Li *et al. One Health Advances* (2023) 1:30
https://doi.org/10.1186/s44280-023-00033-9

One Health Advances

ARTICLE

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Molecular characterization of *bla*$_{NDM}$-harboring plasmids reveal its rapid adaptation and evolution in the Enterobacteriaceae

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Abstract

Carbapenem is one of the few available drugs to treat multidrug-resistance Gram-negative bacteria infections. Recently, the plasmid-mediated spread of the carbapenem resistance gene *bla*$_{NDM}$ poses a significant threat to public health, requiring global monitoring and surveillance. Here, we used both short-read (n = 2461) and long-read (n = 546) sequencing data to characterize the global distribution of *bla*$_{NDM}$. We analyzed the replicon type of *bla*$_{NDM}$-positive plasmids and found that the dominant plasmid type was different in diverse geographical locations. Although *bla*$_{NDM}$ gene has been transferred across diverse countries, its genetic backgrounds are highly conserved, and the mobile genetic element IS*Aba125*, IS*5*, and IS*26* may play an important role in the mobilization of *bla*$_{NDM}$. A significant association was observed between host origin and gene presence/deletion variation on IncX3 plasmid, which may be a key factor in the bacterial adaption to diverse hosts. In this study, we analyzed the diversity, distribution and transmission of *bla*$_{NDM}$-positive plasmids from a global perspective, and emphasize the importance of plasmid analysis for understanding the evolution and adaptation of *bla*$_{NDM}$-positive plasmids and their co-evolution with bacterial genomes (resistome).

Keywords *bla*$_{NDM}$, Plasmids, Bacterial genomics, Carbapenem resistance

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Introduction

Carbapenem resistance has become increasingly prevalent worldwide in the last two decades, resulting in limited therapeutic options for severe clinical infections caused by Gram-negative bacteria [1–3]. New Delhi Metallo-β-lactamase (NDM), a carbapenemase first described in 2009, can hydrolyze nearly all β-lactams, including carbapenems. NDM-encoding gene bla_{NDM} is of particular concern because of its alarming global spread features, including (1) a broad host range and frequent acquisition among *Escherichia coli* and *Klebsiella pneumoniae*; (2) contributing greatly to the prevalence of Carbapenem-resistant Enterobacteriaceae in diverse host and environment, such as livestock, pets, food animal products, and other natural environment; (3) widely transmission in Indian subcontinent, Southeast and East Asia; and (4) co-occurrence with other resistance genes on plasmids, particularly with genes of great public health concern (e.g., *mcr-1*) [4–7]. Recently, many studies have described the above features, however, few researches have focused on the transmission mode and underlying characteristics of bla_{NDM} at the genomic level [8–10].

The vast majority of bla_{NDM} are located on plasmids, which play a key role in the transmission of bla_{NDM} [7]. Three genetic tiers should be considered in understanding plasmid-borne gene molecular epidemiology, including (1) bacterial spread among diverse hosts; (2) inter-bacterial plasmid conjugation; (3) inter-plasmid gene module transposition. Previous researches were usually restricted to a specific region, bacteria, or host, and the number of plasmids involved was usually less than 10 [6, 11, 12]. However, with the accumulation of plasmid sequencing data, there is an urgent need for more comprehensive and intensive studies on the global dissemination of bla_{NDM}.

With the improvements in sequencing technology and reduction in cost, the number of available plasmid sequences in public databases has been increasing. However, numerous repetitive elements complicate the plasmid reconstruction from short-read sequencing data which take up a large portion of databases [13]. To obtain the complete sequences of plasmids, long-read sequencing data (~ 10 kb in one single read) can be a reliable solution [14]. With the faster accumulation of long-read sequencing data, recently, some researchers have built the databases of complete plasmids sequences, such as pATLAS [15], PLSDB [16], COMPASS [17]. This advance contributes significantly to large-scale plasmids comparison.

Previous studies have mostly focused on bla_{NDM}-harboring contigs, which have led to a better understanding of evolution of its genetic contexts. However, a current problem is lacking comprehensive and large-scale analysis based on complete bla_{NDM}-harboring plasmids. Here, we collected all long-reads and short-reads sequencing data of bla_{NDM}-harboring contigs from 2011 to 2021 to understand the global dissemination and genetic characteristics of bla_{NDM}. In this study, we investigated the geographical dominance and source specificity of bla_{NDM}-harboring plasmids, and evaluated their transfer features between different clonal lineages to further understand its evolution and adaptation.

Results

bla_{NDM}-harboring plasmids showed extensive modularity and geographical dominance

In total, 546 complete plasmids (long-read sequencing data) carrying bla_{NDM} retrieved from NCBI database as of 2021 were included in the subsequent analysis, as the remaining were judged as incomplete, mislabeled, repeated or bla_{NDM}-negative (Table 1). These complete bla_{NDM}-harboring plasmids were found to have a diverse length ranging from 46 kb to 131.67 kb (Table 1). Besides, we combined all the bla_{NDM}-carrying contigs (short-read sequencing data) in the NCBI database to make our results more comprehensive and reliable. All long-read and short-read sequencing data collected in this study covered 71 countries (Supplementary Table 1). In total, 1,586 contigs from *E. coli* and 875 contigs from *K. pneumoniae* carrying bla_{NDM} gene were downloaded from the NCBI website (Supplementary Table 2). 1, 225 (77.24%) contigs from *E. coli* were predicted to be located in plasmids and 839 (79.11%) contigs from *K. pneumoniae* were assigned to plasmids.

Table 1 The basic information of complete bla_{NDM}-harboring plasmid sequences

Subtype	Plasmid number	Mean length (kp)	Median length (kp)	Mode length[a] (kp)
$bla_{\mathrm{NDM\text{-}1}}$	325	119.62 ± 76.51	106	54
$bla_{\mathrm{NDM\text{-}3}}$	1	161	161	157
$bla_{\mathrm{NDM\text{-}4}}$	15	66.87 ± 42.49	49	54
$bla_{\mathrm{NDM\text{-}5}}$	164	77.32 ± 54.41	46	46
$bla_{\mathrm{NDM\text{-}6}}$	4	128.00 ± 83.27	106	N/A [b]
$bla_{\mathrm{NDM\text{-}7}}$	17	46.24 ± 6.94	46	46
$bla_{\mathrm{NDM\text{-}9}}$	18	130.67 ± 53.64	110.5	109
$bla_{\mathrm{NDM\text{-}20}}$	1	46	46	46
$bla_{\mathrm{NDM\text{-}21}}$	1	46	46	46
Total	546	103.41 ± 71.33	87	46

[a] Mode length refers to the most common length in each subtype

[b] Not applicable

Among all the bla_{NDM}-harboring plasmids (complete and contigs), a total of 15 Inc types have been identified, suggesting NDM could be easily adapted to multiple plasmid genetic environment, without raising the fitness cost. In total, IncFI (n = 903, 35.98%) was the most dominant maintaining a high proportion during the 11 years (Fig. 1B), followed by IncX3 (n = 749, 29.84%) and IncA/C (n = 225, 8.96%). IncX3 shows an upward trend from 2011 to 2021, while IncA/C shows a downward trend (Fig. 1B). IncX3 is the dominant Inc in countries such as China, India and South Korea, while IncFI has a dominant position in countries such as Canada and the United States (Fig. 1A). Table 2 shows the dominant Inc types of different continents and the number of corresponding plasmids. Significant associations have been observed between several Inc types of bla_{NDM}-harboring plasmids with geographic regions, including America with IncFI, Europe with IncA/C, IncR, and IncX1/X4/X6, East Asia with IncHI2, IncX3, and IncY, South Asia with IncFI, Southeast Asia with IncN and IncFI, and West Asia with IncFI (Table 2). These findings suggested that most transmission of NDM has been limited within the continent region.

