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Diverse flanking sequence types of *bla*$_{NDM}$ in IncX3 and IncB/O/K/Z plasmids in *Escherichia coli* isolated from poultry

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Abstract We identified 33 *bla*$_{NDM}$-harboring *Escherichia coli* in 470 poultry samples from Shandong and Guangdong provinces during 2016 to 2017 and included the subtypes *bla*$_{NDM-1}$, *bla*$_{NDM-5}$ and *bla*$_{NDM-9}$. The *bla*$_{NDM}$ gene possessed by all strains was plasmid-borne and ranged in size from 46 to 265 kb. The plasmids included the replicon types IncX3, IncY, IncB/O/K/Z and IncHI2 and most were transferable and stable in recipient host bacteria. Sequences flanking *bla*$_{NDM}$ in IncX3 and IncB/O/K/Z plasmids were diverse and the mobile element IS*26* played an important role in the evolution of the IncB/O/K/Z *bla*$_{NDM}$ plasmids.

Keywords: carbapenems, *bla*$_{NDM}$, *Escherichia coli*, plasmids, fitness cost, genetic environment

Transmissible carbapenem-resistance and its dissemination in the *Enterobacteriaceae* have posed a major threat to global public health (1). In the existing 3 classes of carbapenemases, New Delhi metallo-beta-lactamases (NDM) encoded by *bla*$_{NDM}$ is most abundant globally and its presence is increasing at an alarming rate in Asia (2, 3). The *bla*$_{NDM}$ gene has been found primarily on plasmids with numerous replicon types including IncF, IncL/M, IncA/C, IncX, IncH, IncN,

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IncR, IncB/O and IncT (4, 5). In addition, this gene is often flanked by mobile genetic elements such as insertion sequences and transposons that are responsible for horizontal genetic exchange and therefore promote the acquisition and spread of resistance genes (4). The presence of carbapenemase-producing *Enterobacteriaceae* (CPE) in livestock animals is of particular concern because this may facilitate gene pool expansion from which pathogenic bacteria can pick up resistance genes, and consumers may subsequently be exposed through the food chain (6). The presence of *bla*$_{NDM}$-harboring isolates is rapidly increasing among food animals in China and this is most likely related to the presence of *bla*$_{NDM}$-harboring plasmids (7-13). We therefore examined *bla*$_{NDM}$-harboring plasmids from poultry in Guangdong and Shandong provinces in China.

We collected 470 organ samples from five unrelated areas during 2016 in Shandong and 2017 in Guangdong (Table S1). *Escherichia coli* strains were isolated on MacConkey agar plates and were further identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequencing (14). The *bla*$_{NDM}$ gene was identified using the PCR method as previously described (15, 16). We found 33 *bla*$_{NDM}$-harboring *E. coli* strains in our sample group and 9 were from Shandong and 24 were from Guangdong. Of the 33 strains, three *bla*$_{NDM}$ subtypes were identified including *bla*$_{NDM-1}$ (1/33), *bla*$_{NDM-5}$ (24/33) and *bla*$_{NDM-9}$ (8/33) (Fig. 1). Antimicrobial susceptibilities of these strains were determined using the agar dilution method according to CLSI guidelines. All 33 isolates were resistant to at least four classes of antibiotics including carbapenems (Table S2). Additional PCR screening for the presence of other resistance genes demonstrated that all *bla*$_{NDM}$-harboring *E. coli* co-harbored other resistance genes and 10 of which co-harbored polymyxin resistance gene *mcr-1* (Table S3 and Fig 1). Pulsed-field gel electrophoresis (PFGE) analysis identified the presence of 19 clusters

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at 85% identity (17) (Fig 1).

