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

5

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23 **Abstract**

24 The New-Delhi metallo- β -lactamase (NDM-1) is one of the most important resistance
25 traits in Enterobacteriaceae. We characterized nine *bla*_{NDM-1} producing *Enterobacteriaceae*
26 recovered from seven patients who have recently travelled or been treated in India (n=1) or
27 mainland China (n=6) during December 2010-May 2012. All the China-linked patients had
28 no links to the Indian subcontinent. The *bla*_{NDM-1} carrying plasmids belonged to the novel
29 IncX3 (~50kb, in seven isolates including two *Escherichia coli*, two *Klebsiella pneumoniae*,
30 one *Citrobacter freundii*, one *Enterobacter aerogenes*, one *E. cloacae*), IncA/C2 (~140 kb, in
31 one *E. coli*) or FII-F1B groups (~110 kb, in one *E. coli*). Restriction fragment length
32 polymorphism analysis of the seven IncX2 plasmids revealed identical pattern in six and two
33 bands difference in the remaining one. The IncX3 plasmids carrying *bla*_{NDM-1} were
34 epidemiologically linked to Guangzhou (n=1), Hunan (n=4), Haifeng (n=1) and Dongguan
35 (n=1) in mainland China. Complete sequencing of the IncX3 plasmid pNDM-HN380 revealed
36 that it was 54,035 bp long and encoded 52 open reading frames. The *bla*_{NDM-1} gene was found
37 in a transposon-like structure flanked by IS*Aba125* and IS26; inserted into the plasmid
38 genetic load region. The sequences of the *bla*_{NDM-1} containing module within the two IS
39 elements were identical to those previously described for *bla*_{NDM-1}-positive Tn*125* in the
40 plasmids or chromosome of Acinetobacter isolates. In summary, this is the first description of
41 IncX3 plasmids carrying *bla*_{NDM-1}. The findings indicate the worrisome involvement of an
42 epidemic plasmid in the dissemination of NDM-1 in China.

43

44 Carbapenem hydrolyzing β -lactamases are a major health threat in the management of
45 Gram-negative infections. In 2008, a novel type of carbapenemases, termed New Delhi
46 Metallo- β -lactamases (NDM-1) was identified in *Escherichia coli* and *Klebsiella pneumoniae*
47 isolated in Sweden from a patient transferred one day previously from India.¹ In 2010, a
48 landmark study identified 37 NDM-1 isolates in the UK, and 143 isolates in different parts of
49 India, Pakistan and Bangladesh and demonstrated an epidemiological link to the Indian
50 subcontinent.² In an environmental study conducted in 2010, NDM-1 producing bacteria of
51 multiple species were grown from 12 of 171 seepage samples and two of 50 water samples
52 collected in New Delhi.³ Taken together, the available findings suggest that the Indian
53 subcontinent is an important reservoir for NDM-1. Recently, small numbers of NDM-1-
54 producing *Enterobacteriaceae* or *Acinetobacter* isolates have been identified in the Balkan
55 states (Bosnia, Kosovo, Montenegro and Serbia),⁴⁻⁷ the Middle East,^{8,9} and China¹⁰⁻¹² among
56 patients without obvious links to the Indian subcontinent.

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

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

78

79 **Materials and Methods**

80 **Bacterial strains, identification and antimicrobial susceptibility testing**

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

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

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

100

101 **Carbapenemase gene detection**

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

107

108 **Multilocus sequence typing**

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

111

112 **Plasmid studies**

113 The transferability of *bla*_{NDM} was tested by filter mating *E. coli* J53 Az^r as the
114 recipient. Transconjugants were selected on MacConkey medium containing sodium azide
115 (100 µg/ml) and meropenem (0.5 µg/ml). In each set of experiment, absence of growth of the
116 parent and the recipients in the selective agar plate was confirmed. Plasmid DNA was
117 extracted with QIAGEN Midi Kit (Qiagen, Hilden, Germany) and introduced to competent *E.*
118 *coli* DH5α (Invitrogen, USA) by electroporation, followed by selection of transformants on

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

121 Replicon typing was conducted on transconjugant or transformant with a single
122 plasmid encoding *bla*_{NDM}. The PCR-based replicon typing (PBRT) scheme was used for
123 recognition of the following plasmid incompatibility groups (Inc): FIA, FIB, FIC, FIIA, HI1,
124 HI2, I1-I7, L/M, N, P, W, T, A/C, K, B/O, X, Y, F.²⁹ The IncF plasmids were subtyped by
125 sequencing.³⁰ The revised IncX plasmid replicon typing procedure was used for detection of
126 the IncX1-IncX4 subtypes.³¹ In all the isolates, the replicon location in the plasmids was
127 confirmed by hybridization with probes specific for *bla*_{NDM} and *rep* amplified by PCR from
128 different samples.