We further predicted the oriT sites of the complete plasmids. Mobility (MOB) types and Mating pair formation (MPF) types of the complete plasmids were also analyzed, which were based on relaxase proteins and T4SS systems, respectively. 39.9% of the complete plasmids (n = 218) predicted to harbor oriT site, MOB and MPF simultaneously, which can be classified as conjugative. Although the typical oriT site were not predicted on the IncX3 plasmids (n = 179, 32.8%), its high conjugation transfer frequency has been confirmed in previous studies [18, 19]. 30 different Inc-MOB-MPF combinations were observed and the top 3 combinations in quantity were highlighted and displayed with date in Supplementary Fig. 1. IncX3-MOB_P-MPF_T was found to be the most popular group (n = 171, 31.3%). Previous study has shown that MOB_P is the most represented of the six relaxases families (MOB_F, MOB_H, MOB_Q, MOB_C, MOB_P, and MOB_V) and usually associated with MPF_T [20, 21]. However, the reasons for this preference are still unclear, possibly because of interactions of the two conjugative modules.

Table 2 The significant associations between continents and plasmid Inc types (n = 882)

Continent [a]	Plasmid Inc type [b]	Plasmid number [c]	p-value [d]
America (396)	IncFI (903)	249	4.29×10^{-23}
Europe (397)	IncA/C (225)	102	2.86×10^{-15}
	IncR (77)	66	1.41×10^{-180}
	IncX1/X4/X6 (93)	53	1.07×10^{-4}
East Asia (812)	IncHI2 (36)	36	2.11×10^{-23}
	IncX3 (749)	574	5.81×10^{-39}
	IncY (29)	23	8.26×10^{-17}
South Asia (202)	IncFI (903)	145	2.02×10^{-20}
Southeast Asia (251)	IncN (59)	36	1.45×10^{-13}
	IncFI (903)	158	2.98×10^{-19}
Middle East (122)	IncFI (903)	73	1.86×10^{-4}

[a] Plasmids that without continent information were excluded. The values in brackets represent the number of plasmids in corresponding continent

[b] Plasmids that could not classified by Inc typing were excluded. The values in brackets represent the number of corresponding Inc type

[c] This number represents the number of plasmids of this Inc type in the continent

[d] Only data with p-value < 0.001 are shown in the table. The p-values were adjusted by Bonferroni correction

Genetic characteristics of bla_{NDM} gene contexts

We recruited 546 complete and putative plasmid sequences and identified the flanking genes of bla_{NDM}. In total, 149 clusters of the flanking of bla_{NDM} in plasmids were identified, among which the largest cluster contained 119 (21.8%) sequences, followed by 13 other clusters contained 10 or more sequences (Fig. 2). Multiple clusters of bla_{NDM} gene contexts have been wide spread across different plasmids, bacteria, host, and geographical regions. IncX3 type plasmids showed significant dominance in the largest two clusters (cluster 1 and 2), and displayed a preference for cluster 1, 2, and 5 (Fig. 2). IncFI plasmids showed the most diverse genetic background (11 of the 14 clusters), followed by IncA/C (n = 8) (Fig. 2). Plasmids collected from *Escherichia* and *Klebsiella* were widely assigned to almost all clusters, indicating the importance of the two genera in the bla_{NDM} transposition. All 14 clusters contained plasmids of human origins and half clusters contained plasmids of animal origins. Besides, 6 clusters covered plasmids from 4 continents (Fig. 2). Plasmids from *Acinetobacter* (serving as the intermediate source for the mobilization of bla_{NDM} into the Enterobacteriaceae [7]), were mainly classified into cluster 6, which also contained plasmids from *Escherichia*, *Klebsiella*, and *Providencia*. This cluster was predominately composed of IncHI1 plasmids and involved multiple host sources and regions. Multiple clusters of bla_{NDM} gene contexts have been wide spread across different host, environments, and even five continents, suggesting its high adaptability to diverse stress or environmental conditions.

The upstream of bla_{NDM} gene is always insertion sequence, while the downstream is always a complete or remnant form of a gene cluster, including ble_{MBL}, *trpF*, *dsbD*, *cutA*, *groES-groEL*, and insertion sequence, which has confirmed in previous study [7]. In this study, gene cluster *"ble_{MBL}, trpF, dsbD"* were identified in 13 clusters (92.9%). Six of 14 clusters harbored IS*Aba125* and

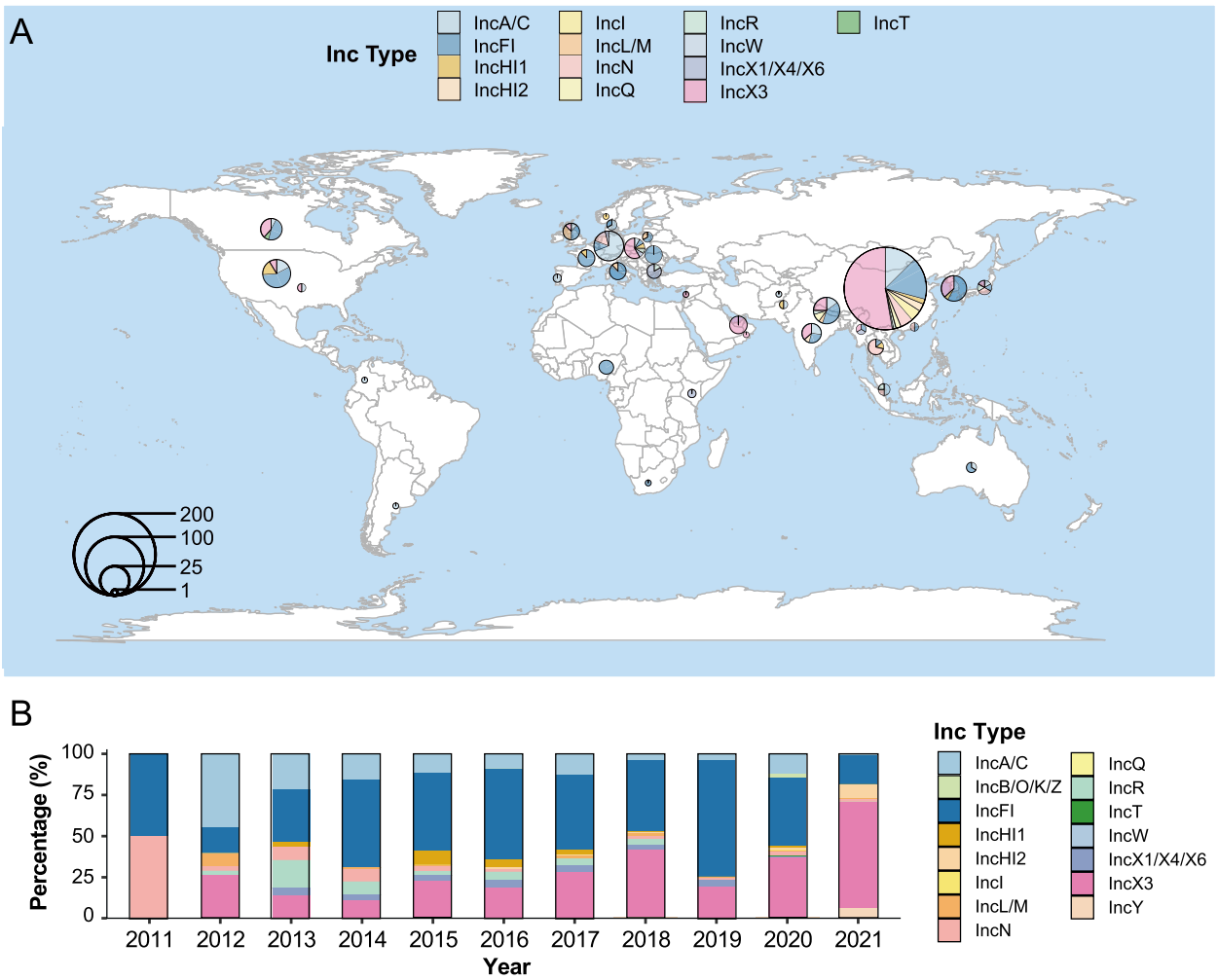

Fig. 1 Distribution of *bla*$_{NDM}$-harboring plasmids worldwide from 2011 to 2021. **A** Geographical distribution of complete *bla*$_{NDM}$-harboring plasmids worldwide (n = 546). The size of circles represented the number of plasmids and the details were annotated in the legend. **B** Temporal changes of all *bla*$_{NDM}$-harboring plasmids (complete plasmids and contigs, n = 2510)

