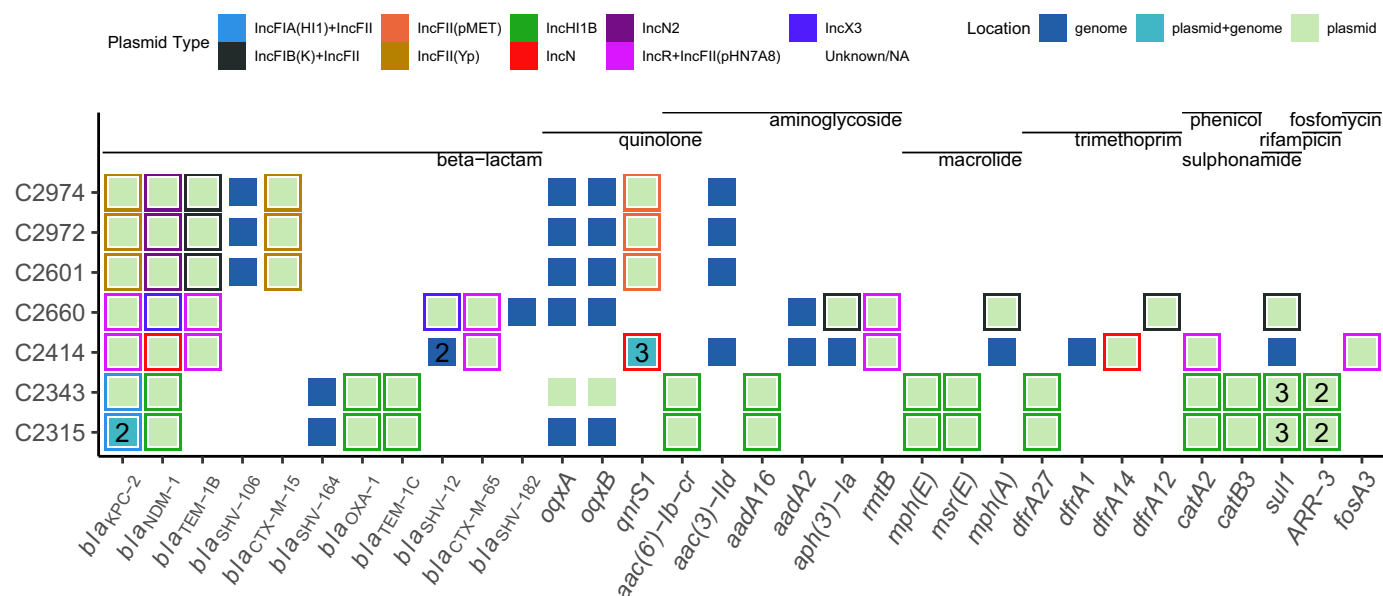


Fig. 2. Antibiotic resistance gene matrix. Border colors represent plasmid replication origin type, and fill colors for gene localizations. The integer in the cell indicates the number of genes in the genome/plasmids.

Though pC2660-NDM was an IncX3 β -lactam resistance plasmid, C2660 carried another IncFIB(K)+IncFII multi-class resistance plasmid. Interestingly, C2601, C2972 and C2974 all carried one IncFII(Yp) β -lactam resistance plasmid harboring *bla*_{CTX-M-15} and *bla*_{KPC-2}, and three orphan AMR resistance plasmids, including IncN2 *bla*_{NDM-1} plasmid, IncFIB(K)+IncFII *bla*_{TEM-1B} plasmid and IncFII(pMET) *qnrS1* plasmid.

Based on the genomic architecture and serotypes, 7 isolates were grouped into 4 clusters. Different isolates in the same cluster had the same or nearly the same plasmids, and related genomes. As expected, isolates in a same cluster were collected from closer temporal and spatial distances: C2315 and C2343 were isolated from the same hospital within 2 months; C2601, C2972 and C2974 were isolated from the same city within 3 months.