129 The plasmids carrying *bla*_{NDM} were further analyzed by restriction fragment length
130 polymorphism (RFLP). Purified plasmid DNA was separately digested with *EcoR1* and *PstI*
131 (Takara, Dalian, China) in accordance with the manufacturer's recommendation.

132

133 **Plasmid sequencing**

134 The complete sequence of the plasmid pNDM-HN380 carrying *bla*_{NDM} in a DH5α
135 transformant (originating from *K. pneumoniae* strain CRE380) was obtained by using the 454
136 GS FLX system (Roche, USA) according to the manufacturer's instruction. Plasmid DNA
137 was prepared as previously described.²⁶ The library yield a total of 70,651 reads with average
138 read length of 500 bp. The reads were assembled by the GS de novo Assembler (version 2.6)
139 into five contigs. The gaps were closed by PCR and Sanger sequencing (Table S1). The
140 complete plasmid sequence was confirmed by comparison of the in silico RFLP and the
141 experimental RFLP using *EcoR1* and *PstI* restriction enzymes. The plasmid was annotated by
142 RAST Server and each predicted open reading frames (ORFs) was further blast against the
143 NCBI non-redundant protein database using BLASTP.³² The WebACT and Mauve (version

144 2.2.0) softwares were used for alignment and comparison of plasmid sequences.^{26,33}
145 XplasMap (version 9.0) was used for construction of a schematic plasmid map.³¹

146

147 **Results**

148 **Patient demographics and strains characteristics**

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

168

169 **RFLP analysis of IncX3 plasmids**

170 The IncX3 plasmids from the seven strains were subjected to RFLP analysis. Six
171 plasmids had identical patterns after *EcoR1* or *Pst1* digestion (Figure 1). The plasmid from *K.*
172 *pneumoniae* strain CRE843 yield results that differed from that for the other strains by two
173 bands for both restriction enzymes.

174

175 **Sequence analysis of pNDM-HN380**

176 The complete sequence of the plasmid, pNDM-HN380 originating from *K.*
177 *pneumoniae* strain CRE380 was obtained (INSDC-GenBank accession JX104760). It is a
178 54,035 bp circular plasmid with an average GC content of 49% and 52 putative open reading
179 frames (ORFs). Figure 2 showed a linear comparison with two other completely sequenced
180 IncX3 plasmids (pEC13_35 and pIncX-SHV). The 30.2 kb backbone structure of pNDM-
181 HN380 is typical of those described for IncX plasmid. The following set of core genes were
182 shared among the three IncX3 plasmids: replication (replication initiation protein, *pir*;
183 replication accessory protein, *bis*), partitioning (*parA*), plasmid maintenance (a putative
184 DNA-binding protein, *hns*; a putative type III topoisomerase, *topB*), conjugation/type IV
185 secretion system (T4SS, with 11 genes, *pilX1* to *pilX11*), transcriptional activator (*actX*) and
186 putative DNA transfer proteins (*taxA* and *taxC*). The 30.2 kb backbone of pNDM-HN380 is
187 highly homologous to pIncX-SHV (100% coverage and 99% nucleotide identity); the
188 similarity with that in pEC14_35 was lower (89% coverage and 98% identity).

189 The genetic load region between the resolvase, *res* gene and the *hns* gene is 23.9 kb in
190 length. This region is mosaic with areas of high and low GC contents, suggesting it arose
191 from multiple genetic events. The genetic load region of pNDM-HN380 contained 22 ORFs,
192 of which nine were found in pIncX-SHV. The nine ORFs with high homology in the two
193 plasmids include one resistance gene (*bla_{SHV}*), three mobile genetic elements (IS26, Tn3 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 *ISAbal25*
199 and *IS26* 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 transposase, *Tn3*. The *ISAbal25* element upstream of *bla*_{NDM-1} was
202 interrupted by an *IS5* element and a 5-bp target site duplication (CCTAA) was identified at
203 the point of insertion between the 5' end of the *IS5* element and the *ISAbal25* 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 *ISAbal25* (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 *IS26* 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 *IS26* element in the *bla*_{NDM-1} downstream region and the interruption of
215 *ISAbal25* by *IS5*), 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).