an insert element was observed between IS*Aba125* and *bla*$_{NDM}$ in three clusters (Fig. 2). For putative plasmid contigs assembled from short-read sequencing data, ~ 50% (n = 584) included Tn*3* family transposase, suggesting Tn*3* as an important transposon of *bla*$_{NDM}$ genes among diverse plasmids. IS*Aba125* (371 contigs), IS*5* (338 contigs), and IS*26* (75 contigs) are the three most common mobile genetic elements identified (Fig. 2, Supplementary Table 3), indicating their important role in the mobilization of *bla*$_{NDM}$. The genetic environment of *bla*$_{NDM}$ gene is highly conserved which can be transferred in different host bacteria through different mobile genetic elements.

Few studies have been conducted to reveal the variation of *bla*$_{NDM}$-harboring plasmid, and its evolution during the transmission of *bla*$_{NDM}$ among different host. IncX3 type plasmids occupied a large fraction in recent years, even up to 64.36% in 2021, exceeding IncFI (Fig. 1B). In this study, we extracted the corresponding nucleotide sequences of the coding gene regions where core plasmid-genome SNPs located in (complete IncX3 plasmids). Most of the *bla*$_{NDM}$-IncX3 plasmids were highly similar, and the overall identity was over 99%, which has been confirmed by several epidemiological studies [22–24]. Only a few point mutations were observed in adjacent insertion elements. Specially, two adjacent base substitutions were observed in the IS*26* transposase Tnp26 in 3 complete plasmids (Accession Number: NZ_CP048028, NZ_MK033579, NZ_MK033583), which resulted in G184N substitutions, as previously reported [25]. The G184N substitutions can generate IS*26***, the third IS*26* variant, which showed a 10-fold increase in the cointegration frequency in previous study [25], suggesting that point mutations may be important in the adaption and persistence of plasmids in different host.

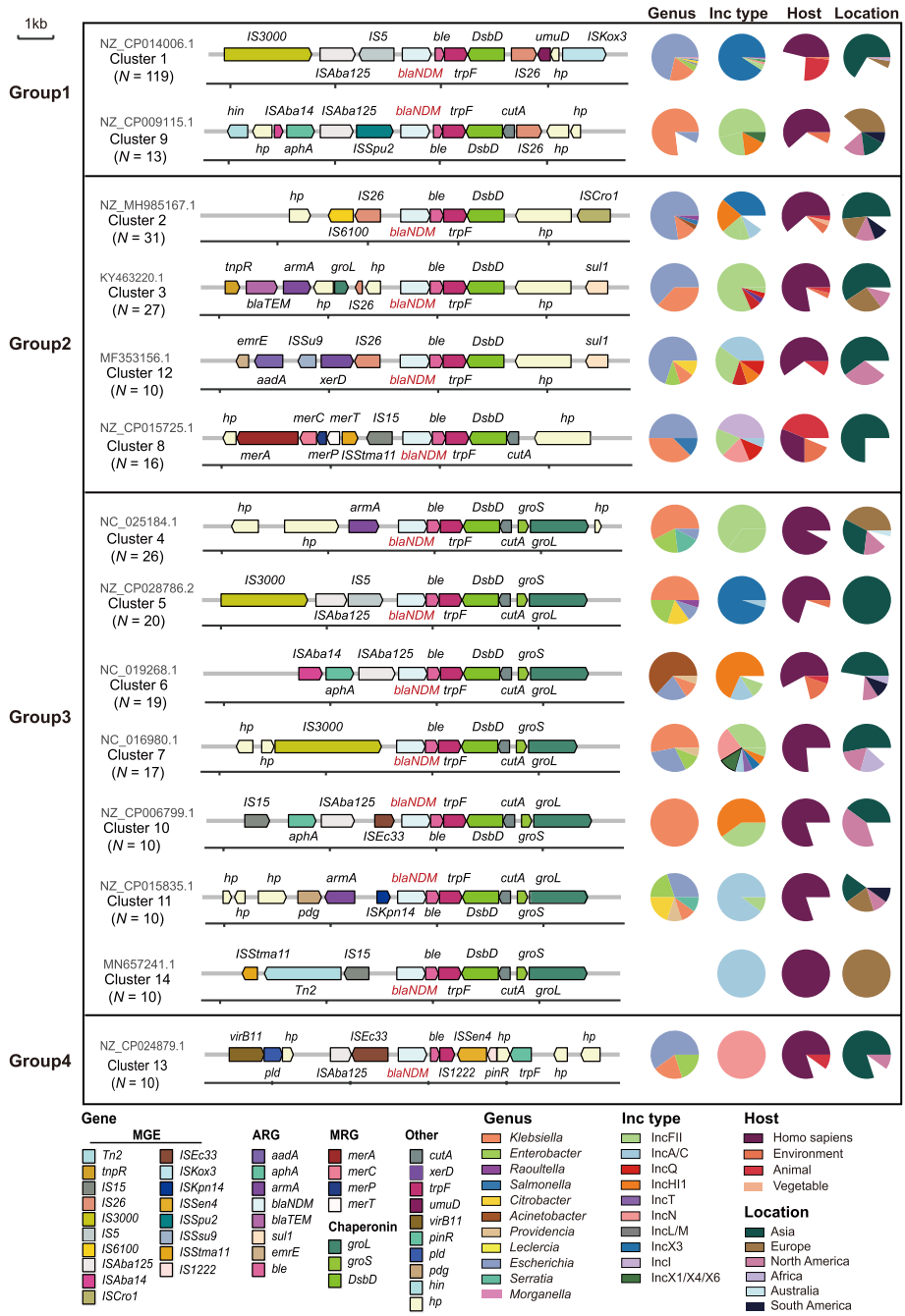

Fig. 2 Clusters of diverse bla_{NDM} genetic contexts on plasmids. The left listed the representative genetic contexts of every cluster ($n \geq 10$). ORFs are color-coded and the direction of transcription indicated by arrowheads. The right pie graph showed the distribution of genus, Inc type, host source, and location situation of plasmids in the corresponding cluster. The missing part of circles represented the loss of information

Dissemination characteristics of bla_{NDM}-harboring plasmids across diverse host lineages and environment

We focused on complete plasmids from Enterobacteriaceae species, which make up over 50% of the complete plasmids. 66 distinct sequence types (STs) and 25 STs were identified in *E. coli* ($n = 192$) and *K. pneumoniae* ($n = 55$) (Fig. 3). 19 shared core plasmid-genome SNPs were identified from *E. coli*-harboring IncX3 plasmids (core plasmid-genome: 16,600 bp, 36%) and 72 shared SNPs were detected among *K. pneumoniae*-harboring IncX3 plasmids (core plasmid-genome: 18,967bp, 41.1%) (Fig. 3), which further confirmed the high conservation