A total of 4 different *bla*_{KPC-2} plasmids were identified in the 7 isolates. Coverage of homologous sequence inter-cluster among these

plasmids was only 6%–44%, with an exception that pC2414-KPC shared 92.39% homologous sequence with pC2660-KPC, and 99.51% vice versa, although there were several structural variants between pC2414-KPC and pC2660-KPC. Searching in NCBI nt and RefSeq assembly database, only pC2414-KPC and pC2660-KPC had similar plasmids reported before, and those remaining were relatively new plasmids (Supplementary Fig. 2). In details, there were 17 resembled plasmids (coverage of homologous sequence bi-directional $\geq 90\%$) for pC2414-KPC, and 15 resembled plasmids for pC2660-KPC. All those resembled plasmids were carried by *K. pneumoniae*. On the contrary, pC2315-KPC/pC2343-KPC shared only 48.29% homologous sequences with p2-OXA (KY913898.1), a *bla*_{KPC-2} absent plasmid from a *K. oxytoca* strain, and 64.31% vice versa. pC2601-KPC/pC2972-KPC/pC2974-KPC shared only 59.29% homologous sequences with pKPC2_020019 (CP028554.1) from a *K. variicola* strain, and 64.27% vice versa.

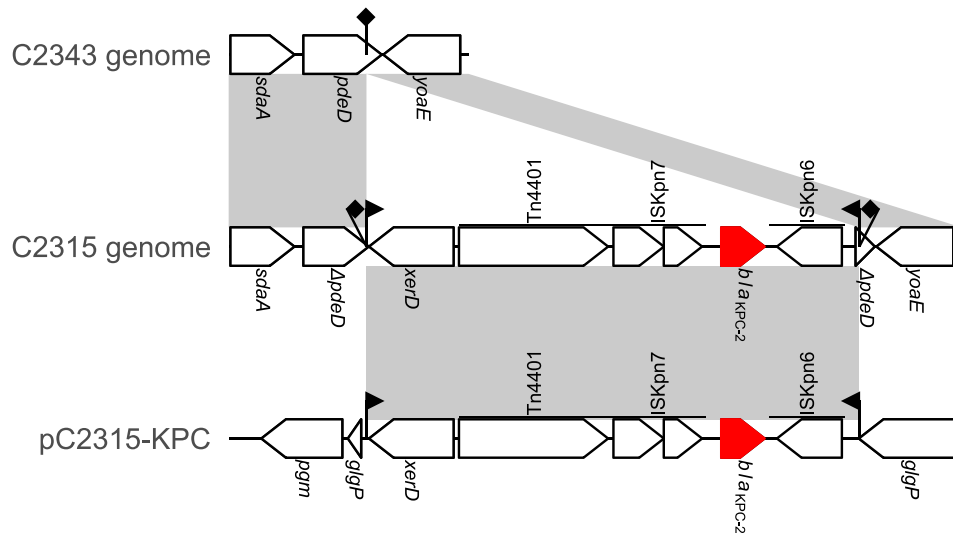


Fig. 3. Sequence comparison among the regions surrounding *bla*_{KPC-2} from pC2315-KPC, C2315 genome and C2343 genome. Open reading frames are portrayed by arrows/triangles, with red for resistance genes *bla*_{KPC-2}. Horizontal line marks transposon or insertion sequence. Gray shading represents homology region. Triangles flag inverted repeats, and diamonds flag direct repeats.

A total of 4 different *bla*_{NDM-1} plasmids were identified in these 7 isolates. Coverage of homologous sequence inter-cluster among these plasmids was only 1%–34%. Searching in NCBI nt and RefSeq assembly database (Supplementary Fig. 3), pC2315-NDM/pC2343-NDM shared 78.14% homologous sequences with pMH16-390M_1 (AP018583.1) from a *K. pneumoniae* strain, and 71.37% vice versa. pC2414-NDM shared 97% homologous sequences with pNDM-BTR (NC_022375.1) from an *E. coli* strain and pNDM1_LL34 (CP025965.2) from a *K. pneumoniae* strain, and 94% vice versa. There were 9 resembled plasmids for pC2601-NDM/pC2972-NDM/pC2974-NDM, and 14 resembled plasmids for pC2660-NDM. Interestingly, most those resembled plasmids were carried by species other than *K. pneumoniae*.