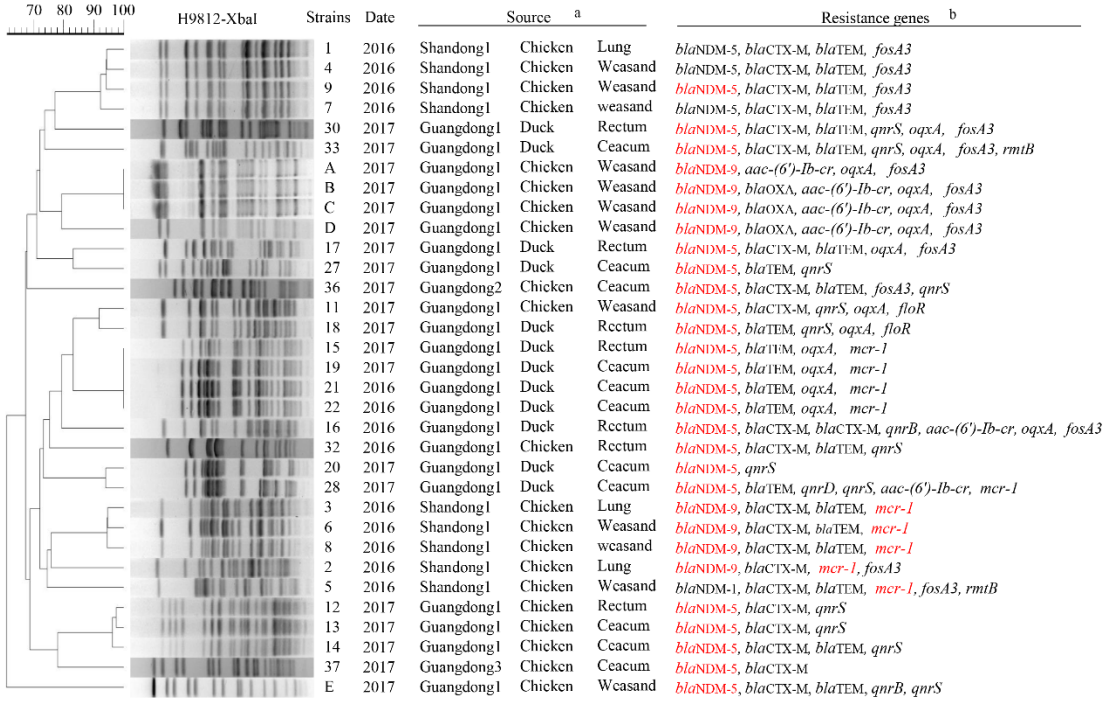

Figure 1. PFGE patterns and resistance genes of 33 *bla*$_{NDM}$-harboring *E. coli* strains. [a] Shandong1, Guangdong1, Guangdong2 and Guangdong3 represent four different areas in Shandong and Guangdong provinces. [b] Genes shown in red color represent that it could be transferred through conjugation.

Conjugation assays were performed with the 33 *bla*$_{NDM}$ strains as previously described using *E. coli* J53 as the recipient (18). Transconjugants harboring *bla*$_{NDM}$ and *mcr-1* were selected on MacConkey agar / sodium azide (150 μg/mL) plates that also contained either meropenem (0.3 μg/mL) or colistin (0.5 μg/mL), respectively. All transconjugants were confirmed by PCR and Enterobacterial repetitive intergenic consensus (ERIC) PCR assays (19). The *bla*$_{NDM}$ gene from 29/33 strains was successfully transferred, as was *mcr-1* in 5/10 of the *mcr-1*-harboring strains (Fig. 1 and Table 1). The plasmids carrying *bla*$_{NDM}$ were characterized by PCR-based replicon typing (PBRT) (20, 21), S1 nuclease PFGE and Southern hybridization (18, 22). The *bla*$_{NDM}$ genes were all located on plasmids of different replicon types that included IncB/O, IncY, IncX3 and

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IncHI2 with sizes from 46 to 265 kb (Table 1).

The fitness of different *bla*$_{NDM}$-harboring plasmids was tested *via* stability and competition experiments as previously described (23, 24). Plasmid DNA from transconjugants were extracted using plasmid minikits (Qiagen, Hilden, Germany) and then electroporated into *E. coli* DH5α except for the plasmid from strain 33 that failed. Each transformant was confirmed to contain a single plasmid by S1 nuclease PFGE. Transconjugants harboring *bla*$_{NDM}$ were selected on Luria-Bertani agar plates containing cefotaxime (1 μg/mL) and incubated 8 days for stability assays and 12 days for competition assays. Except for the replicon HI2 type plasmids, the remainders were stable and persistent in their host strains without reduced fitness (Table 1), these are conditions conducive to the dissemination of *bla*$_{NDM}$.

Table 1. Characteristics of *bla*$_{NDM}$-harboring plasmids among *E. coli* isolates

Replicon type	Size (~kb)	*bla*$_{NDM}$	Conjugation transfer	Conjugation frequency [a]	Host strains [b]	Plasmid stability	Relative competitive fitness	
							Value	SD [c]
IncB/O/K/Z	105	-9	+	1.93×10^{-2}	2	100%	1.36	0.0875
IncB/O/K/Z	115	-9	+	8.62×10^{-4}	3, 6, 8	100%	1.21	0.1420
IncY	120	-1	-	N.A	5	100%	0.99	0.0128
IncX3	46	-5	-	N.A	1, 4, 7	100%	0.98	0.0427
IncX3	46	-5	+	7.14×10^{-4}	9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 27, 28, 30, 32, E, 36, 37	100%	0.98	0.0369
IncHI2	265	-5	+	N.D	33	N,D	N.D	N.D
IncHI2	260	-9	+	7.59×10^{-6}	A, B, C, D	93.3%	0.84	0.0894

