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Identification and characterization of a novel incompatibility group X3 plasmid carrying *bla*$_{NDM-1}$ in *Enterobacteriaceae* isolates with epidemiological links to multiple geographical areas in China

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Abstract

The New-Delhi metallo-β-lactamase (NDM-1) is one of the most important resistance traits in Enterobacteriaceae. We characterized nine *bla*$_{NDM-1}$ producing *Enterobacteriaceae* recovered from seven patients who have recently travelled or been treated in India (n=1) or mainland China (n=6) during December 2010-May 2012. All the China-linked patients had no links to the Indian subcontinent. The *bla*$_{NDM-1}$ carrying plasmids belonged to the novel IncX3 (~50kb, in seven isolates including two *Escherichia coli*, two *Klebsiella pneumoniae*, one *Citrobacter freundii*, one *Enterobacter aerogenes*, one *E. cloacae*), IncA/C2 (~140 kb, in one *E. coli*) or FII-F1B groups (~110 kb, in one *E. coli*). Restriction fragment length polymorphism analysis of the seven IncX2 plasmids revealed identical pattern in six and two bands difference in the remaining one. The IncX3 plasmids carrying *bla*$_{NDM-1}$ were epidemiologically linked to Guangzhou (n=1), Hunan (n=4), Haifeng (n=1) and Dongguan (n=1) in mainland China. Complete sequencing of he IncX3 plasmid pNDM-HN380 revealed that it was 54,035 bp long and encoded 52 open reading frames. The *bla*$_{NDM-1}$ gene was found in a transposon-like structure flanked by IS*Aba125* and IS*26*; inserted into the plasmid genetic load region. The sequences of the *bla*$_{NDM-1}$ containing module within the two IS elements were identical to those previously described for *bla*$_{NDM-1}$-positive Tn*125* in the plasmids or chromosome of Acinetobacter isolates. In summary, this is the first description of IncX3 plasmids carrying *bla*$_{NDM-1}$. The findings indicate the worrisome involvement of an epidemic plasmid in the dissemination of NDM-1 in China.

Carbapenem hydrolyzing β-lactamases are a major health threat in the management of Gram-negative infections. In 2008, a novel type of carbapenemases, termed New Delhi Metallo-β-lactamases (NDM-1) was identified in *Escherichia coli* and *Klebsiella pneumoniae* isolated in Sweden from a patient transferred one day previously from India.[1] In 2010, a landmark study identified 37 NDM-1 isolates in the UK, and 143 isolates in different parts of India, Pakistan and Bangladesh and demonstrated an epidemiological link to the Indian subcontinent.[2] In an environmental study conducted in 2010, NDM-1 producing bacteria of multiple species were grown from 12 of 171 seepage samples and two of 50 water samples collected in New Delhi.[3] Taken together, the available findings suggest that the Indian subcontinent is an important reservoir for NDM-1. Recently, small numbers of NDM-1-producing *Enterobacteriaceae* or *Acinetobacter* isolates have been identified in the Balkan states (Bosina, Kosovo, Montenegro and Serbia),[4-7] the Middle East,[8,9] and China [10-12] among patients without obvious links to the Indian subcontinent.

The progenitor of $bla_{\text{NDM-1}}$ remains undefined although similarity with the β-lactamase II from *Erythrobacter litoralis* has lead to proposal for an environmental reservoir but this is disputed by others.[13,14] Organisms that naturally produce carbapenems and plant pathogens are additional possibilities but further work is required for confirmation.[15,16] $bla_{\text{NDM-1}}$ has always been found in association with an upstream insertion sequence IS*Aba125* which provides the -35 promoter sequence.[17] The dissemination of NDM-1 mainly involves mobile genetic elements rather than clonal spread. In *Enterobacteriaceae*, $bla_{\text{NDM-1}}$ has been identified on plasmids with a narrow (IncF1B, IncFII) or broad (IncA/C, IncH, IncL/M and IncN) host range and rarely in the chromosome.[18-21] The first plasmid to be completely sequenced was pHK-NDM-1 (IncL/M, INSDC-GenBank accession HQ451074). The other plasmids that have been completely sequenced and deposited in the INSDC-GenBank were pNDM-1_Dok01 (IncA/C2, AP012208), pNDM-KN (IncA/C2, JN157804), pNDM10505

(IncA/C2, JF503991), pNDM10469 (IncA/C2, JN861072), pNDM102337 (IncA/C2, JF714412), pMR0211 (IncA/C2, JN687470), p271A (IncN2, JF785549), pNDM-MAR (IncH1B-F1B, JN420336), pGUE-NDM (IncFII, JQ364967). Complete sequencing of plasmids provides information for the analysis of the genetic environment of *bla*$_{NDM-1}$ and for a better understanding of the epidemiological aspects of plasmids. Previous studies have indicated that the *bla*$_{NDM-1}$ gene was sometimes carried by untypeable plasmids.[2,11] In this study, we characterized untypeable plasmids carrying *bla*$_{NDM-1}$ in *Enterobacteriaceae* strains recovered from patients with an epidemiological link to mainland China. The results indicate the emergence of a novel plasmid carrying *bla*$_{NDM-1}$ in multiple provinces in China.