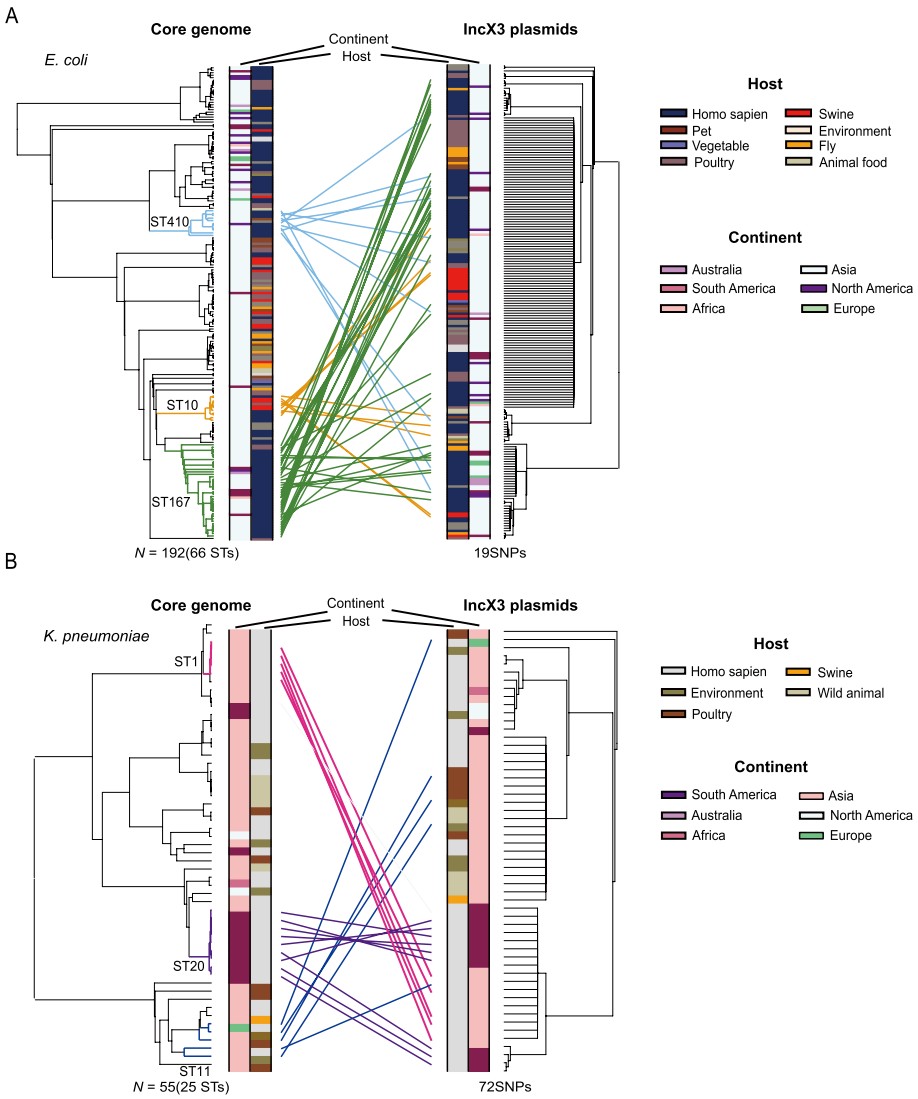

Fig. 3 Tanglegram links phylogenetic trees constructed using core genome SNPs and IncX3 plasmid. **A** A tanglegram of IncX3 plasmid conserved sequence (16,600bp, 36.0%) phylogeny with the core genome phylogeny of *E. coli* (*n* = 192). Lines have been drawn between tips in the trees representing the same isolate. **B** A tanglegram of IncX3 plasmid conserved sequence (18,967bp, 41.1.0%) phylogeny with the core genome phylogeny of *K. pneumoniae* (*n* = 55). The color of tree nodes and links represented the ST type of the isolates. The inner bar showed the host sources of the isolates and the outer bar showed the continent they were collected from

of bla_{NDM}-positive plasmids. bla_{NDM}-harboring IncX3 plasmids from *K. pneumoniae* had higher SNPs ($n = 72$) than those from *E. coli* ($n = 19$) (Fig. 3).

For *E. coli*, many IncX3 plasmids were associated with clone expansions of isolates, which were described as two or more isolates of the same ST clustered in the phylogenetic tree based on core plasmid-genome SNPs. In particular, 105 (54.7%) bla_{NDM}-harboring IncX3 plasmids belonged to *E. coli* isolates of 11 clonal expansions and isolates belonged to ST167, ST410, and ST10 accounted for the majority (19.8, 5.2, and 4.7%, respectively). For *K. pneumoniae*, ST20 (16.4%), ST1 (5.4%), ST11 (5.4%) were the most common lineages. IncX3 plasmids from *E. coli* ST167 showed little variation with other 27 STs ($G_{ST} = 6.6\times10^{-4}$) and large amount of variation was found among 414 ST pairs ($G_{ST} = 1$). The pairwise SNP differences of core-genome of IncX3 plasmids from different *E. coli* STs were typically low, from 0 to 3. The IncX3 plasmids from ST16 *Klebsiella pneumoniae* was extremely similar to IncX3 plasmids from ST147 *Klebsiella pneumoniae* ($G_{ST} = 0$) and large amount of variation was found among 134 ST pairs ($G_{ST} = 1$). The maximum pairwise SNP difference of IncX3 plasmids from *Klebsiella pneumoniae* was 6