219

220 **Discussion**

221 The present study revealed the presence of *bla*_{NDM-1} in multiple *Enterobacteriaceae*
222 isolates recovered from returning travelers who have been treated in different parts of China.
223 With the exception of two patients who were of the same family,¹¹ the other patients were not
224 epidemiologically related to each other. Since the isolates were identified by active
225 surveillance upon admission, we concluded that they represent *bla*_{NDM-1} importations. In
226 Hong Kong, a territory-wide surveillance for carbapenemases has been implemented since
227 the last quarter of 2008.²⁶ Beside admission screening of at risk patients, microbiology
228 laboratories routinely refer all carbapenem-resistant *Enterobacteriaceae* (CRE) isolates to a
229 centralized laboratory for molecular testing.²⁶ During the study period, over 500 CRE isolates
230 have been tested by PCR assays. Up to May 2012, a total of ten *bla*_{NDM-1}-carrying isolates,
231 including one previously reported by us,²⁶ were identified. The findings suggest that the
232 spread of *bla*_{NDM-1} in China is much wider than previously realized. Previous studies have
233 identified *bla*_{NDM-1} among clinical isolates of *A. baumannii* and *non-baumannii* isolates in
234 Beijing and six provinces (Guangdong, Zhejiang, Hainan, Anhui, Liaoning, Shandong) from
235 patients without history of foreign travel^{10,12} and in a chicken strain of *A. lwoffii* in Shandong
236 province.³⁴ In Hong Kong, the existing strategy only tests patients with a history of recent
237 hospitalization or surgery abroad, those who have traveled to NDM-endemic countries but
238 without hospitalization are not screened.^{11,26} Given that foreign travel alone has been shown
239 to be an important risk factor for acquisition of antibiotic-resistant enteric bacteria, such as
240 CTX-M producing *Enterobacteriaceae*,^{35,36} the number of NDM-positive patients reported
241 here may be an underestimation.

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

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

269 This is the first characterized *bla*_{NDM-1}-carrying IncX3 plasmid, in which the *bla*_{NDM-1}
270 was identified inside a composite transposon-like structure flanked by IS26 and IS*Aba125*. It
271 seems that the 10.8 kb *bla*_{NDM-1} containing module was integrated *en bloc* into the IncX3
272 resistance load region by a recombination event involving IS26 and possibly the other mobile
273 elements flanking the junctions. Our findings were in agreement with the horizontal transfer
274 of the entire module (comprising the IS*Aba125* fragment with the -35 promoter region, the
275 *bla*_{NDM-1} gene, the bleomycin resistance gene, the truncated *trpF* gene, followed by the *tat*
276 and *dct*, the chaperonin subunits, *groES* and *groEL*, and the transposase, *insE*) from the
277 genus *Acinetobacter* to *Enterobacteriaceae*, as suggested previously.¹⁰ In *Acinetobacter*,
278 transposon Tn*I25* appeared to be the main vehicle for dissemination of *bla*_{NDM-1}.^{10,38} This and
279 previous studies indicates that further transfer to *Enterobacteriaceae* requires other mobile
280 elements, such as IS26 (pMR0211, JN687470; pGUE-NDM, JQ364967; and pNDM-HK,
281 HQ451074), IS903 (pNDM-1_Dok01, AP012208), IS*kpn14* (pNDM-KN, JN157804 and
282 pNDM10505, JF503991), IS1 (pNDM10469, JN861072), IS*Ec33* (p271A, JF785549) and
283 Tn3 (pNDM-MAR, JN420336 and pKpANDM-1, FN396876).^{8,16,18,19,26,39,40} The IncX
284 plasmids were thought to be narrow host range plasmids of *Enterobacteriaceae* but the ability
285 of transfer to *Pseudomonas aeruginosa* has been demonstrated.⁴¹ In the future, it would be
286 interesting to investigate the transferability of IncX plasmids to the genus *Acinetobacter*
287 which would be expected to facilitate the inter-genera flow of resistance genes.