Materials and Methods

Bacterial strains, identification and antimicrobial susceptibility testing

The isolates included in this study were identified through a national program introduced since December 2010 for surveillance of carbapenem-resistant *Enterobacteriaceae* in Hong Kong, China. In short, admission screening was implemented for all inpatients with a recent history of hospitalization or surgery abroad. Fecal samples or rectal swabs were collected at admission and were plated onto MacConkey plates supplemented with 1 μg/ml meropenem (MCA-M). Colonies on the MCA-M were identified to species level. The combined disc method was used to screen for possible carbapenemase production by testing with ertapenem, imipenem and meropenem alone and in combination with EDTA (Sigma) or phenylboronic acid (Sigma).[22] An increase of ≥5 mm in presence of EDTA or phenylboronic acid was used to indicate the possible presence of metallo-β-lactamase and class A carbapenemase, respectively. Isolates tested positive in the phenotypic assays were referred to a centralized laboratory for carbapenemase genes detection including *bla*$_{NDM}$. During December 2011-May2012, the program identified nine *bla*$_{NDM}$ positive

Enterobacteriaceae isolates from seven patients. The nine isolates were included in the present study. Four of the isolates were recovered from two members of the same family.[11]

The VITEK GNI system (bioMerieux Vitek Inc., Hazelwood, MO, USA) was used for bacterial identification. Susceptibility testing of the isolates was performed by disk diffusion assay and E-test (AB Biodisk, Solna, Sweden) and result interpreted according to the CLSI.[23]

Carbapenemase gene detection

The major carbapenemase gene (*bla*$_{NDM}$, *bla*$_{IMP}$ *bla*$_{VIM}$ *bla*$_{KPC}$ and *bla*$_{OXA-48}$) were detected by PCR using previously described primers.[11,24-26] The entire coding sequence of *bla*$_{NDM}$ was amplified and sequenced using the following primer pairs: NDM-FW-10319 5'-GCC ATG TCA CTG AAT ACT CGT -3 and NDM-RV-11450, 5'- GCG ATC CTT CCA ACT CGT -3',

Multilocus sequence typing

The sequence type (ST) of *K. pneumoniae* and *E. coli* isolates was determined using the Pasteur Institute and University College Cork scheme, respectively.[27,28]

Plasmid studies

The transferability of *bla*$_{NDM}$ was tested by filter mating *E. coli* J53 Az^{r} as the recipient. Transconjugants were selected on MacConkey medium containing sodium azide (100 μg/ml) and meropenem (0.5 μg/ml). In each set of experiment, absence of growth of the parent and the recipients in the selective agar plate was confirmed. Plasmid DNA was extracted with QIAGEN Midi Kit (Qiagen, Hilden, Germany) and introduced to competent *E. coli* DH5α (Invitrogen, USA) by electroporation, followed by selection of transformants on

Luria Bertani (LB) agar supplemented with meropenem (0.5 μg/ml). The size of plasmids in the transconjugants or transformants was sized by S1-PFGE.

Replicon typing was conducted on transconjugant or transformant with a single plasmid encoding bla_{NDM}. The PCR-based replicon typing (PBRT) scheme was used for recognition of the following plasmid incompatibility groups (Inc): FIA, FIB, FIC, FIIA, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F.[29] The IncF plasmids were subtyped by sequencing.[30] The revised IncX plasmid replicon typing procedure was used for detection of the IncX1-IncX4 subtypes.[31] In all the isolates, the replicon location in the plasmids was confirmed by hybridization with probes specific for bla_{NDM} and *rep* amplified by PCR from different samples.

The plasmids carrying bla_{NDM} were further analyzed by restriction fragment length polymorphism (RFLP). Purified plasmid DNA was separately digested with *EcoR*1 and *Pst*I (Takara, Dalian, China) in accordance with the manufacturer's recommendation.