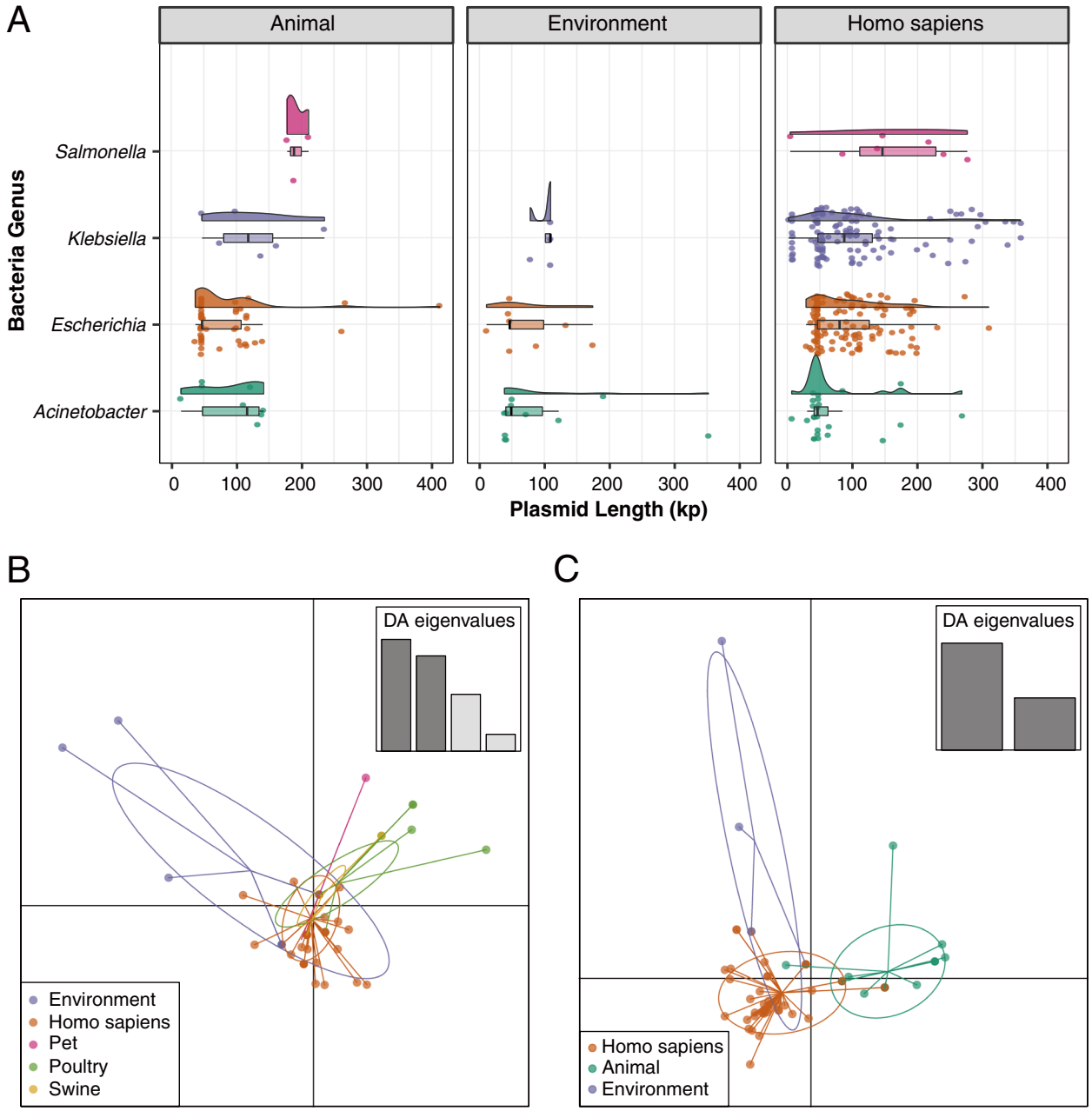

Fig. 4 Source specificity of *bla*$_{NDM}$-harboring plasmids. **A** "violin + boxplot + jittered" composite graph showed the probability distribution, medians and confidence intervals of plasmid length derived per isolation source and bacteria genus. **B** DAPC (Discriminant analysis of principal components) based on the conserved region SNPs among all IncX3 plasmids (n = 192, both complete plasmids and putative plasmid contigs from *E. coli*). Inset shows the histogram of discriminant analysis eigenvalues. **C** DAPC based on the genetic content matrix (n = 98, complete plasmids from *E. coli*) constructed by the gene annotation of whole IncX3 plasmids

(ST20), 2 (ST1), and 5 (ST11). Our results indicated that plasmid-mediated *bla*$_{NDM}$ disseminated by a multiple plasmids/multiple host lineages pattern.

Extensive differences in plasmid length were observed among different bacteria hosts (Fig. 4A), suggesting correlations between the host and plasmid types. *bla*$_{NDM}$-harboring plasmid has been identified in a variety of bacteria host, except Enterobacteriaceae in most complex environment, further indicating the capability of adaptation to multiple environments (Fig. 4A). Further, we built Discriminant Analysis Components (DAPC) models based on single-nucleotide polymorphism (SNPs) in conserved regions and genes presence/absence variations, respectively, to

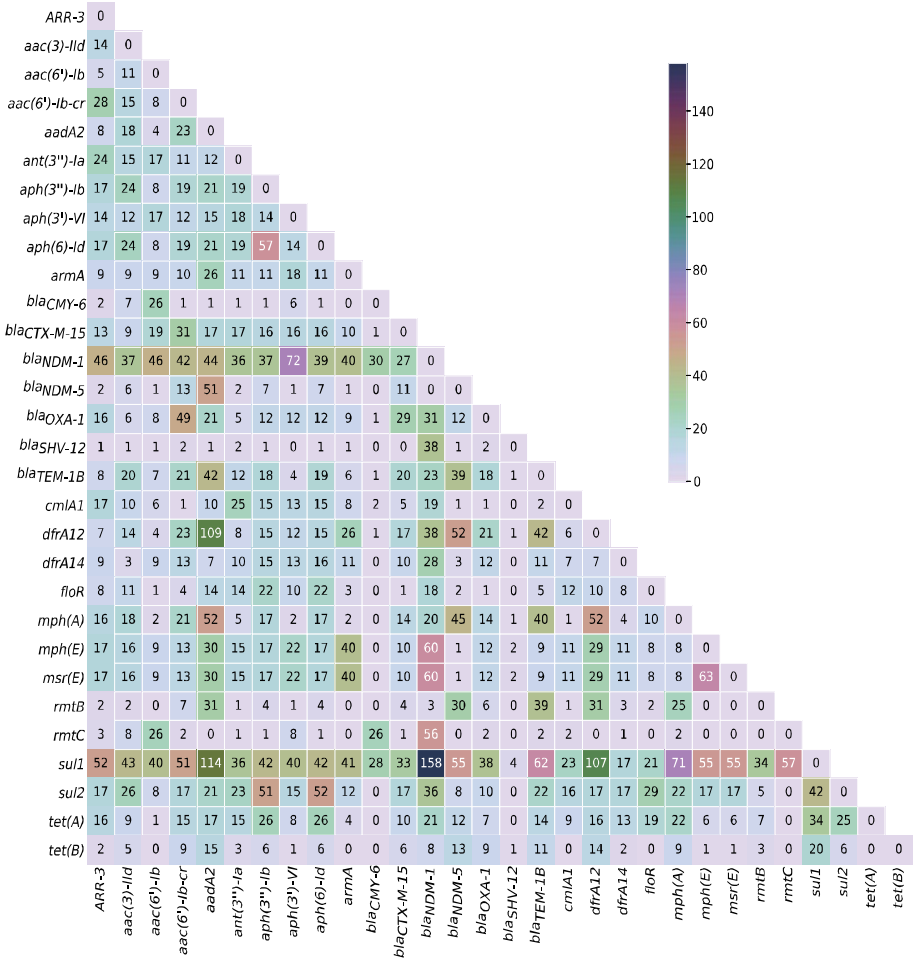

Fig. 5 Co-occurrence of bla_{NDM} and other ARGs. We exhibited pairwise co-occurrence matrix of all ARGs detected in strains carrying bla_{NDM}-harboring plasmids. The colors and numbers in boxes indicate the cases of two ARGs co-existent

investigate the genomic characterization of different host origins. No significant association were observed between core-genome SNPs and host origins for IncX3 plasmids (Fig. 4B). Only complete IncX3 plasmids were recruited for genes presence/absence variation analysis to avoid the errors introduced by draft contigs. DAPC suggested that the genes content variation in plasmids could be used to identify its original host source (Fig. 4C). Gain and loss of genetic material has long been recognized to be an important process in bacterial evolution [26], our results indicates that gain or loss of genes in bla_{NDM}-positive IncX3 plasmids may be a key factor in adaption improvement to different environments.

Co-existence of bla_{NDM} and other ARGs on various Inc-type plasmids

Carbapenem-resistant gene, bla_{NDM}, was always found co-existing with other antimicrobial resistance genes (ARGs), most of which conferring resistance to clinically relevant antimicrobials, including fluoroquinolones, third-generation cephalosporins, and aminoglycosides, posing an increasing threat to global public health [27]. Figure 5 illustrated the pairwise co-occurrence matrix of ARGs with bla_{NDM} in strains carrying bla_{NDM}-harboring plasmids. 122 (93.1%) ARGs were found to co-exist with at least one other ARG within the same host, strongly suggesting the co-transmission of bla_{NDM} and other ARGs between different bacteria. *sul1* (which encodes sulfonamide-resistant dihydropteroate synthase) was the most frequent gene that co-existed with both $bla_{NDM\text{-}1}$ (158, 48.9%) and $bla_{NDM\text{-}5}$ (55, 34.0%).