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

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

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

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

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317

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325

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327

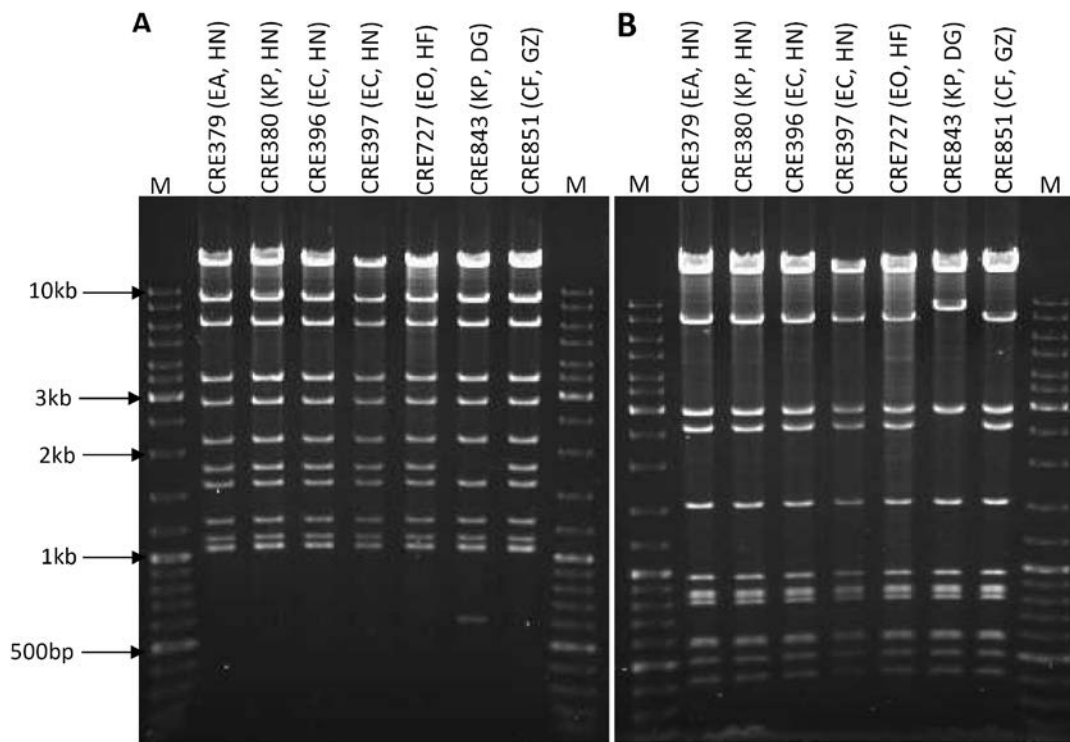
328 Table 1. Patient demographics, bacterial strains and features of plasmids carrying *bla*_{NDM-1}

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	<i>E. coli</i>	Gen, Amk, Sxt, Tet, Cip, Chl	ST101	FII _Y -FIB _S ^c	110
March 2011	CRE851	M/60	Guangzhou	<i>C. freundii</i>	Gen, Amk, Sxt, Cip	-	X3	50
July 2011	CRE379	F/1 ^a	Hunan	<i>E. aerogenes</i>	Fot	-	X3	50
Aug 2011	CRE380	F/1 ^a	Hunan	<i>K. pneumoniae</i>	Gen, Sxt, Tet, Cip, Chl	ST483	X3	50
Aug 2011	CRE396	F/1 ^a	Hunan	<i>E. coli</i>	Sxt, Tet, Cip, Chl	ST744	X3	50
Aug 2011	CRE397	F/26 ^a	Hunan	<i>E. coli</i>	Sxt, Tet, Cip, Chl	ST744	X3	50
Nov 2011	CRE866	M/74	Fujian	<i>E. coli</i>	Gen, Amk, Sxt, Tet, Cip, Chl, Fot	ST101	A/C2	140
Feb 2012	CRE727	M/82	Haifeng	<i>E. cloacae</i>	Gen, Amk, Sxt, Tet, Cip, Chl, Fot	-	X3	50
May 2012	CRE843	M/1	Dongguan	<i>K. pneumoniae</i>	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 ^bFor the following drugs, Gen, gentamicin; Amk, amikacin; Sxt, cotrimoxazole; Tet, tetracycline; Cip, ciprofloxacin; Chl, chloramphenicol; Fot, fosfomycin.331 ^cPositive for both FII_Y (allele Y3) and FIB_S (Salmonella FIB) replicons.³⁰

333 Figure 1. Restriction analysis of IncX3 plasmids carrying *bla*_{NDM-1}. Plasmids were digested
334 with (A) *EcoRI* and (B) *PstI* and separated by electrophoresis in 1% agarose. M,
335 GeneRuler™ DNA ladder. The labels above each lane show the strain number, bacterial
336 species origin (EA, *E. aerogenes*; KP, *K. pneumoniae*; EC, *E. coli*; EO, *E. cloacae*; CF, *C.*
337 *freundii*) and the geographic source of importation (HN, Hunan; HF, Haifeng; DG, Dongguan;
338 GZ, Guangzhou).