Plasmid sequencing

The complete sequence of the plasmid pNDM-HN380 carrying bla_{NDM} in a DH5∝ transformant (originating from *K. pneumoiae* strain CRE380) was obtained by using the 454 GS FLX system (Roche, USA) according to the manufacturer's instruction. Plasmid DNA was prepared as previously described.[26] The library yield a total of 70,651 reads with average read length of 500 bp. The reads were assembled by the GS de novo Assembler (version 2.6) into five contigs. The gaps were closed by PCR and Sanger sequencing (Table S1). The complete plasmid sequence was confirmed by comparison of the in silico RFLP and the experimental RFLP using *EcoR*1 and *Pst*I restriction enzymes. The plasmid was annotated by RAST Server and each predicted open reading frames (ORFs) was further blast against the NCBI non-redundant protein database using BLASTP.[32] The WebACT and Mauve (version

2.2.0) softwares were used for alignment and comparison of plasmid sequences.[26,33] XplasMap (version 9.0) was used for construction of a schematic plasmid map.[31]

Results

Patient demographics and strains characteristics

The patient history and characteristics of the bacterial strains were summarized in Table 1. All patients had travel history and all but one of them had recently been hospitalized in mainland China before the *bla*$_{NDM}$-producing strains were detected in Hong Kong. One isolate (CRE727) was identified in a urine sample. All the other isolates were identified in rectal swab or stool samples. All strains exhibited resistance to cephalosporins (ceftriaxone, ceftazidime), carbapenems (ertapenem, imipenem, meropenem), β-lactam/β-lactamase inhibitors combinations (amoxicillin-clavulanate, piperacillin-tazobactam). Co-resistance involving multiple non-β-lactam drugs was common. Combined disc testing revealed that all had a MBL phenotype. In all the strains, PCR and sequencing confirmed presence of the *bla*$_{NDM-1}$ allele (100% identity to INSDC-GenBank HQ451074). Plasmid replicon typing showed that the *bla*$_{NDM-1}$-carrying plasmids (50-140 kb in sizes) were of IncFII$_Y$/FIB$_S$ IncX3, or IncA/C. In seven strains originating from five patients with history of medical care in Guangdong (Guangzhou, Haifeng and Dongguan) and Hunan provinces of China, the *bla*$_{NDM-1}$ genes were localized to IncX plasmids of the same size (50kb). In conjugation experiments, the plasmids harbouring *bla*$_{NDM-1}$ in all nine strains could be transferred at frequencies of 10^{-1} to 10^{-5} transconjugants per donor cells. Transfer of the IncFII$_Y$-FIB$_S$ and IncA/C2 carrying *bla*$_{NDM-1}$ was associated with co-resistance to gentamicin, amikacin and/or tetracyclines in the recipients. No coresistance to non-β-lactam agents was found in recipients of the IncX3 plasmids carrying *bla*$_{NDM-1}$.

RFLP analysis of IncX3 plasmids

The IncX3 plasmids from the seven strains were subjected to RFLP analysis. Six plasmids had identical patterns after *EcoR*1 or *Pst*1 digestion (Figure 1). The plasmid from *K. pneumoniae* strain CRE843 yield results that differed from that for the other strains by two bands for both restriction enzymes.

Sequence analysis of pNDM-HN380

The complete sequence of the plasmid, pNDM-HN380 originating from *K. pneumoniae* strain CRE380 was obtained (INSDC-GenBank accession JX104760). It is a 54,035 bp circular plasmid with an average GC content of 49% and 52 putative open reading frames (ORFs). Figure 2 showed a linear comparison with two other completely sequenced IncX3 plasmids (pEC13_35 and pIncX-SHV). The 30.2 kb backbone structure of pNDM-HN380 is typical of those described for IncX plasmid. The following set of core genes were shared among the three IncX3 plasmids: replication (replication initiation protein, *pir*; replication accessory protein, *bis*), partitioning (*parA*), plasmid maintenance (a putative DNA-binding protein, *hns*; a putative type III topoisomerase, *topB*), conjugation/type IV secretion system (T4SS, with 11 genes, *pilX1* to *pilX11*), transcriptional activator (*actX*) and putative DNA transfer proteins (*taxA* and *taxC*). The 30.2 kb backbone of pNDM-HN380 is highly homologous to pIncX-SHV (100% coverage and 99% nucleotide identity); the similarity with that in pEC14_35 was lower (89% coverage and 98% identity).