Of note, C2315 had additional copy of *bla*_{KPC-2} on the genome. Detailed sequence comparison revealed that there was a 10 Kbp identical region around each *bla*_{KPC-2}. The identical region had a 4 bp polymer invert repeats at each terminal, and was flanked by a 6 bp direct repeats on the genome (Fig. 3). There were an ISKpn6 and an ISKpn7 located hundreds of base pairs away from the nearest breakpoint, thus the identical region looked like a non-composite transposon. Interestingly, C2343 belonged to the same cluster as C2315 but had no *bla*_{KPC-2} on the genome. Thus, it is likely that the *bla*_{KPC-2}-harboring transposon was copied from the plasmid pC2315-KPC into C2315 genome. Although stability assay indicated the *bla*_{KPC-2} on C2315 genome showed low activity, this transposition event still raised the concern about the fixation of carbapenem resistance by adopting *bla*_{KPC-2} on to the genome. This kind of intrinsic CRKP would be more easily to form KPC-2-NDM-1-CRKP, as those isolates only need to acquire a *bla*_{NDM-1} plasmid.

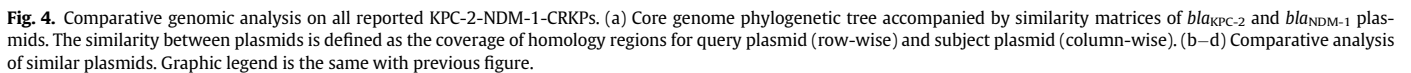
3.5. Evolutionary pathway in forming KPC-2-NDM-1-CRKP

How KPC-2-NDM-1-CRKPs emerged remains largely unknown. Since we had all 7 KPC-2-NDM-1-CRKPs been sequenced, combining with another two sequenced and released KPC-2-NDM-1-CRKPs, we first attempted to investigate the evolutionary relationship of the genomes and evolution concordance between plasmids and genomes. We found their genomes were highly divergent (Fig. 4(a)), indicating that those KPC-2-NDM-1-CRKPs may have multiple origins. We also noticed the similarity of *bla*_{KPC-2} plasmids was concordant to the genome evolution. Specifically, the *bla*_{KPC-2} plasmid from

CP034327 showed >80% coverage of homology sequences to pC2414-KPC and pC2660-KPC. The main differences were insertion sequences mediated inversions and transpositions. Of note, the *bla*_{KPC-2} plasmid from CP026586 was found as part of pC2414-KPC, which had not been integrated with the IncR backbone (Fig. 4(b)). It remains unclear whether CP026586 and C2414/C2660/CP034327 originated from a common KPC-2-CRKP ancestor, though it's also likely that they were independently acquired related *bla*_{KPC-2} plasmids. Another interesting observation was the discordance of similarity of *bla*_{NDM-1} plasmids to the genome evolution. The *bla*_{NDM-1} plasmid from CP026586 showed >85% coverage of homology sequences to pC2414-NDM (Fig. 4(c)). Though the *bla*_{NDM-1} plasmid from CP034327 was almost identical to pC2660-NDM (Fig. 4(d)), they were quite divergent to pC2414-NDM, even with different replication origins. This discordance indicated it's a most recent event to adopt a different type of *bla*_{NDM-1} plasmid for at least one strain (C2414 or C2660/CP034327).

The conjugation assays reflected the higher transferability of *bla*_{NDM-1} plasmid than *bla*_{KPC-2} plasmid, and reminded us a possible evolutionary pathway that it's KPC-2-CRKP that acquired an extra highly transferable *bla*_{NDM-1} plasmid to form KPC-2-NDM-1-CRKP. And, the species preference of the public resembled plasmids of *bla*_{KPC-2} or *bla*_{NDM-1} plasmids also supported this speculation. For example, the resembled plasmids of pC2660-KPC were restricted to *K. pneumoniae*, while the resembled plasmids of pC2660-NDM displayed diversity on host species (Fig. 5).