ARG co-occurrence networks were constructed with different thresholds of co-existing frequency (Fig. 6A). The largest clusters of different ARGs combination ($n \geq 3$, bla_{NDM} included) were listed for every pairwise network (Fig. 6B). For $bla_{NDM\text{-}1}$, ARG combinations co-existed across ten bacteria genera

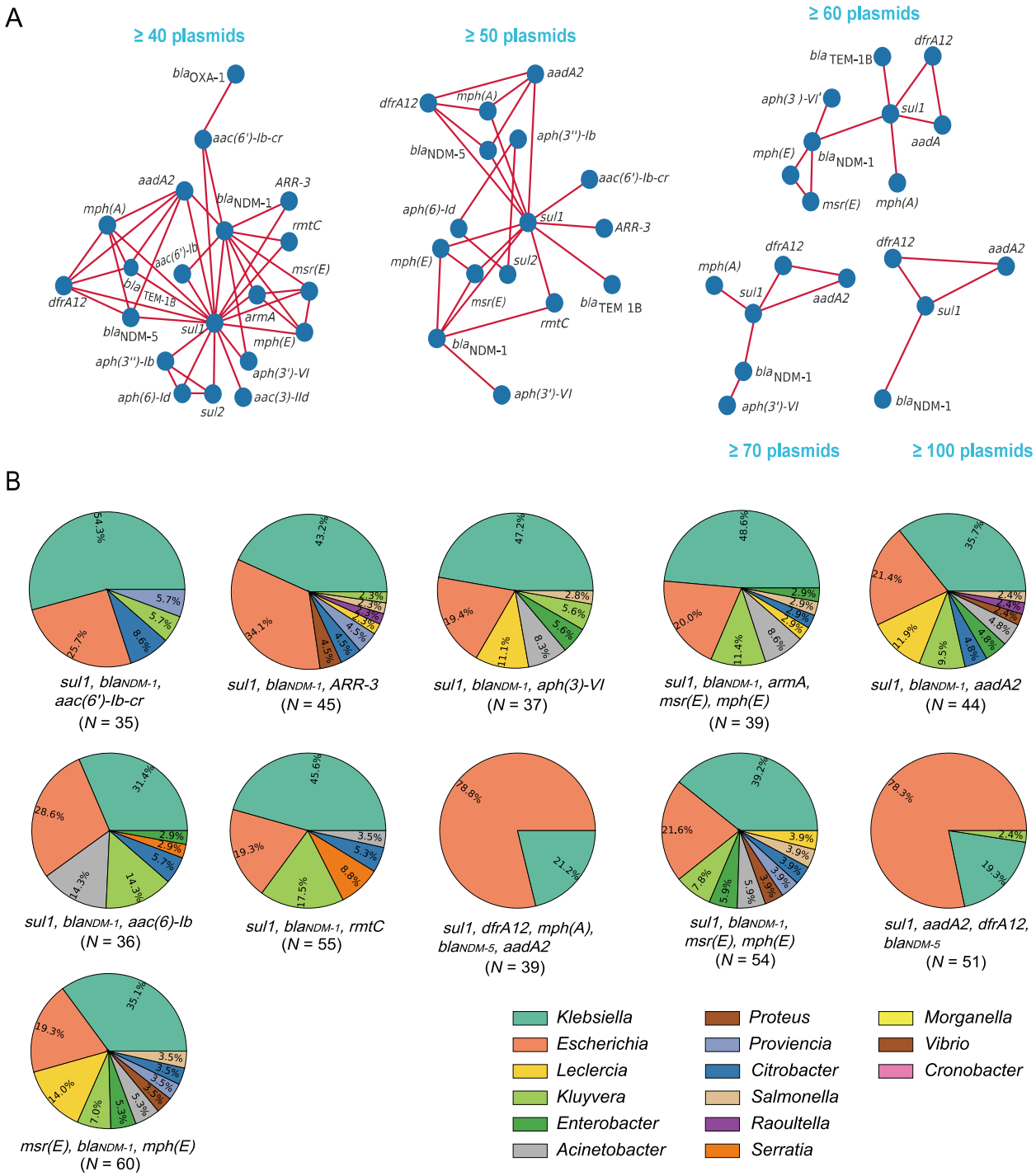

Fig. 6 ARGs co-occurrence networks among bla_{NDM}-harboring plasmids. **A** Visualization of ARGs co-occurrence networks among all strains carrying bla_{NDM}-harboring plasmids. Nodes represent different ARGs. Edge widths represent connection weight calculated by Roary. The threshold was set at ≥40, ≥50, ≥60, ≥70, ≥100 plasmids. **B** For all pairwise ARGs co-occurrence network, the largest clusters of bla_{NDM} were listed. A total of 11 ARGs clusters are listed. The pie chart shows the proportion of different bacteria genera in each cluster, and each color represents a bacteria genus

including bla_{NDM-1}-*msrC-mphC* (60 plasmids, 18.6%), bla_{NDM-1}-*sul1-aadA2* (44 plasmids, 13.6%) and bla_{NDM-1}-*sul1-msrC-mphC* (54 plasmids, 16.7%). The largest cluster of bla_{NDM-1} was the combination of bla_{NDM-1}, *sul1*, *msrC*, *mphC*, and *armA* genes, covering seven bacteria genera, followed by combination of bla_{NDM-1}-*sul1*

with *aac(6')-lb-cr* (35 plasmids, 10.8%), *ARR-3* (45 plasmids, 13.9%), *aph(3)-VI* (37 plasmids, 11.5%), *aac(6)-lb* (36 plasmids, 11.1%) and *rmtC* (55 plasmids, 17.0%).

More Inc types were identified in the $bla_{\text{NDM-1}}$ involved clusters than $bla_{\text{NDM-5}}$ involved clusters. All $bla_{\text{NDM-1}}$ involved clusters were related to more than seven Inc types, except for the combination of $bla_{\text{NDM-1}}$-*sul1* with *aph(3)-VI*, *rmtC* or *aac(6)-lb*, which was only occurred in four Inc types. Among other $bla_{\text{NDM-1}}$ involved clusters, IncA/C and IncX3 type plasmids account for over 60%, especially, 88.2% of plasmids harboring $bla_{\text{NDM-1}}$-*sul1-aac(6)-lb* belonged to IncA/C. For $bla_{\text{NDM-5}}$ involved networks, IncA/C (>85%) was the predominant Inc type, followed by IncX3 (~5%) and IncFI. The co-existence of bla_{NDM} with multiple ARGs and plasmid Inc type in the same host indicated its strong ability of adaption to the multiple drug resistance environment, without significantly rising the fitness cost.

Discussion

Plasmids are the primary carriers of ARGs which usually are significant threats to global public health. Horizontal gene transfer via plasmids is widely recognized as one of the most important ways for the transmission of ARGs, such as bla_{NDM}. Recently, investigation on ARGs is mainly based on genetic backgrounds and clonally evolving lineages, and analysis of plasmids is usually excluded or only evaluated by low-resolution techniques (such as Inc typing). In this study, we collected 546 bla_{NDM}-harboring plasmids with complete sequence and 2,352 bla_{NDM}-harboring CRE draft genomes (putative plasmid contigs, n = 2,064; putative chromosome contigs, n = 397), to investigate the diversity, distribution, and transmission of bla_{NDM}-harboring plasmids from 2011 to 2021 in a global perspective. Our study highlighted the importance of analyzing bla_{NDM}-harboring plasmids to understand the evolution and adaptation of bla_{NDM}-harboring plasmids and its coevolution of with bacteria genome (resistome).

First, plasmid classification enables better understanding of the characterization and transmission mode of diverse global plasmids. Transferable plasmids can be spread between stains and even species, thus, understanding global geographic distribution of bla_{NDM}-harboring plasmids is pivotal to study the mobilization events [28]. Inc type was still selected as the classification criteria for bla_{NDM}-harboring plasmids. In total, several Inc types were found to be significantly associated with specific continents. bla_{NDM}-harboring IncFI-type plasmid was first identified in an *E. coli* strain isolated from a patient from India in 2009, and its transmission was highly identical with population migration, which was primary dominant type in south, southeast, west Asia, and America (Fig. 1A). The discovery of the dominant plasmids (Inc type) in different countries can help to trace the emergence and global spread of the bla_{NDM} gene.