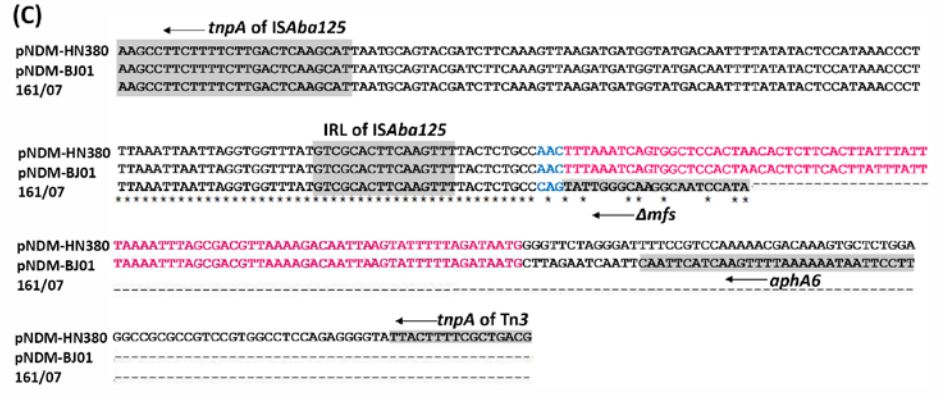
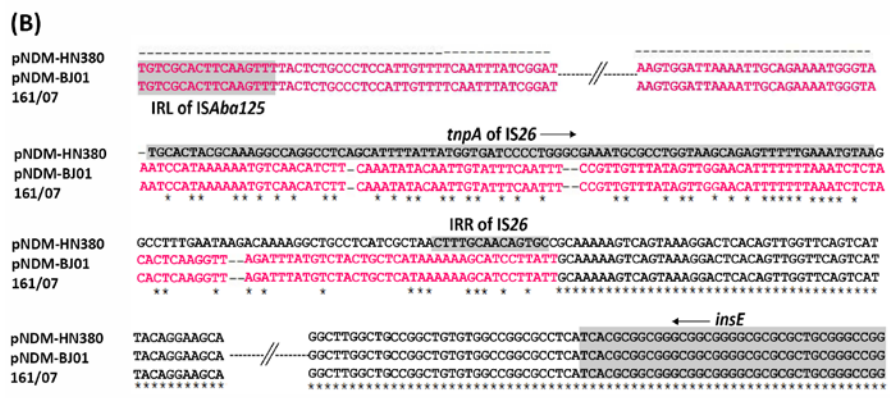
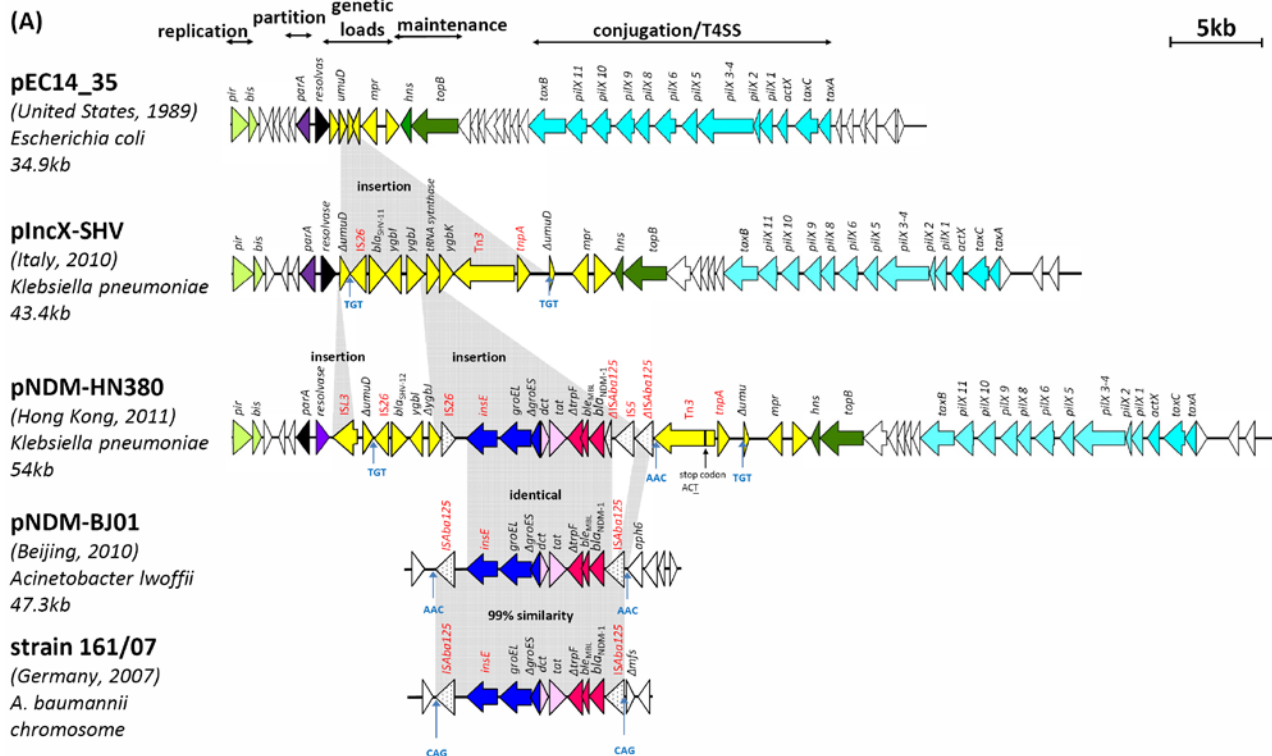
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342 **Figure 2.** Comparative analysis of (A) linear plasmid maps for three IncX3 plasmids, pEC14_35, pIncX-SHV, pNDM-HN380 and two *bla*_{NDM-1}-
343 carrying transposon sequences in pNDM-BJ01 and *A. baumannii* strain 161/07, (B) sequences downstream of *insE* and (C) sequences upstream
344 of the *ISAbal25* in the 5' end of *bla*_{NDM-1}.
345 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
346 ORFs. Homologous ORFs in the plasmid maps are represented in the same colour. Direct repeats and mobile elements are labelled in blue and
347 red, respectively. (B) and (C) Consensus regions in the aligned sequences of pNDM-HN380, pNDM-BJ01 and 161/07 are marked with asterisk.
348 The sequences identical in pNDM-HN380 and pNDM-BJ01 are coloured green. The ORFs are indicated by gray shading and the arrow next to
349 the label indicates the ORF orientation. The accession numbers were: pEC14_35 (JN935899); pIncX-SHV (JN247852); pNDM-HN380
350 (JX104760), pNDM-BJ01 (JQ001791) and *Acinetobacter baumannii* strain 161/07 (HQ857107).
351