[a] N.D, not detected. N.A, not applicable. [b] Flanking sequences of *bla*$_{NDM}$ in strains 3 and 8 were similar to each other but differed from isolate 6. Flanking sequences of *bla*$_{NDM}$ in strains 15, 19, 21, 22 were also similar to each other. The flanking sequences of *bla*$_{NDM}$ in the other IncX3 plasmid-harboring strains were similar. [c] SD, standard deviation.

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We sequenced the *bla*$_{NDM}$-harboring plasmids of the replicon IncB/O type (105 and 115 kb) and the IncX3 type using the Illumina Hiseq platform (Majorbio, Shanghai) and assembled data using SOAP denovo. Gaps were closed through PCR and Sanger sequencing. Plasmid sequences were analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and plasmid replicon types were analyzed using the PlasmidFinder tool (https://cge.cbs.dtu.dk/services/ PlasmidFinder/). PCR primers were designed to detect the *bla*$_{NDM}$ genetic environment of similar plasmids (Tables S4 and S5).

Two types of sequences flanking *bla*$_{NDM}$ were found in the IncX3 plasmids that were assigned the names pNDM-T16 and pNDM-T21. Plasmid pNDM-T16 was 46161 bp and almost 100% identical to plasmid pNDM5_IncX3 (KU761328) with only 6 single-base changes, in which the IS*Aba125* sequence adjacent to *bla*$_{NDM-5}$ was interrupted by IS*5* and split into two fragments. The sequence of pNDM-T21 has not been reported previously and downstream of IS*5* was a truncated IS*3000* transposase gene that most likely was the product of intramolecular recombination (Figure S2). This discovery gives further evidence for the diversity of the sequence types for IncX3 *bla*$_{NDM}$-harboring plasmids as reported previously (25-27).

The IncB/O plasmids were identified as IncB/O/K/Z plasmids using the PlasmidFinder tool. Three kinds of sequences flanking *bla*$_{NDM}$ were found and the plasmids were assigned the names pNDM-T2, pNDM-T3 and pNDM-T6. The major genetic organization surrounding *bla*$_{NDM-9}$ in these plasmids was similar to that in the plasmids pHNTH02-1 (MG196294) and p5CRE51-NDM-9 (CP021177). All 5 plasmids possessed IS*26*-*intI1*-*dfrA12*-*qacEΔ1*-*sul1* -IS*CR1*-(ΔIS*1294*)-Δ*dct*-*tat*-*trpF*-*ble*$_{MBL}$-*bla*$_{NDM}$-ΔIS*Aba125*-IS*26* and this IS*26* composite transposon was most likely responsible for *bla*$_{NDM}$ transfer (28) (Fig. 2).

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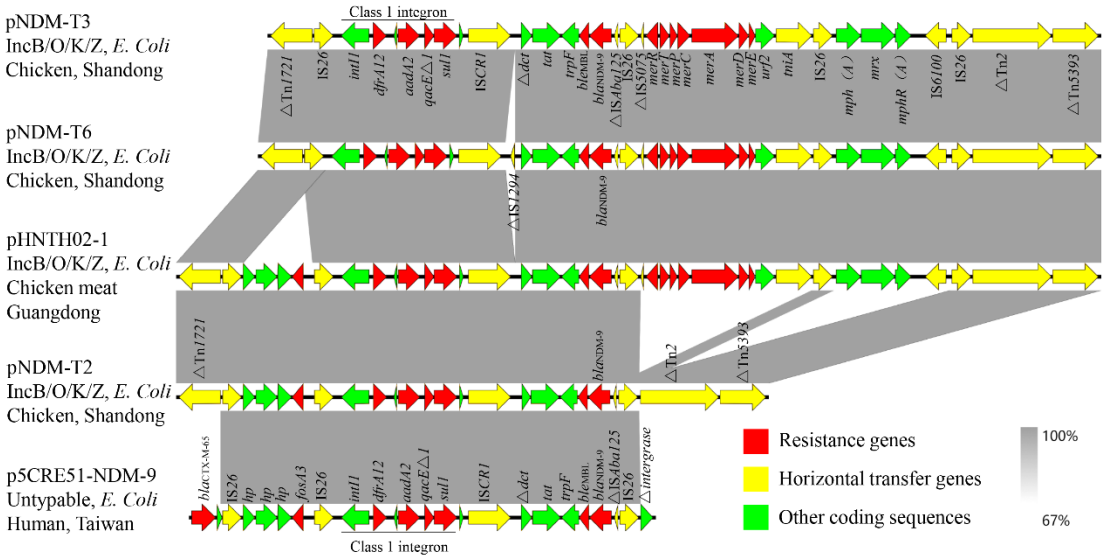

Figure 2. Comparison of the *bla*$_{NDM}$ genetic environment in pNDMT-2 and pNDMT-6 with other plasmids.