To further verify this hypothesis, all public data on *K. pneumoniae* strains carrying similar *bla*_{KPC-2} or *bla*_{NDM-1} plasmids were pulled together to assess the co-evolution of the genomes and plasmids (Fig. 6(a)). If our hypothesis was true, we could observe the precursor of KPC-2-NDM-1-CRKP, a KPC-2-CRKP which carried resembled *bla*_{KPC-2} plasmid, and at the same time, had related genome. As expected, the *K. pneumoniae* strains carrying resembled plasmids to pC2660-KPC have close genomes to C2660, while the genomes of the strains carrying resembled plasmids to pC2660-NDM displayed far distance from C2660 (Fig. 6(b)). Thus, these findings highly suggested that C2660 originated from a pC2660-KPC-bearing *K. pneumoniae* that acquired pC2660-NDM later. Similar evolutionary pathway was also identified in C2414, as well as a newly released KPC-2-NDM-1-CRKP strain KSH203 (CP034327).



In this study, we identified an increased incidence, high stability and non-inferior fitness of KPC-2-NDM-1-CRKPs. In addition, for the first time we revealed the evolutionary pathway of KPC-2-NDM-1-CRKP formation. Importantly, we found that ceftazidime/avibactam, a compound just being introduced to China, displayed little effect on KPC-2-NDM-1-CRKP. Thus, our data raise an alarm on the increased prevalence of KPC-2-NDM-1-CRKP, especially after the widespread use of ceftazidime/avibactam, which may provide a selective advantage for KPC-2-NDM-1-CRKP. Further, we suspected at least two transmission events of KPC-2-NDM-1-CRKPs within the same hospital and even across different hospitals. Thus, our findings highlight the importance of infection control and prevention the potential outbreak of KPC-2-NDM-1-CRKPs.

also supports our proposed model that KPC-2-NDM-1-CRKPs were derived from KPC-2-CRKPs progenitors that acquired *bla*_{NDM-1} plasmids. In this study, we found that *bla*_{KPC-2} preferred to IncFII plasmid, while *bla*_{NDM-1} located on diverse plasmids. Thus, the restriction of a certain plasmid to a certain bacterial host may contribute to the distinct pattern of *bla*_{KPC-2} vs. *bla*_{NDM-1} to adopt to different STs. Currently, the mechanisms why certain plasmid favors specific host (i.e. IncFII plasmid to CRKP ST11) remain unclear. It has been suggested that certain successful high-risk CRKP clones are linked to specific narrow-host-range IncF plasmids [20]. It is likely that this fine-tuning association may confer epidemiological advantages over other clones that carry wide-host-range plasmids, as the coevolution of a pandemic clone with a specific plasmid may provide rapid and continual adaptation opportunities and additional ability to outcompete other CRKP clones [21].

Previously reported KPC-2-NDM-1-CRKPs were sporadic isolates. Thus, it remains unclear whether KPC-2-NDM-1-CRKPs are stable in the environment and whether they have the ability to transmit among patients. In our study, we found 2 clusters of isolates, C2315/2343 and C2601/C2972/C2974, which providing us a unique opportunity for understanding how KPC-2-NDM-1-CRKPs adapt to the environment and their inter-host transmission capacity. We observed an interpolation event of *bla*_{KPC-2} from plasmid to genome in C2315/