The genetic load region between the resolvase, *res* gene and the *hns* gene is 23.9 kb in length. This region is mosaic with areas of high and low GC contents, suggesting it arose from multiple genetic events. The genetic load region of pNDM-HN380 contained 22 ORFs, of which nine were found in pIncX-SHV. The nine ORFs with high homology in the two plasmids include one resistance gene (bla_{SHV}), three mobile genetic elements (IS*26*, Tn*3* and

194 *tnpA)* and five ORFs of unknown functions (Δ*umuD*, *ygbI*, Δ *ygbJ*, *mpr* and *orf29*). However,
195 pIncX and pNDM-HN380 had two different alleles of bla_{SHV} that differed from each other by
196 five nucleotides and two amino acids (Gly234 and Glu235 in SHV-11 versus Ser234 and
197 Lys235 in SHV-12).

198 In pNDM-HN380 (Figure 2, panel A), the bla_{NDM-1} gene was flanked by IS*Aba125*
199 and IS*26* in the 5' and 3' regions, respectively. This 10.8 kb bla_{NDM-1}-containing transposon-
200 like structure was inserted between the truncated *ygbj* gene (encoding a putative
201 dehydrogenase) and the transponase, *Tn3*. The IS*Aba125* element upstream of bla_{NDM-1} was
202 interrupted by an IS*5* element and a 5-bp target site duplication (CCTAA) was identified at
203 the point of insertion between the 5' end of the IS*5* element and the IS*Aba125* fragment. In
204 the bla_{NDM-1} upstream region, there was a 3-bp target site duplication (AAC) at the point of
205 insertion between *Tn3* and IS*Aba125* (Figure 2, panel C), suggesting that this was a
206 transposition event. The 3-bp (AAC) target site duplication was identical to that described for
207 pNDM-BJ01 (accession number JQ001791) but different from that for strain 161/07
208 (accession number HQ857107). No target site duplication repeats could be identified in the
209 sequence adjacent to the IS*26* element in the 3' region. The genes found downstream of
210 bla_{NDM-1} include the bleomycin resistance gene (ble_{MBL}) and a truncated *trpF* gene, followed
211 by two ORFs displaying homology (~70%) with the genome of *Stenotrophomonas*
212 *maltophilia* K279a (accession number AM743169), and genes encoding chaperonin subunits
213 (truncated *groS* and *groEL*) and the transposase *insE*. The genetic structure of this transposon
214 (except for the IS*26* element in the bla_{NDM-1} downstream region and the interruption of
215 IS*Aba125* by IS*5*), including part of the sequences spanning the junctions (Figure 2, panel B
216 and C) was identical to those described in the *Acinetobacter lowffii* plasmid pNDM-BJ01
217 (INSDC-GenBank accession JQ001791) and in the chromosome of *A. baumannii* 161/07
218 (INSDC-GenBank accession HQ857107).

Discussion

The present study revealed the presence of *bla*$_{NDM-1}$ in multiple *Enterobacteriaceae* isolates recovered from returning travelers who have been treated in different parts of China. With the exception of two patients who were of the same family,[11] the other patients were not epidemiologically related to each other. Since the isolates were identified by active surveillance upon admission, we concluded that they represent *bla*$_{NDM-1}$ importations. In Hong Kong, a territory-wide surveillance for carbapenemases has been implemented since the last quarter of 2008.[26] Beside admission screening of at risk patients, microbiology laboratories routinely refer all carbapenem-resistant *Enterobacteriaceae* (CRE) isolates to a centralized laboratory for molecular testing.[26] During the study period, over 500 CRE isolates have been tested by PCR assays. Up to May 2012, a total of ten *bla*$_{NDM-1}$-carrying isolates, including one previously reported by us,[26] were identified. The findings suggest that the spread of *bla*$_{NDM-1}$ in China is much wider than previously realized. Previous studies have identified *bla*$_{NDM-1}$ among clinical isolates of *A. baumannii* and *non-baumannii* isolates in Beijing and six provinces (Guangdong, Zhejiang, Hainan, Anhui, Liaoning, Shandong) from patients without history of foreign travel [10,12] and in a chicken strain of *A. lwoffi* in Shandong province.[34] In Hong Kong, the existing strategy only tests patients with a history of recent hospitalization or surgery abroad, those who have traveled to NDM-endemic countries but without hospitalization are not screened.[11,26] Given that foreign travel alone has been shown to be an important risk factor for acquisition of antibiotic-resistant enteric bacteria, such as CTX-M producing *Enterobacteriaceae*,[35,36] the number of NDM-positive patients reported here may be an underestimation.