The sequences surrounding the *bla*$_{NDM-9}$ genes were diverse and was most likely the result of the action of the IS*26* mobile element. We therefore designed specific PCR primers to detect circular intermediates (circle A-D) and other possible structures (structure A-G) mediated by IS*26* in three IncB/O/K/Z plasmids (Table S6 and Fig3). The PCR products were confirmed by Sanger sequencing and we found only one cyclic intermediate formed by IS*26*-*mph(A)*-*mrx*-*mphR(A)*-IS*6100*-IS*26*. Interestingly, the A-G structures were all detected although no direct repeats were found adjacent to the IS*26* insertions. These results demonstrated there were various plasmids possessed different genetic organizations surrounding *bla*$_{NDM-9}$ in the host bacteria, and all were derived from a larger plasmid *via* IS*26* transposition. Remarkably, even though the circular intermediates containing *bla*$_{NDM}$ were not detected in these plasmids, IS*26*-*intI1*-*dfrA12*-*qacEΔ1*-*sul1*-IS*CR1*-Δ*dct*-*tat*-*trpF*-*ble*$_{MBL}$-*bla*$_{NDM}$-ΔIS*Aba125*-IS*26* has also been found in IncA/C2 (pNDM-2248, CP021177), IncHI2-N (pC629, CP015725) and IncFII(Y)-like (pKPGJ-1a, CP017283.1) plasmids that originated from *E. coli*, *Salmonella* Indiana, and *Klebsiella variicola*,

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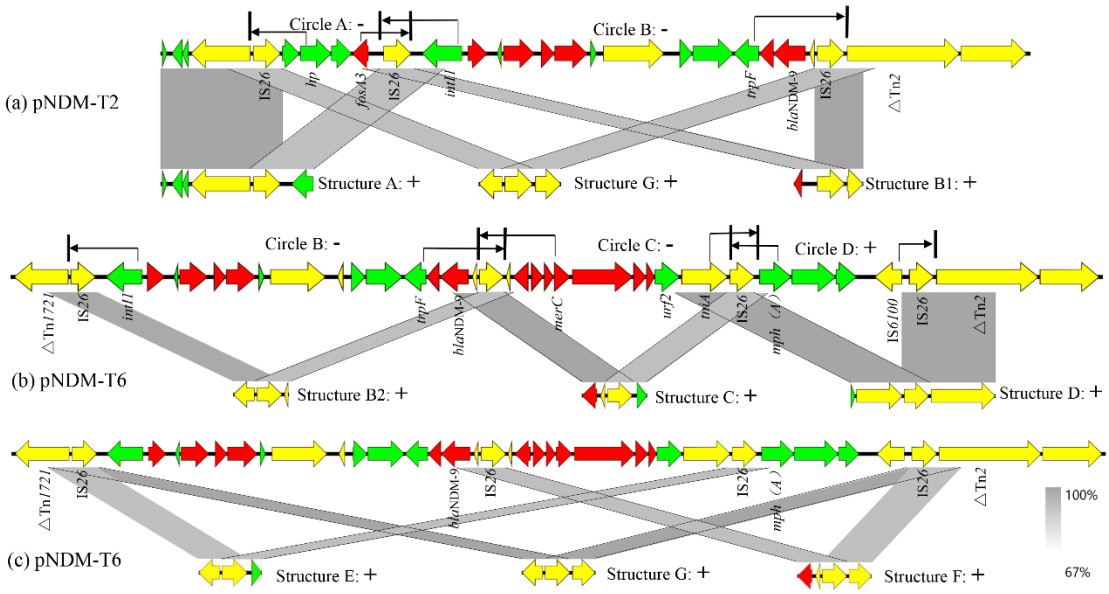
114 respectively (28, 29). This suggests the possible transfer of *bla*$_{NDM}$ between different plasmids

115 mediated through IS*26*-mediated composite transposon.



116

117 **Figure 3**. Circular intermediates and possible structures present in the IncB/O/K/Z plasmids found

118 in this study.

119 In conclusion, this study demonstrated that the dissemination of *bla*$_{NDM}$ among poultry is

120 closely related to diverse plasmids, especially the IncX3 and IncB/O/K/Z conjugative plasmids.