353 References

354

355 1. Yong D, Toleman MA, Giske CG *et al*. Characterization of a new metallo-beta-
356 lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a
357 unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India.
358 *Antimicrob Agents Chemother* 2009; **53**, 5046-54.

359 2. Kumarasamy KK, Toleman MA, Walsh TR *et al*. Emergence of a new antibiotic
360 resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and
361 epidemiological study. *Lancet Infect Dis* 2010; **10**, 597-602.

362 3. Walsh TR, Weeks J, Livermore DM, & Toleman MA. Dissemination of NDM-1
363 positive bacteria in the New Delhi environment and its implications for human health:
364 an environmental point prevalence study. *Lancet Infect Dis* 2011; **11**, 355-62.

365 4. Bogaerts P, Bouchahrouf W, de Castro RR *et al*. Emergence of NDM-1-producing
366 Enterobacteriaceae in Belgium. *Antimicrob Agents Chemother* 2011; **55**, 3036-8.

367 5. Halaby T, Reuland AE, Al Naiemi N *et al*. A case of New Delhi metallo-beta-
368 lactamase 1 (NDM-1)-producing *Klebsiella pneumoniae* with putative secondary
369 transmission from the Balkan region in the Netherlands. *Antimicrob Agents*
370 *Chemother* 2012; **56**, 2790-1.

371 6. Mazzariol A, Bosnjak Z, Ballarini P *et al*. NDM-1-producing *Klebsiella pneumoniae*,
372 Croatia. *Emerg Infect Dis* 2012; **18**, 532-4.

373 7. Poirel L, Schrenzel J, Cherkaoui A, Bernabeu S, Renzi G, & Nordmann P. Molecular
374 analysis of NDM-1-producing enterobacterial isolates from Geneva, Switzerland. *J*
375 *Antimicrob Chemother* 2011; **66**, 1730-3.