We described here a novel conjugative, *bla*$_{NDM-1}$-carrying plasmid in multiple *Enterobacteriaceae* strains. The findings from the RFLP analysis demonstrated that the

IncX3 plasmid has disseminated among multiple enterobacterial species (*E. coli*, *K. pneumoniae*, *C. freundii* and *E. cloacae*) originating from patients with epidemiological links to multiple geographic areas in China. Since most of the patients had contacts with hospitals, nosocomial dissemination of $bla_{\text{NDM-1}}$ involving the horizontal transfer of the plasmid among hospitals in different areas of China is likely. Recently, two studies have demonstrated limited nosocomial transmission of $bla_{\text{NDM-1}}$ -producing isolates in non-endemic areas.[21,37] We previously showed that the two $bla_{\text{NDM-1}}$-carrying *E. coli* strains (CRE396 and CRE397) in the infant and her mother were clonally related.[11] Here, we confirmed that there was *in vivo* transfer of the $bla_{\text{NDM-1}}$-carrying IncX3 plasmid among *E. coli*, *K. peumoniae* and *E. aerogenes* strains carried by the infant. Although plasmids have been implicated to play a major role in the dissemination of $bla_{\text{NDM-1}}$ in *Enterobacteriaceae*, the plasmids in the same or different geographic areas either belonged to different incompatibility groups or were different from each other.[2,20] Therefore, this is the first time that an epidemic plasmid is implicated in $bla_{\text{NDM-1}}$ dissemination. Since the IncX3 subgroup of family could not be amplified with the initially described PBRT scheme,[29] our current understanding of the epidemiology of this group of plasmids is limited. According to the type of associated resistance, genes previously localized on IncX plasmids included: β-lactams ($bla_{\text{TEM-1}}$, $bla_{\text{TEM-52}}$, $bla_{\text{SHV-1}}$) quinolones (*qnrS1*), amonoglycoisdes (*aphA1*), olaquindox (*oqxAB*) and bleomycin (*blmS*).[31] In general, the resistance genes were recruited into a variable genetic load region by IS elements and transposons while the other plasmid scaffolds were conserved.[31] In a collection of 47 *E. coli* isolates from cases of porcine post-weaning diarrhoea, up to 34% of them were found to be positive for different subgroups of the IncX plasmids not carrying $bla_{\text{NDM-1}}$. Since resistance in food animals could disseminate explosively, future studies should explore possible roles play by animal pathogens and commensal in the dissemination of $bla_{\text{NDM-1}}$.[34]

This is the first characterized *bla*$_{NDM-1}$-carrying IncX3 plasmid, in which the *bla*$_{NDM-1}$ was identified inside a composite transposon-like structure flanked by IS*26* and IS*Aba125*. It seems that the 10.8 kb *bla*$_{NDM-1}$ containing module was integrated *en bloc* into the IncX3 resistance load region by a recombination event involving IS*26* and possibly the other mobile elements flanking the junctions. Our findings were in agreement with the horizontal transfer of the entire module (comprising the IS*Aba125* fragment with the -35 promoter region, the *bla*$_{NDM-1}$ gene, the bleomycin resistance gene, the truncated *trpF* gene, followed by the *tat* and *dct*, the chaperonin subunits, *groES* and *groEL*, and the transponase, *insE*) from the genus *Acinetobacter* to *Enterobacteriaceae*, as suggested previously.[10] In *Acinetobacter*, transposon Tn*125* appeared to be the main vehicle for dissemination of *bla*$_{NDM-1}$.[10,38] This and previous studies indicates that further transfer to *Enterobacteriaceae* requires other mobile elements, such as IS*26* (pMR0211, JN687470; pGUE-NDM, JQ364967; and pNDM-HK, HQ451074), IS*903* (pNDM-1_Dok01, AP012208), IS*kpn14* (pNDM-KN, JN157804 and pNDM10505, JF503991), IS*1* (pNDM10469, JN861072), IS*Ec33* (p271A, JF785549) and Tn*3* (pNDM-MAR, JN420336 and pKpANDM-1, FN396876).[8,16,18,19,26,39,40] The IncX plasmids were thought to be narrow host range plasmids of *Enterobacteriaceae* but the ability of transfer to *Pseudomonas aeruginosa* has been demonstrated.[41] In the future, it would be interesting to investigate the transferability of IncX plasmids to the genus *Acinetobacter* which would be expected to facilitate the inter-genera flow of resistance genes.