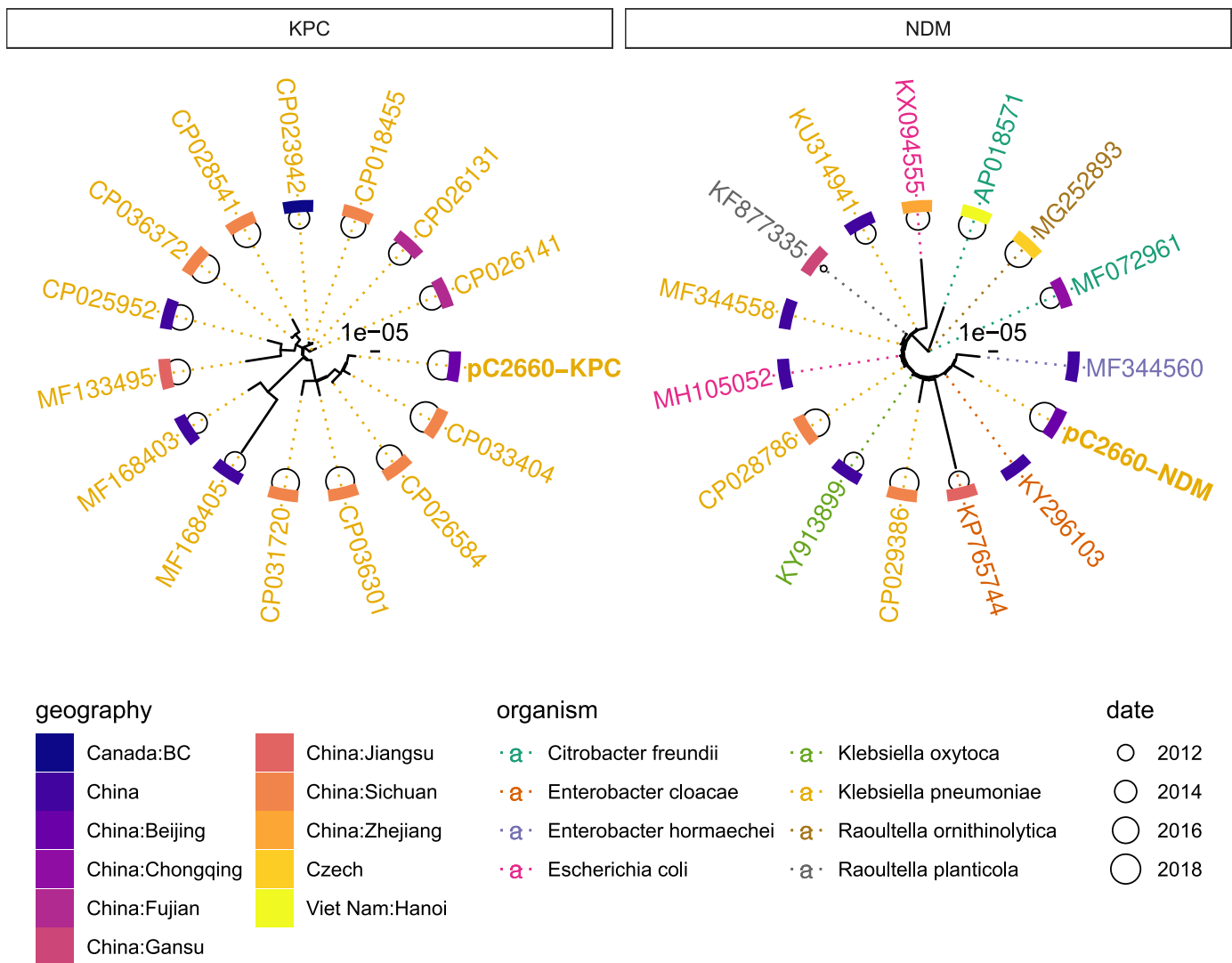


Fig. 5. Comparison of evolutionary characteristics between pC2660-KPC and pC2660-NDM. Plasmid label is colored according to organism taxonomy, filled bar indicates geographical location, and the size of circle represents collection date, the larger the more recently.