- 376 8. McGann P, Hang J, Clifford RJ *et al.* Complete sequence of a novel 178-kilobase
377 plasmid carrying bla(NDM-1) in a *Providencia stuartii* strain isolated in Afghanistan.
378 *Antimicrob Agents Chemother* 2012; **56**, 1673-9.
- 379 9. Poirel L, Ozdamar M, Ocampo-Sosa AA, Turkoglu S, Ozer UG, & Nordmann P.
380 NDM-1-producing *Klebsiella pneumoniae* now in Turkey. *Antimicrob Agents*
381 *Chemother* 2012; **56**, 2784-5.
- 382 10. Fu Y, Du X, Ji J, Chen Y, Jiang Y, & Yu Y. Epidemiological characteristics and
383 genetic structure of blaNDM-1 in non-baumannii *Acinetobacter* spp. in China. *J*
384 *Antimicrob Chemother* 2012.
- 385 11. Ho PL, Li Z, Lai EL, Chiu SS, & Cheng VC. Emergence of NDM-1-producing
386 *Enterobacteriaceae* in China. *J Antimicrob Chemother* 2012; **67**, 1553-5.
- 387 12. Hu H, Hu Y, Pan Y *et al.* Novel plasmid and its variant harboring both a bla(NDM-1)
388 gene and type IV secretion system in clinical isolates of *Acinetobacter lwoffii*.
389 *Antimicrob Agents Chemother* 2012; **56**, 1698-702.
- 390 13. Girlich D, Poirel L, & Nordmann P. Diversity of naturally occurring Ambler class B
391 metallo-beta-lactamases in *Erythrobacter* spp. *J Antimicrob Chemother* 2012.
- 392 14. Zheng B, Tan S, Gao J *et al.* An unexpected similarity between antibiotic-resistant
393 NDM-1 and beta-lactamase II from *Erythrobacter litoralis*. *Protein Cell* 2011; **2**, 250-
394 8.
- 395 15. Derzelle S, Duchaud E, Kunst F, Danchin A, & Bertin P. Identification,
396 characterization, and regulation of a cluster of genes involved in carbapenem
397 biosynthesis in *Photobacterium luminescens*. *Appl Environ Microbiol* 2002; **68**, 3780-9.

- 398 16. Sekizuka T, Matsui M, Yamane K *et al.* Complete sequencing of the bla(NDM-1)-
399 positive IncA/C plasmid from *Escherichia coli* ST38 isolate suggests a possible origin
400 from plant pathogens. *PLoS One* 2011; **6**, e25334.
- 401 17. Walsh TR. Emerging carbapenemases: a global perspective. *Int J Antimicrob Agents*
402 2010; **36 Suppl 3**, S8-14.
- 403 18. Villa L, Poirel L, Nordmann P, Carta C, & Carattoli A. Complete sequencing of an
404 IncH plasmid carrying the blaNDM-1, blaCTX-M-15 and qnrB1 genes. *J Antimicrob*
405 *Chemother* 2012.
- 406 19. Poirel L, Bonnin RA, & Nordmann P. Analysis of the resistome of a multidrug-
407 resistant NDM-1-producing *Escherichia coli* strain by high-throughput genome
408 sequencing. *Antimicrob Agents Chemother* 2011; **55**, 4224-9.
- 409 20. Nordmann P, Poirel L, Walsh TR, & Livermore DM. The emerging NDM
410 carbapenemases. *Trends Microbiol* 2011; **19**, 588-95.
- 411 21. Kim MN, Yong D, An D *et al.* Nosocomial clustering of NDM-1-producing
412 *Klebsiella pneumoniae* sequence type 340 strains in four patients at a South Korean
413 tertiary care hospital. *J Clin Microbiol* 2012; **50**, 1433-6.
- 414 22. Cohen SJ & Leverstein-Van Hall MA. Guideline for phenotypic screening and
415 confirmation of carbapenemases in *Enterobacteriaceae*. *Int J Antimicrob Agents* 2010;
416 **36**, 205-10.
- 417 23. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial*
418 *susceptibility testing: twenty-first informational supplement M100-S21*. CLSI,
419 Wayne, PA, USA, 2011.