The backbone of pNDM-HN380 is organized similarly to the backbone of IncX plasmids.[31] The tandem genes *topB-hns*, which act as a conserved stealth module that stabilizes plasmid DNA, is present in all but one (pLN126_33) of the completely sequenced IncX plasmids.[31,42] The *topB* gene is a paralogue of a chromosomally encoded topoisomerase III gene in *E. coli*.[42] In Gram-negative bacteria, the H-NS protein is a global repressor of transcription which modulates diverse functions that include biogenesis of flagella and

expression of genes acquired horizontally.[43] It has been proposed that H-NS binds to curved AT-rich DNA. Therefore, changes in the DNA bend as a result of increase in temperature would weaken the binding, thereby providing a mechanism for dynamic modulation of gene expression in relation to changes in environmental temperature.[44] Recently, the plasmid-encoded Sfh protein, which is an H-NS homologue, has been found to allow plasmids to be transmitted to new bacterial hosts with minimal effects on their fitness.[45]

This study does not have enough data to determine the origin of the *bla*$_{\text{NDM-1}}$-carrying bacteria with links to China. Those cases had not travelled to the Indian subcontinent, but we cannot exclude the possibility that *bla*$_{\text{NDM-1}}$-carrying bacteria were acquired from contacts with other people with such travel history. Since the sequences flanking *bla*$_{\text{NDM-1}}$ in pNDM-HN380 were identical to those having links to the Indian subcontinent, an independent gene escape seems less likely. Nonetheless, it might be speculated that the IncX3 plasmid could be a specific vehicle for *bla*$_{\text{NDM-1}}$ in China.

In conclusion, this study identified a novel *bla*$_{\text{NDM-1}}$-carrying IncX3 plasmid disseminated among multiple species of *Enterobacteriaceae* originating from patients with links to widely separated areas in China. The emergence of NDM-1 in China has likely been contributed by inadequate surveillance, misuse of antimicrobial agents and an incomplete infection control infrastructure in the hospitals. These issues should be addressed as a matter of national healthcare priority. Further studies will be necessary to unveil the full extent of NDM-1 in the country and to investigate the prevalence of this novel plasmid among Gram-negative bacteria.

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Competing Interests: All authors have no conflicts of interest to declare.

328 Table 1. Patient demographics, bacterial strains and features of plasmids carrying *bla*$_{NDM-1}$

Collection date	Strain no.	Sex /age	Place of medical care abroad	Bacterial species	Resistance patterns[b]	MLST	Plasmid replicon type	Plasmid size (kb)
Dec 2010	172924	F/54	India	*E. coli*	Gen, Amk, Sxt, Tet, Cip, Chl	ST101	FII_Y-FIB_S[c]	110
March 2011	CRE851	M/60	Guangzhou	*C. freundii*	Gen, Amk, Sxt, Cip	-	X3	50
July 2011	CRE379	F/1[a]	Hunan	*E. aerogenes*	Fot	-	X3	50
Aug 2011	CRE380	F/1[a]	Hunan	*K. pneumoniae*	Gen, Sxt, Tet, Cip, Chl	ST483	X3	50
Aug 2011	CRE396	F/1[a]	Hunan	*E. coli*	Sxt, Tet, Cip, Chl	ST744	X3	50
Aug 2011	CRE397	F/26[a]	Hunan	*E. coli*	Sxt, Tet, Cip, Chl	ST744	X3	50
Nov 2011	CRE866	M/74	Fujian	*E. coli*	Gen, Amk, Sxt, Tet, Cip, Chl, Fot	ST101	A/C2	140
Feb 2012	CRE727	M/82	Haifeng	*E. cloacae*	Gen, Amk, Sxt, Tet, Cip, Chl, Fot	-	X3	50
May 2012	CRE843	M/1	Dongguan	*K. pneumoniae*	Gen, Sxt, Tet, Cip	ST476	X3	50

329 [a] Strain CRE79, CRE380 and CRE396 were recovered from the same patient. The two patients (F/1 and F/26) were of the same family.
330 [b]For the following drugs, Gen, gentamicin;, Amk, amikacin; Sxt, cotrimoxazole; Tet, tetracycline; Cip, ciprofloxacin; Chl, chloramphenicol; Fot,
331 fosfomycin.
332 [c]Positive for both FII_Y (allele Y3) and FIB_S (Salmonella FIB) replicons.[30]

Figure 1. Restriction analysis of IncX3 plasmids carrying $bla_{\text{NDM-1}}$. Plasmids were digested with (A) *EcoR*I and (B) *Pst*I and separated by electrophoresis in 1% agarose. M, GeneRulerTM DNA ladder. The labels above each lane show the strain number, bacterial species origin (EA, *E. aerogenes*; KP, *K. pneumoniae*; EC, *E. coli*; EO, *E. cloacae*; CF, *C. freundii*) and the geographic source of importation (HN, Hunan; HF, Haifeng; DG, Dongguan; GZ, Guangzhou).