Multiple studies had described bla_{NDM} genetic contexts, while mostly aimed to characterize the transmission of bla_{NDM} in limited areas [29–31]. As we know, investigation of the association between plasmid backbones and antimicrobial resistance gene modules is vital important. In our study, we identified 14 dominant clusters of bla_{NDM} genetic contexts. The upstream of bla_{NDM} contained a high number of ISs (insertion sequences), while the downstream presents a high structural diversity (Fig. 2). The constitution of genetic environment of ARGs may be the result of pressure from local ecological and evolutionary pressures [32]. Here, we further explored the geographical dominance, host source specificity, bacterial genera, and dominant Inc type of plasmid for a better understanding of complex transfer modes of specific gene background. According to the observations of currently available data, IncX3 plasmid occupied a large fraction of the dominant cluster, and was identified in diverse geographical locations, host sources, and bacterial genera suggesting the wide spread and low fitness cost of IncX3 plasmid, as previous reported [7, 18, 19, 33, 34]. We hypothesized that IncX3 could be the dominant bla_{NDM}-harboring plasmids in the next a few years. Among 14 dominant clusters, clusters 4, 7, 10, 11, and 14 were only found in clinical origin, illustrating host sources are also key factors restricting the transfer of ARGs. Cluster 4 and cluster 5 were discovered only in Asia and cluster 14 was discovered only in Europe, indicating that country boundaries may limit the co-transfer of ARGs and genetic background in flanking regions. Besides, multiple plasmid fusion events were observed in this study, especially in IncFI-type plasmids. An important evolutionary feature of plasmids is that they contain multiple transposable elements and undergo frequent genetic transposition, leading to plasmid fusions and possibly better adaptation to diverse stress (such as, antibiotics) and bacterial host [35].

We found that plasmid-mediated bla_{NDM} disseminated by a multiple plasmids/multiple lineages pattern. NDM-harboring plasmids are acquired by diverse lineages, such as the IncX3 plasmid that was found in 66 *E. coli* STs and 25 *K. pneumoniae* STs. Few SNP differences were identified among the conserved region of IncX3, IncFI, and IncA/C plasmids (three dominant types), respectively, suggesting the fast horizontal transfer of bla_{NDM}-harboring plasmids, as a previous Europen-wide study showed [36]. IncX3 plasmids from ST167 *E. coli* had the minimum SNP differences with those from the other 27 STs,

which may suggest its expediated dissemination among different host lineages. Previous studies had reported that the *E. coli* ST167 type was emerging all around the world as a high-risk clone [37, 3839]. Our results also suggested the important role of ST167 *E. coli* in the global transmission of bla_{NDM}-harboring plasmids. It seemed that ST167 *E. coli* acts as a powerful mediator to promote the horizontal transfer of plasmids carrying bla_{NDM} among diverse clones.

bla_{NDM} had been widely reported in clinical, animal, food, and environmental samples, and the Inc types of bla_{NDM}-harboring plasmids showed no obvious bias to diverse samples types [404142]. These findings indicated the broad transfer of bla_{NDM}-harboring plasmids across humans, animals and environments, which is an alarming public health concern. According to our results, IncFI was the most dominant Inc type of all bla_{NDM}-harboring plasmid, while most of them were incomplete contig sequences. The second dominant type was IncX3, which occupied a large fraction in recent years, even up to 64.36% in 2021, exceeding IncFI (Fig. 1B). Here, we chose the widely concerned IncX3 plasmids to investigate the genetic characterization which might contribute to host source specificity. Although we recruited data as much as possible, the SNPs identified were very few and could not be used to distinguish host sources. Notably, we found that differences in gene content across plasmids appear to make a higher contribution to host-source specificity than SNPs in conserved regions. These results suggested that gain or loss of genes in bla_{NDM}-harboring IncX3 plasmids may play a key role in adaption improvement when transferred across different hosts. Acquisition and loss of genetic material has been recognized as an important process in bacterial evolution [43]. We speculate that some dominant plasmids may show the popularity advantage by acquiring some key components through evolution, such as some conjugation components and Type IV secretion system, which could help them fit it the certain bacteria host. Liu et al. showed that outer-membrane core complex (OMCCF) of a T4SS plays an important role in IncF plasmid dissemination and F fimbrial biogenesis [44]. H-NS-like Protein on bla_{NDM}-positive IncX3 plasmid can also affect the transmission of plasmid among different bacterial hosts [34]. Study on the gene content of transferable plasmid enables better understanding of the fitness and evolution process of antimicrobial resistance.

The co-existence of multiple ARGs and bla_{NDM} enables bacteria to adapt to different stresses or niches, and is an important driver of co-evolution of microbial populations. In theory, the co-existence of multiple ARGs might increase the fitness cost of bacteria. However, bacteria might take compensatory measures to reduce this cost, allowing the antimicrobial resistance trait to be maintained. In previous studies, low fitness cost was observed in carriage of bla_{NDM} [18, 19, 34, 35], indicating that bla_{NDM} might be a dominant gene in bacterial adaptive evolution. The low fitness cost of bla_{NDM} provide potential opportunities for its co-occurrence with other ARGs. In this study, we found diverse co-existence situations of other ARGs with bla_{NDM} in strains carrying bla_{NDM}-harboring plasmids, and identified the most common co-occurrence pattern, which had never been explored from a worldwide perspective before. The co-existence of bla_{NDM-1} and other ARGs were occurred in multiple genera and the network combinations were different from bla_{NDM-5}, which was mainly identified in *E. coli* and *K. pneumoniae*. IncA/C was the predominant type in the co-occurrence networks we identified, especially for bla_{NDM-5} involved one, indicating its important role in the appearance and development of MDR bla_{NDM}-harboring strains. IncA/C belonged to broad-host-range (BHR) plasmid families [46], and monitoring of MDR IncA/C plasmids needs to be strengthened in the future studies. Exploration of other ARGs co-existed with bla_{NDM} provided a clearer insight into the risk of multi-drug resistance (MDR) in bla_{NDM} positive isolates.

We acknowledge several limitations of this study. Firstly, to ensure the accuracy, most analyses were based on complete plasmid sequences and short-read assemblies were added as supplementary. Although the data was far beyond other similar studies, the bias was still inevitable. Secondly, we tried to investigate the transmission characterization of bla_{NDM} based on the metadata (sample source, location, collection date, etc.) we collected. However, some information was missed and relevant papers or records were unavailable. Besides, although we had selected statistical models or methods which could reduce the influence from other factors, the bias would not be eliminated. Finally, as we focused on the most popular types whether about other ARGs or genetic contexts associated with bla_{NDM}, other situations (even though partly listed and submitted) were not detailed depicted.

In conclusion, we have highlighted major characterizations of bla_{NDM}-harboring plasmids, which will continue to interact and co-evolve with strains and other genetic elements. Our findings advanced the understanding of the global spread of bla_{NDM}. Strains, plasmids, and other key mobile genetic elements should be included in the continuously monitoring of carbapenem resistance gene bla_{NDM} in the future.

Materials and methods

Complete *bla*$_{NDM}$-harboring plasmids

bla$_{NDM}$-harboring plasmid records were searched in the NCBI nucleotide database by typing the query "NDM plasmid", and then filtered by the PLSDB database [16] to remove incomplete or mislabeled chromosomal records. The relevant gb and fasta files were downloaded based on the filtered accession numbers and parsed using the module Biopython (v1.78) in Python (v3.8.5). All sequences were searched by BLASTn (v2.9.0+) to ensure the carry of *bla*$_{NDM}$ gene. Metadata including length, topology, organism, strain, isolate, country, host, isolation source, journal, etc. were extracted from the gb file. The corresponding paper title and abstract were obtained for missing entries based on the PMID numbers, and information was extracted and supplemented using custom python scripts.