121 The sequences flanking *bla*$_{NDM}$ in IncB/O/K/Z as well as in IncX3 plasmids, tended to be diverse

122 and was most likely the result of intramolecular recombination and frequent activity of mobile

123 elements such as IS*26*. These events resulted in new types of *bla*$_{NDM}$-harboring plasmids and are

124 related to the process of evolution for the *bla*$_{NDM}$-harboring plasmids over time. Given the

125 continued spread of *bla*$_{NDM}$ gene and the significant role that mobile elements and cyclic

126 intermediates played in the evolution and dissemination of resistance plasmids (2, 30), further

127 studies concerning the molecular mechanisms behind the dissemination of *bla*$_{NDM}$ are urgently

128 needed.

129

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Accession numbers The complete nucleotide sequences of the three *bla*$_{NDM}$-carrying plasmids characterized in this study were submitted to GenBank under accession numbers MN335919 (pNDM-T2), MN335921 (pNDM-T6), MN335922 (pNDM-T16), and the annotated sequences of the *bla*$_{NDM}$ genetic environment identified from strain 3 and strain 21 have been submitted to GenBank under accession number MN335920 (pNDM-T3), MN307121 (pNDM-T21), respectively.

SUPPLEMENTAL MATERIAL

Supplemental file 1, PDF file, ……MB.

ACKNOWLEDGMENTS

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FUNDING INFORMATION

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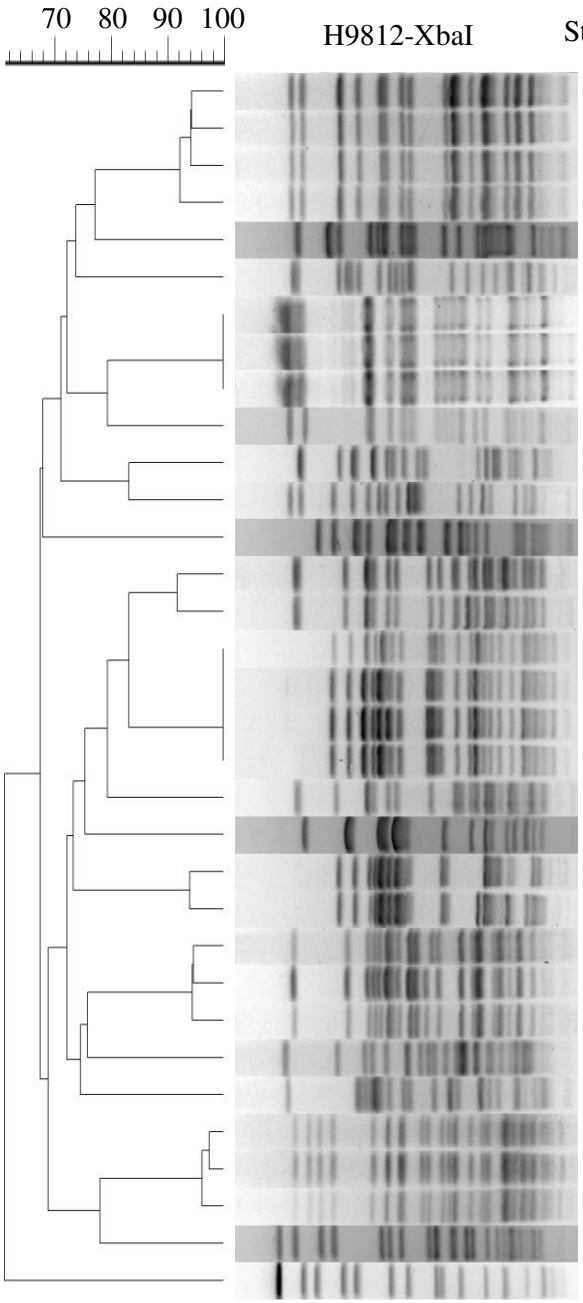
Figure 1