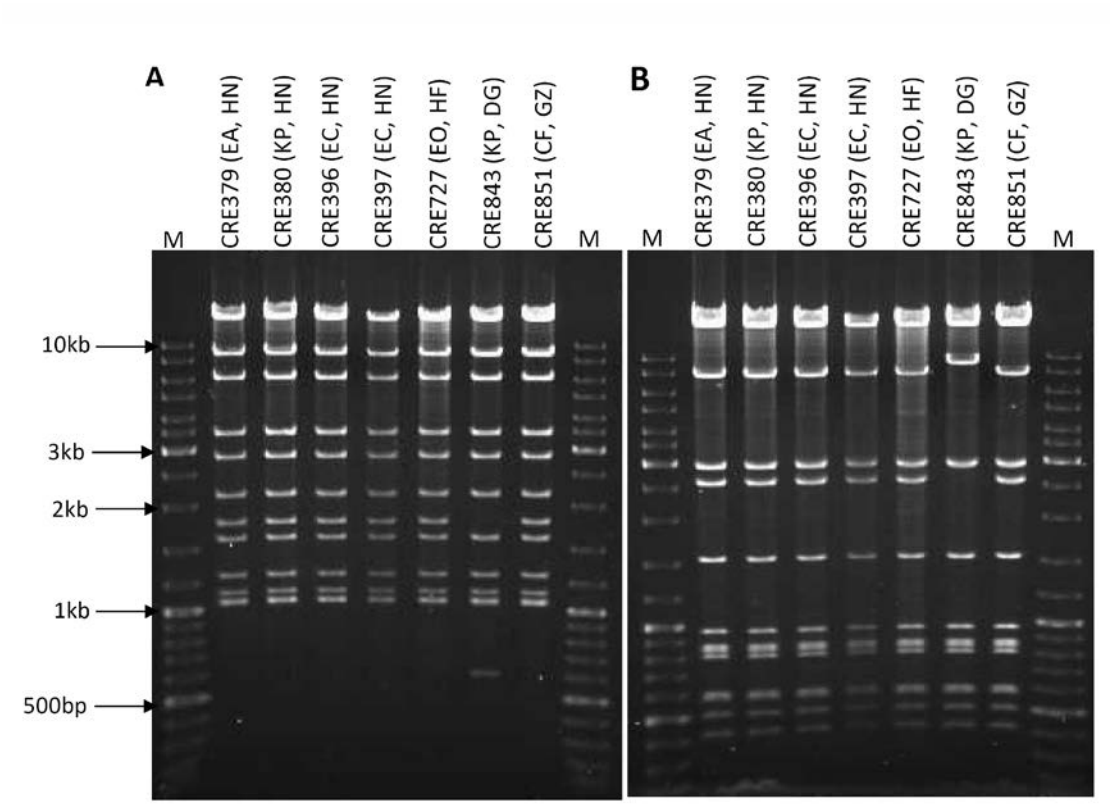
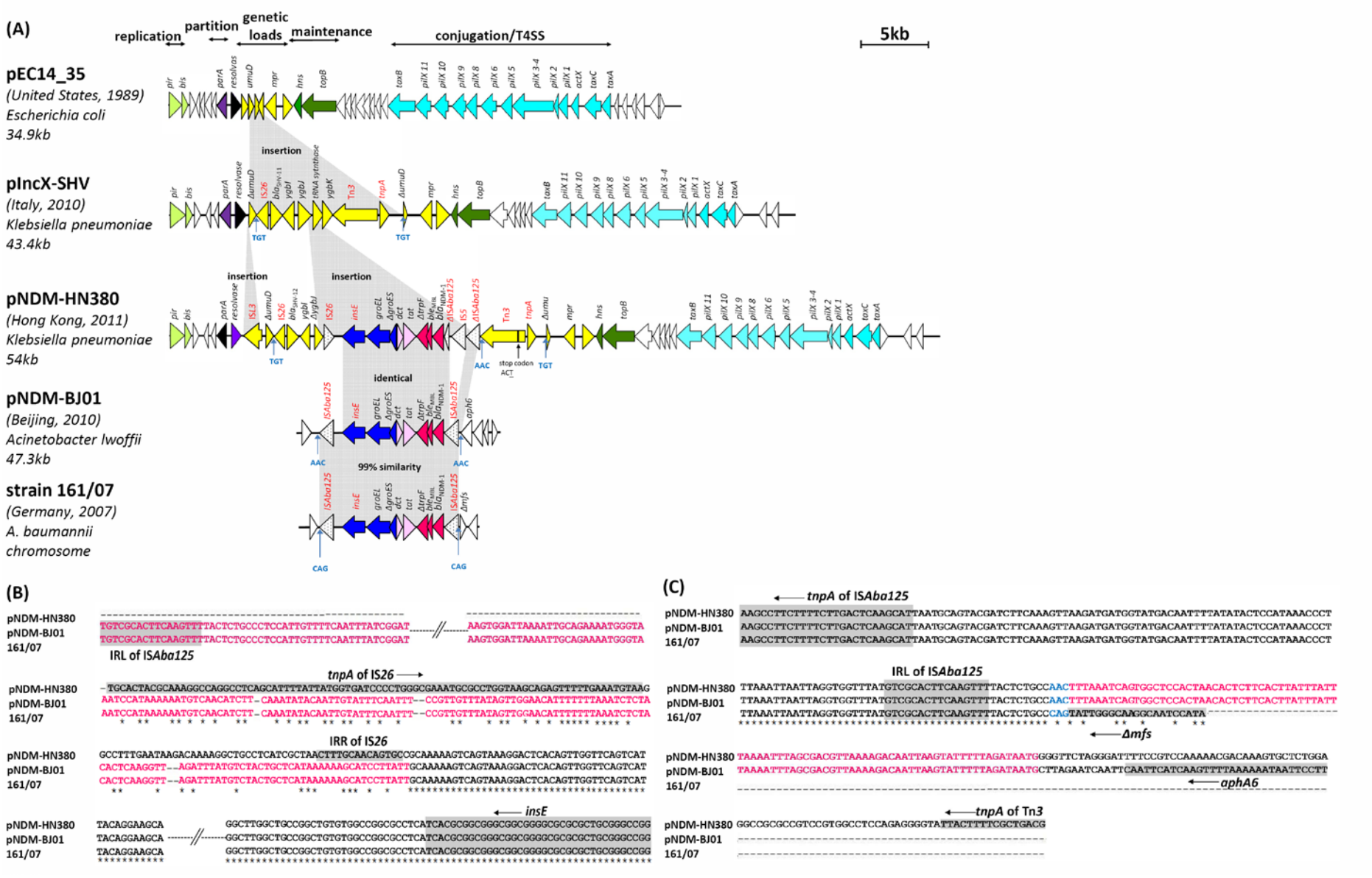