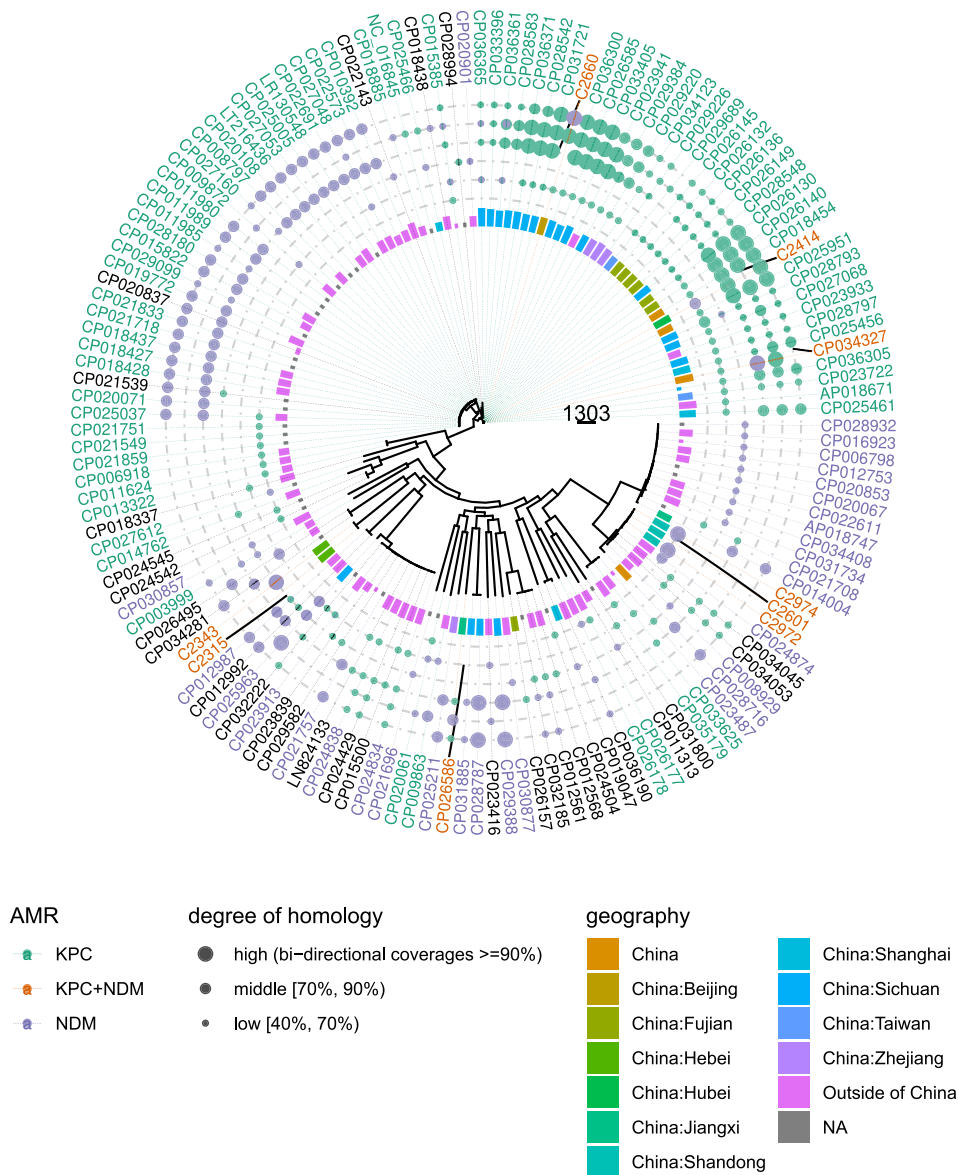
C2343 (Fig. 3), and plasmid breakage events (Supplementary Fig. 4) in C2601/C2972/C2974 where larger plasmid was fragmented into smaller plasmids. However, it should be noted that, although plasmids in KPC-2-NDM-1-CRKP undergo rapid dynamics during their possible adaption to the environment and inter-host transmission, these KPC-2-NDM-1-CRKP did keep both *bla*_{KPC-2} and *bla*_{NDM-1} plasmids during the long time, indicating that these *bla*_{KPC-2} and *bla*_{NDM-1} plasmids can be stably transmitted. Further support for this notion came from our in vitro experiments that KPC-2-NDM-1-CRKP maintained both *bla*_{KPC-2} and *bla*_{NDM-1} plasmids during 10-day serial passage. Thus, our study, for the first time, implies that KPC-2-NDM-1-CRKP are stable and have inter-host transmission capacity in the real world, which highlights the importance of control the spread of KPC-2-NDM-1-CRKP.