H9812-XbaI	Strains	Date	Source [a]			Resistance genes [b]
	1	2016	Shandong1	Chicken	Lung	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *fosA3*
	4	2016	Shandong1	Chicken	Weasand	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *fosA3*
	9	2016	Shandong1	Chicken	Weasand	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *fosA3*
	7	2016	Shandong1	Chicken	weasand	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *fosA3*
	30	2017	Guangdong1	Duck	Rectum	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *qnrS*, *oqxA*, *fosA3*
	33	2017	Guangdong1	Duck	Ceacum	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *qnrS*, *oqxA*, *fosA3*, *rmtB*
	A	2017	Guangdong1	Chicken	Weasand	*bla*NDM-9, *aac-(6')-Ib-cr*, *oqxA*, *fosA3*
	B	2017	Guangdong1	Chicken	Weasand	*bla*NDM-9, *bla*OXA, *aac-(6')-Ib-cr*, *oqxA*, *fosA3*
	C	2017	Guangdong1	Chicken	Weasand	*bla*NDM-9, *bla*OXA, *aac-(6')-Ib-cr*, *oqxA*, *fosA3*
	D	2017	Guangdong1	Chicken	Weasand	*bla*NDM-9, *bla*OXA, *aac-(6')-Ib-cr*, *oqxA*, *fosA3*
	17	2017	Guangdong1	Duck	Rectum	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *oqxA*, *fosA3*
	27	2017	Guangdong1	Duck	Ceacum	*bla*NDM-5, *bla*TEM, *qnrS*
	36	2017	Guangdong2	Chicken	Ceacum	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *fosA3*, *qnrS*
	11	2017	Guangdong1	Chicken	Weasand	*bla*NDM-5, *bla*CTX-M, *qnrS*, *oqxA*, *floR*
	18	2017	Guangdong1	Duck	Rectum	*bla*NDM-5, *bla*TEM, *qnrS*, *oqxA*, *floR*
	15	2017	Guangdong1	Duck	Rectum	*bla*NDM-5, *bla*TEM, *oqxA*, *mcr-1*
	19	2017	Guangdong1	Duck	Ceacum	*bla*NDM-5, *bla*TEM, *oqxA*, *mcr-1*
	21	2016	Guangdong1	Duck	Ceacum	*bla*NDM-5, *bla*TEM, *oqxA*, *mcr-1*
	22	2016	Guangdong1	Duck	Ceacum	*bla*NDM-5, *bla*TEM, *oqxA*, *mcr-1*
	16	2016	Guangdong1	Duck	Rectum	*bla*NDM-5, *bla*CTX-M, *bla*CTX-M, *qnrB*, *aac-(6')-Ib-cr*, *oqxA*, *fosA3*
	32	2016	Guangdong1	Chicken	Rectum	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *qnrS*
	20	2017	Guangdong1	Duck	Ceacum	*bla*NDM-5, *qnrS*
	28	2017	Guangdong1	Duck	Ceacum	*bla*NDM-5, *bla*TEM, *qnrD*, *qnrS*, *aac-(6')-Ib-cr*, *mcr-1*
	3	2016	Shandong1	Chicken	Lung	*bla*NDM-9, *bla*CTX-M, *bla*TEM, *mcr-1*
	6	2016	Shandong1	Chicken	Weasand	*bla*NDM-9, *bla*CTX-M, *bla*TEM, *mcr-1*
	8	2016	Shandong1	Chicken	weasand	*bla*NDM-9, *bla*CTX-M, *bla*TEM, *mcr-1*
	2	2016	Shandong1	Chicken	Lung	*bla*NDM-9, *bla*CTX-M, *mcr-1*, *fosA3*
	5	2016	Shandong1	Chicken	Weasand	*bla*NDM-1, *bla*CTX-M, *bla*TEM, *mcr-1*, *fosA3*, *rmtB*
	12	2017	Guangdong1	Chicken	Rectum	*bla*NDM-5, *bla*CTX-M, *qnrS*
	13	2017	Guangdong1	Chicken	Ceacum	*bla*NDM-5, *bla*CTX-M, *qnrS*
	14	2017	Guangdong1	Chicken	Ceacum	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *qnrS*
	37	2017	Guangdong3	Chicken	Ceacum	*bla*NDM-5, *bla*CTX-M
	E	2017	Guangdong1	Chicken	Weasand	*bla*NDM-5, *bla*CTX-M, *bla*TEM, *qnrB*, *qnrS*