Figure 2. Comparative analysis of (A) linear plasmid maps for three IncX3 plasmids, pEC14_35, pIncX-SHV, pNDM-HN380 and two bla_{NDM-1}-carrying transposon sequences in pNDM-BJ01 and *A. baumannii* strain 161/07, (B) sequences downstream of i*nsE* and (C) sequences upstream of the IS*Aba125* in the 5' end of bla_{NDM-1}.

The function blocks of the plasmids are indicated above the linear maps. The lengths of the ORFs are drawn in proportion to the size of the ORFs. Homologous ORFs in the plasmid maps are represented in the same colour. Direct repeats and mobile elements are labelled in blue and red, respectively. (B) and (C) Consensus regions in the aligned sequences of pNDM-HN380, pNDM-BJ01 and 161/07 are marked with asterisk. The sequences identical in pNDM-HN380 and pNDM-BJ01 are coloured green. The ORFs are indicated by gray shading and the arrow next to the label indicates the ORF orientation. The accession numbers were: pEC14_35 (JN935899); pIncX-SHV (JN247852); pNDM-HN380 (JX104760), pNDM-BJ01 (JQ001791) and Acinetobacter baumannii strain 161/07 (HQ857107).


(A)
replication
partition
genetic loads
maintenance
conjugation/T4SS
5kb
pEC14_35
(United States, 1989)
Escherichia coli
34.9kb
pIncX-SHV
(Italy, 2010)
Klebsiella pneumoniae
43.4kb
pNDM-HN380
(Hong Kong, 2011)
Klebsiella pneumoniae
54kb
pNDM-BJ01
(Beijing, 2010)
Acinetobacter lwoffii
47.3kb
strain 161/07
(Germany, 2007)
A. baumannii
chromosome
insertion
identical
99% similarity
(B)
IRL of ISAba125
tnpA of IS26
IRR of IS26
insE
(C)
tnpA of ISAba125
IRL of ISAba125
Δmfs
aphA6
tnpA of Tn3
pNDM-HN380
pNDM-BJ01
161/07

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