Both conjugation assay and phylogenetic analysis reveal the high transferability of *bla*_{NDM-1} plasmids, not only within species, but also among different species. This raises the necessity to monitor the prevalence of *bla*_{NDM-1} plasmid and warn the high probability of *bla*_{NDM-1} incorporating with native Enterobacteriaceae to extend

their resistant profile, not merely with KPC-2-CRKP [22]. On the other hand, highly diverse plasmid background of *bla*_{NDM-1} brings obstacle for the clinical prevention and control.

Our analysis, for the first time, uncovered an evolutionary pathway in forming the KPC-2-NDM-1-CRKP. Our inference method heavily relied on the public complete genome sequences to construct the phylogenetic tree of genomes and to calculate the relatedness of plasmids. Unfortunately, it's impossible to sample and sequence all related isolates. And currently only assemblies from NCBI RefSeq database had been used, and some potential isolates from NCBI SRA database could have been overlooked. Although we found that C2601, C2972 and C2974 possibly originated from an NDM-1-CRKP that acquired *bla*_{KPC-2} plasmid later, this reciprocal evolutionary pathway was less confident because no *K. pneumoniae* isolate was found to carry a plasmid with higher degree of homology to the *bla*_{NDM-1} plasmid. What's more, it's hard to draw any conclusion for remaining KPC-2-NDM-1-CRKP and the recently reported hypervirulent KPC-2-NDM-1-CRKP strain NUHL30457 [9]. More complete genomes are needed for further confirmation.

a



b

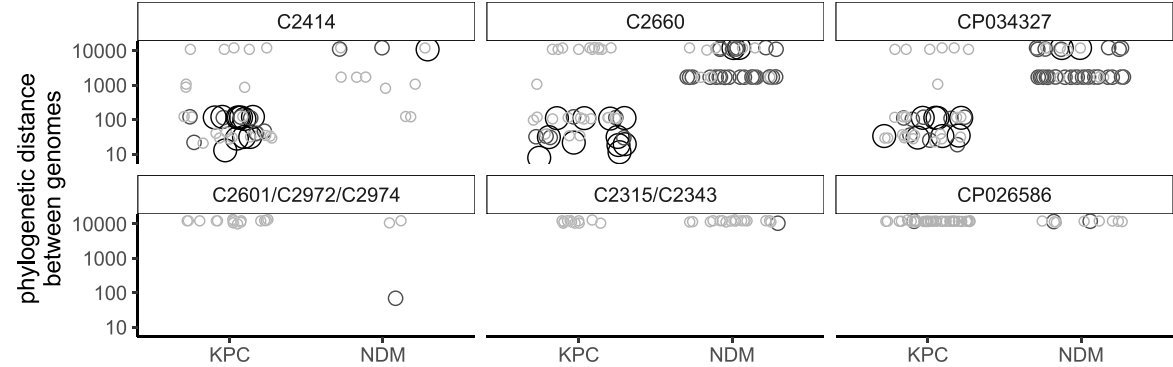


Fig. 6. Evolutionary pathway inference for KPC-2-NDM-1-CRKP. (a) Phylogenetic tree for subject *K. pneumoniae* genomes (inner tree track), with annotations of geographical location (fill color of middle bar track), collection date (height of middle bar track), and similarity to query *bla*_{KPC-2}/*bla*_{NDM-1} plasmids (outer polar scatter-plot tracks). Genome label is colored according to whether the isolate harbors *bla*_{KPC-2} and *bla*_{NDM-1} plasmids. The outer six tracks represent relationship between each genome and query genome to which a black line linked. One point indicates a plasmid in subject genome is similar to the *bla*_{KPC-2} or *bla*_{NDM-1} plasmid in query genome. Point size denotes the degree of similarity. (b) Scatter-plot of phylogenetic distance of genomes (y axis) versus similarity of plasmids (point size), grouped by *bla*_{KPC-2} or *bla*_{NDM-1} plasmid.

Declaration of competing interest

All authors have nothing to disclose.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.ebiom.2019.102599](https://doi.org/10.1016/j.ebiom.2019.102599).

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