Figure 2

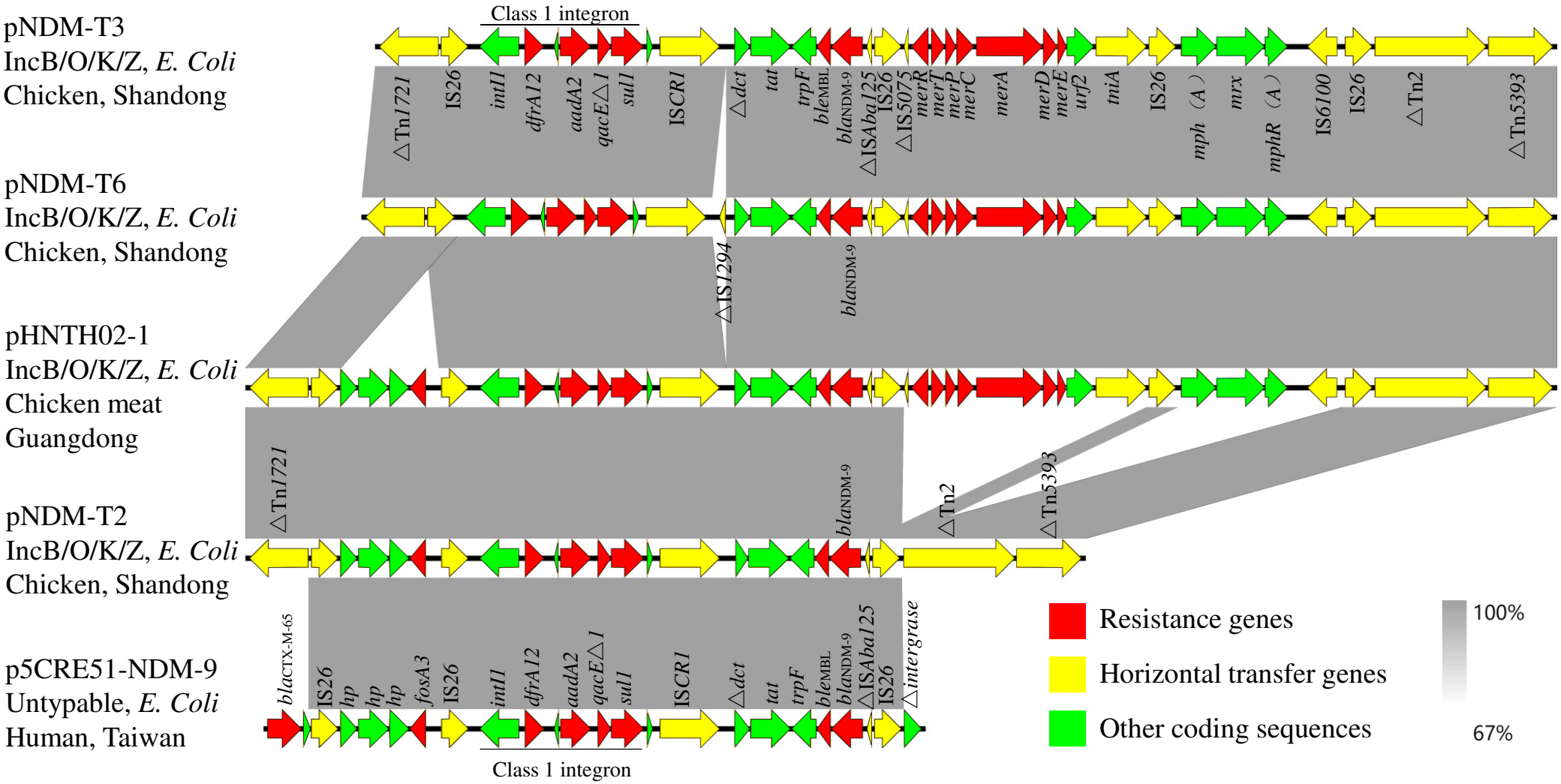

pNDM-T3
IncB/O/K/Z, E. Coli
Chicken, Shandong
pNDM-T6
IncB/O/K/Z, E. Coli
Chicken, Shandong
pHNTH02-1
IncB/O/K/Z, E. Coli
Chicken meat
Guangdong
pNDM-T2
IncB/O/K/Z, E. Coli
Chicken, Shandong
p5CRE51-NDM-9
Untypable, E. Coli
Human, Taiwan
Class 1 integron
△Tn1721
IS26
intI1
dfrA12
aadA2
qacE△1
sul1
ISCR1
△dct
tat
trpF
bleMBL
blaNDM-9
△ISAba125
IS26
△IS5075
merR
merT
merP
merC
merA
merD
merE
urf2
tniA
IS26
mph（A）
mrx
mphR（A）
IS6100
IS26
△Tn2
△Tn5393
△IS1294
blaNDM-9
△Tn1721
blaNDM-9
△Tn2
△Tn5393
blaCTX-M-65
IS26
hp
hp
hp
fosA3
IS26
intI1
dfrA12
aadA2
qacE△1
sul1
ISCR1
△dct
tat
trpF
bleMBL
blaNDM-9
△ISAba125
IS26
△intergrase
Class 1 integron
Resistance genes
Horizontal transfer genes
Other coding sequences
100%
67%