- 420 24. Endimiani A, Carias LL, Hujer AM *et al.* Presence of plasmid-mediated quinolone
421 resistance in *Klebsiella pneumoniae* isolates possessing blaKPC in the United States.
422 *Antimicrob Agents Chemother* 2008; **52**, 2680-2.
- 423 25. Poirel L, Heritier C, Tolun V, & Nordmann P. Emergence of oxacillinase-mediated
424 resistance to imipenem in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2004;
425 **48**, 15-22.
- 426 26. Ho PL, Lo WU, Yeung MK *et al.* Complete sequencing of pNDM-HK encoding
427 NDM-1 carbapenemase from a multidrug-resistant *Escherichia coli* strain isolated in
428 Hong Kong. *PLoS One* 2011; **6**, e17989.
- 429 27. Diancourt L, Passet V, Verhoef J, Grimont PA, & Brisse S. Multilocus sequence
430 typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol* 2005; **43**,
431 4178-82.
- 432 28. Wirth T, Falush D, Lan R *et al.* Sex and virulence in *Escherichia coli*: an
433 evolutionary perspective. *Mol Microbiol* 2006; **60**, 1136-51.
- 434 29. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, & Threlfall EJ. Identification of
435 plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005; **63**, 219-28.
- 436 30. Villa L, Garcia-Fernandez A, Fortini D, & Carattoli A. Replicon sequence typing of
437 IncF plasmids carrying virulence and resistance determinants. *J Antimicrob*
438 *Chemother* 2010; **65**, 2518-29.
- 439 31. Johnson TJ, Bielak EM, Fortini D *et al.* Expansion of the IncX plasmid family for
440 improved identification and typing of novel plasmids in drug-resistant
441 *Enterobacteriaceae*. *Plasmid* 2012.

- 442 32. Aziz RK, Bartels D, Best AA *et al.* The RAST Server: rapid annotations using
443 subsystems technology. *BMC Genomics* 2008; **9**, 75.
- 444 33. Rissman AI, Mau B, Biehl BS, Darling AE, Glasner JD, & Perna NT. Reordering
445 contigs of draft genomes using the Mauve aligner. *Bioinformatics* 2009; **25**, 2071-3.
- 446 34. Wang Y, Wu C, Zhang Q *et al.* Identification of New Delhi metallo-beta-lactamase 1
447 in *Acinetobacter lwoffii* of food animal origin. *PLoS One* 2012; **7**, e37152.
- 448 35. Tangden T, Cars O, Melhus A, & Lowdin E. Foreign travel is a major risk factor for
449 colonization with *Escherichia coli* producing CTX-M-type extended-spectrum beta-
450 lactamases: a prospective study with Swedish volunteers. *Antimicrob Agents*
451 *Chemother* 2010; **54**, 3564-8.
- 452 36. Kennedy K & Collignon P. Colonisation with *Escherichia coli* resistant to "critically
453 important" antibiotics: a high risk for international travellers. *Eur J Clin Microbiol*
454 *Infect Dis* 2010; **29**, 1501-6.
- 455 37. Denis C, Poirel L, Carricajo A *et al.* Nosocomial transmission of NDM-1-producing
456 *Escherichia coli* within a non-endemic area in France. *Clin Microbiol Infect* 2012; **18**,
457 E128-E130.
- 458 38. Poirel L, Bonnin RA, Boulanger A, Schrenzel J, Kaase M, & Nordmann P. Tn125-
459 related acquisition of blaNDM-like genes in *Acinetobacter baumannii*. *Antimicrob*
460 *Agents Chemother* 2012; **56**, 1087-9.
- 461 39. Bonnin RA, Poirel L, Carattoli A, & Nordmann P. Characterization of an IncFII
462 plasmid encoding NDM-1 from *Escherichia coli* ST131. *PLoS One* 2012; **7**, e34752.

- 463 40. Carattoli A, Villa L, Poirel L, Bonnin RA, & Nordmann P. Evolution of IncA/C
464 blaCMY-2-carrying plasmids by acquisition of the blaNDM-1 carbapenemase gene.
465 *Antimicrob Agents Chemother* 2012; **56**, 783-6.
- 466 41. Tardif G & Grant RB. Transfer of plasmids from *Escherichia coli* to *Pseudomonas*
467 *aeruginosa*: characterization of a *Pseudomonas aeruginosa* mutant with enhanced
468 recipient ability for enterobacterial plasmids. *Antimicrob Agents Chemother* 1983; **24**,
469 201-8.
- 470 42. Norman A, Hansen LH, She Q, & Sorensen SJ. Nucleotide sequence of pOLA52: a
471 conjugative IncX1 plasmid from *Escherichia coli* which enables biofilm formation
472 and multidrug efflux. *Plasmid* 2008; **60**, 59-74.
- 473 43. Ko M & Park C. Two novel flagellar components and H-NS are involved in the motor
474 function of *Escherichia coli*. *J Mol Biol* 2000; **303**, 371-82.
- 475 44. Dorman CJ. H-NS, the genome sentinel. *Nat Rev Microbiol* 2007; **5**, 157-61.
- 476 45. Doyle M, Fookes M, Ivens A, Mangan MW, Wain J, & Dorman CJ. An H-NS-like
477 stealth protein aids horizontal DNA transmission in bacteria. *Science* 2007; **315**, 251-
478 2.
- 479
480
481