Short-read assembly *bla*$_{NDM}$-harboring contigs in *E. coli* and *Klebsiella pneumoniae* genomes

All entries of *bla*$_{NDM}$-harboring contigs of *E. coli* (n = 2,048) and *K. pneumoniae* (n = 989) were fetched from the NDARO database (National Database of Antibiotic Resistant Organisms, https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/) (Supplementary Table 2). All corresponding *E. coli* (n = 1,380) and *K. pneumoniae* (n = 972) genome sequences were downloaded using the tool ncbi-genome-download (v0.3.0) (https://github.com/kblin/ncbi-genome-download) based on the unique assembly numbers. For each genome, the contig with the *bla*$_{NDM}$ gene was identified using BLASTn. All *bla*$_{NDM}$-harboring contigs were extracted from the short-read assemblies. The machine-learning classifier mlplasmids (v1.0.0) [47] was run to determine the plasmid- and chromosome-derived contigs. Predicted contigs with a posterior probability lower than 0.7 were discarded.

Geographical distribution of the *bla*$_{NDM}$-harboring plasmids

The Geographical distribution of complete *bla*$_{NDM}$-harboring plasmids were visualized using ggplot (v3.3.6) package in R (v3.6.3) (https://www.r-project.org/). Constructed contingency tables were constructed using both complete plasmids and contigs to display the geographical distribution frequency of each Inc type. We performed the Fisher exact test (alternative = "greater") and adjusted naive p values using the Bonferroni correction which can avoid Type I errors (false positives) and chosen the adjusted p-value threshold of 0.001 to determine enrichment of Inc types for five continents.

Characterization of *bla*$_{NDM}$-harboring sequences

Replicon and relaxase typing of complete *bla*$_{NDM}$-harboring plasmids and mobility prediction was performed using MOB-suite (v3.0.0) [20]. For putative *bla*$_{NDM}$-harboring plasmid contigs, incompatibility (Inc) groups were first identified using Abricate (v1.0.1) with the PlasmidFinder database [48]. The contigs without outcomes were then mapped to every complete *bla*$_{NDM}$-harboring plasmid using BLASTn and the genome coverage for each reference plasmid was calculated. The reference plasmid with the highest genome coverage (>90%) was defined as the most similar plasmid and its Inc type was identified as the Inc type of the contig. The combinations of different features were visualized using the UpSetR (v1.4.0) package in R (v3.6.3).

Genetic context of *bla*$_{NDM}$

Complete plasmids from fifteen genera, including *Escherichia* (n = 230), *Klebsiella* (n = 160), *Acinetobacter* (n = 42), *Enterobacter* (n = 42), *Citrobacter* (n = 13), *Salmonella* (n = 10), *Providencia* (n = 11), *Proteus* (n = 5), *Raoultella* (n = 5), *Serratia* (n = 5), *Vibrio* (n = 3), *Leclercia* (n = 32), *Cronobacter* (n = 3), *Morganella* (n = 2), and *Kluyvera* (n = 1), were analyzed to understand the co-transfer modules of *bla*$_{NDM}$ and its flanking regions among different bacteria species. Twenty genes upstream and downstream of *bla*$_{NDM}$ genes were extracted respectively and assigned to different groups based on the genera (Supplementary Table 5). We further extracted 5 kb sequences upstream and downstream of *bla*$_{NDM}$ genes, and then clustered them based on sequence identity cut-off point of 85%. All *bla*$_{NDM}$-harboring sequences were annotated using Prokka (v1.5) [49], including complete plasmids, predicted plasmid contigs, and predicted chromosome contigs. Genes flanking the *bla*$_{NDM}$ loci were manually retrieved from the .gff files of contigs and grouped by different NDM subtypes and bacteria species they belonged to. Significantly, for complete plasmids, 5kb sequences each upstream and downstream of *bla*$_{NDM}$ were extracted manually and then clustered at sequence identity cut-off of 85% using CD-HIT [50]. The gene context of *bla*$_{NDM}$ for different clusters were displayed using the R package gggenes (v0.4.1).

Phylogenetic analyses

The core genome-based phylogenetic of *bla*$_{NDM}$-harboring isolates from *E. coli* and *K. pneumoniae* was constructed using Parsnp (v1.5.3) [51]. In silico multilocus sequence typing (MLST) of *E. coli* and *K. pneumoniae* genomes was performed with the tool mlst (https://github.com/tseemann/mlst). Tanglegrams linking the

core genome and plasmid phylogenies were generated using the dendextend package (v2.1.3) in R. Adobe Illustrator CC (v22.1) was used to add additional annotations and merge different parts of the figures.

Discriminant analysis of principal components

The .vcf file of 192 IncX3 plasmids (including complete plasmids and predicted contigs) from *E. coli* produced by Parsnp and HarvestTools [51] was imported into R using the package vcfR (v1.12.0). Core genome SNPs were counted. The package adegenet (v2.1.3) in R was used to implement discriminant analysis of principal components (DAPC) analysis based on the core genome SNPs. The .gff files of 98 complete IncX3 plasmids from *E. coli* produced by Prokka were used as input for Roary (v3.13.0) [52] with default settings. DAPC was done using a gene presence/absence matrix from Roary output.

Construction of ARGs co-occurrence network

ARGs on all bla_{NDM}-harboring strains (complete plasmids) were detected using ResFinder (v4.0) [3] with minimal identity and coverage of 95%. The results were transformed into a binary matrix in Python to indicate presence/absence. A pairwise co-occurrence matrix of ARGs was constructed from the binary ARG presence/absence matrix. The co-occurrence relationships between all pairs of ARGs were visualized using the heatmap function from the seaborn package (v0.11.0) in Python. The co-occurrence networks, in which the nodes represent ARGs and edges represent a frequency of pairwise co-occurrence (the threshold was set at ≥40, ≥50, ≥60, ≥70, ≥100 plasmids), were constructed by the networkx package (v2.4) in Python. For every network subgraph, the co-existence of multiple ARGs (≥ 3) was explored using the find_clusters function from the networkx package and the corresponding frequency of occurrence was calculated.

Abbreviations

NDM	New Delhi metallo-beta lactamase
ST	Sequence type
Inc	Incompatibility
ARG	Antimicrobial resistance gene
SNP	Single nucleotide polymorphism
IS	Insertion sequence

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44280-023-00033-9.

Additional file 1: Supplementary Table 1. All information of complete bla_{NDM}-harboring plasmids.

Additional file 2: Supplementary Table 2. All information of complete bla_{NDM}-harboring contigs.

Additional file 3: Supplementary Table 3. bla_{NDM} genetic contexts on putative plasmid contigs assembled from short-read sequencing data.

Additional file 4: Supplementary Table 4. Genetic background types of putative bla_{NDM}-harboring plasmid contigs.

Additional file 5: Supplementary Table 5. Genetic background types of complete bla_{NDM}-harboring plasmids based on different genuses.

Additional file 6: Supplementary Figure 1. Intersection plot of the combination of Inc, MOB, and MPF types found in the set of completed plasmid sequences ($n = 546$). The top 3 combinations in quantity were highlighted (blue: IncX3-MOBP-MPFT, green: IncA/C-MOBH-MPFF, and orange: IncFI-MOBF-MPFF).

Authors' contributions

Y.L.: Investigation, Methodology, Formal analysis, Writing — original draft. Y.Y.: Investigation, Visualization, Writing — original draft. Y.W.: Methodology. T.R.W.: Conceptualization. S.W.: Review & editing, Funding acquisition, Project administration, Conceptualization. C.C.: Review & editing, Conceptualization. All authors read and approved the final manuscript.

Funding

This work was supported by the Laboratory of Lingnan Modern Agriculture Project (NT2021006), and National Natural Science Foundation of China (32273054).

Availability of data and materials

The complete code used to generate the analysis reported in the manuscript will be available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

Received: 9 August 2023 Revised: 4 December 2023 Accepted: 5 December 2023
Published online: 20 December 2023

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