Figure 3

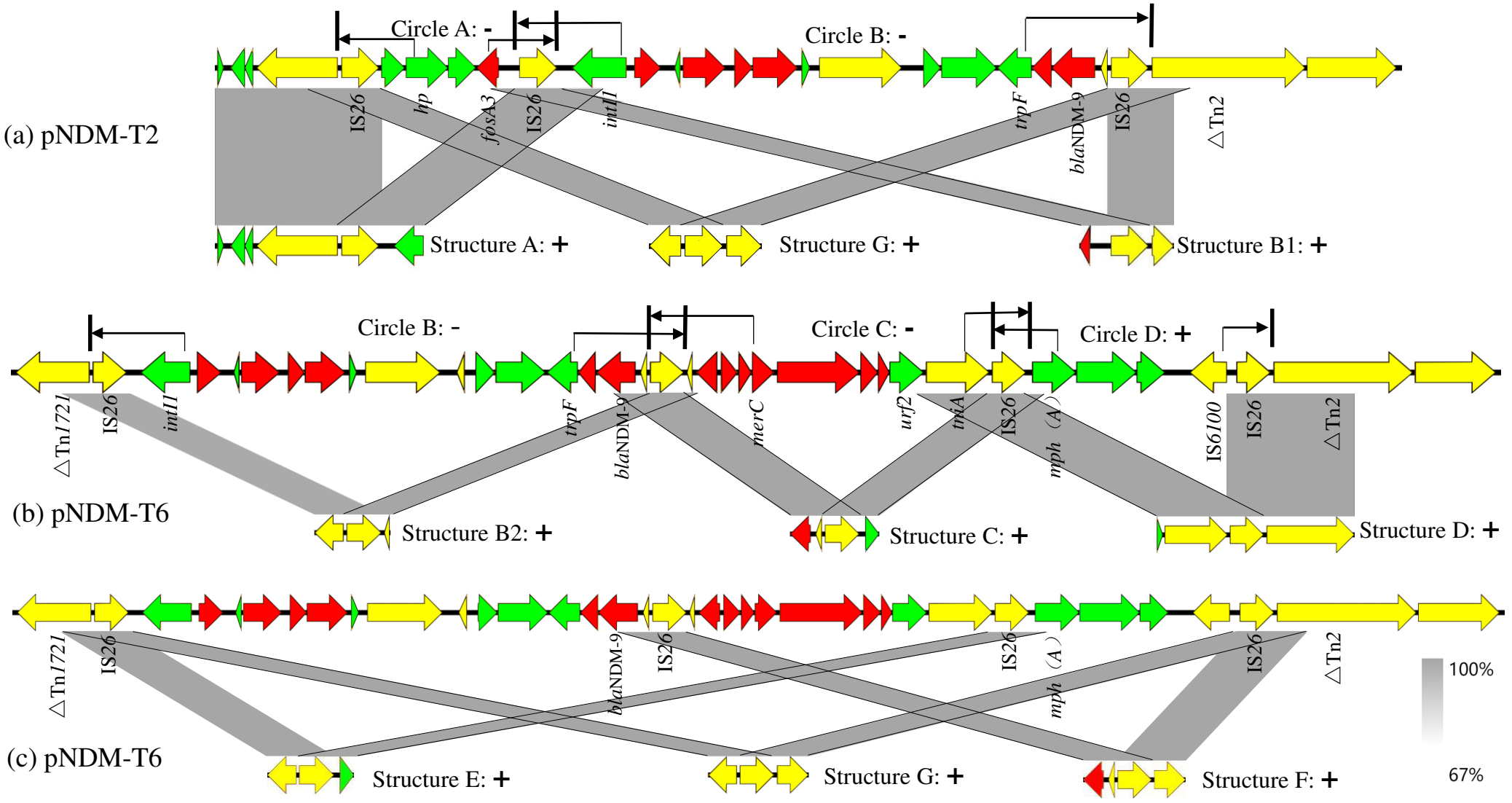

(a) pNDM-T2
Circle A: -
Circle B: -
IS26
hp
fosA3
IS26
intI1
tnpF
blaNDM-9
IS26
△Tn2
Structure A: +
Structure G: +
Structure B1: +
(b) pNDM-T6
Circle B: -
Circle C: -
Circle D: +
△Tn1721
IS26
intI1
tnpF
blaNDM-9
merC
urf2
tniA
IS26
mph（A）
IS6100
IS26
△Tn2
Structure B2: +
Structure C: +
Structure D: +
(c) pNDM-T6
△Tn1721
IS26
blaNDM-9
IS26
IS26
mph（A）
IS26
△Tn2
100%
67%
Structure E: +
Structure G: +
Structure F: +

Figure S1

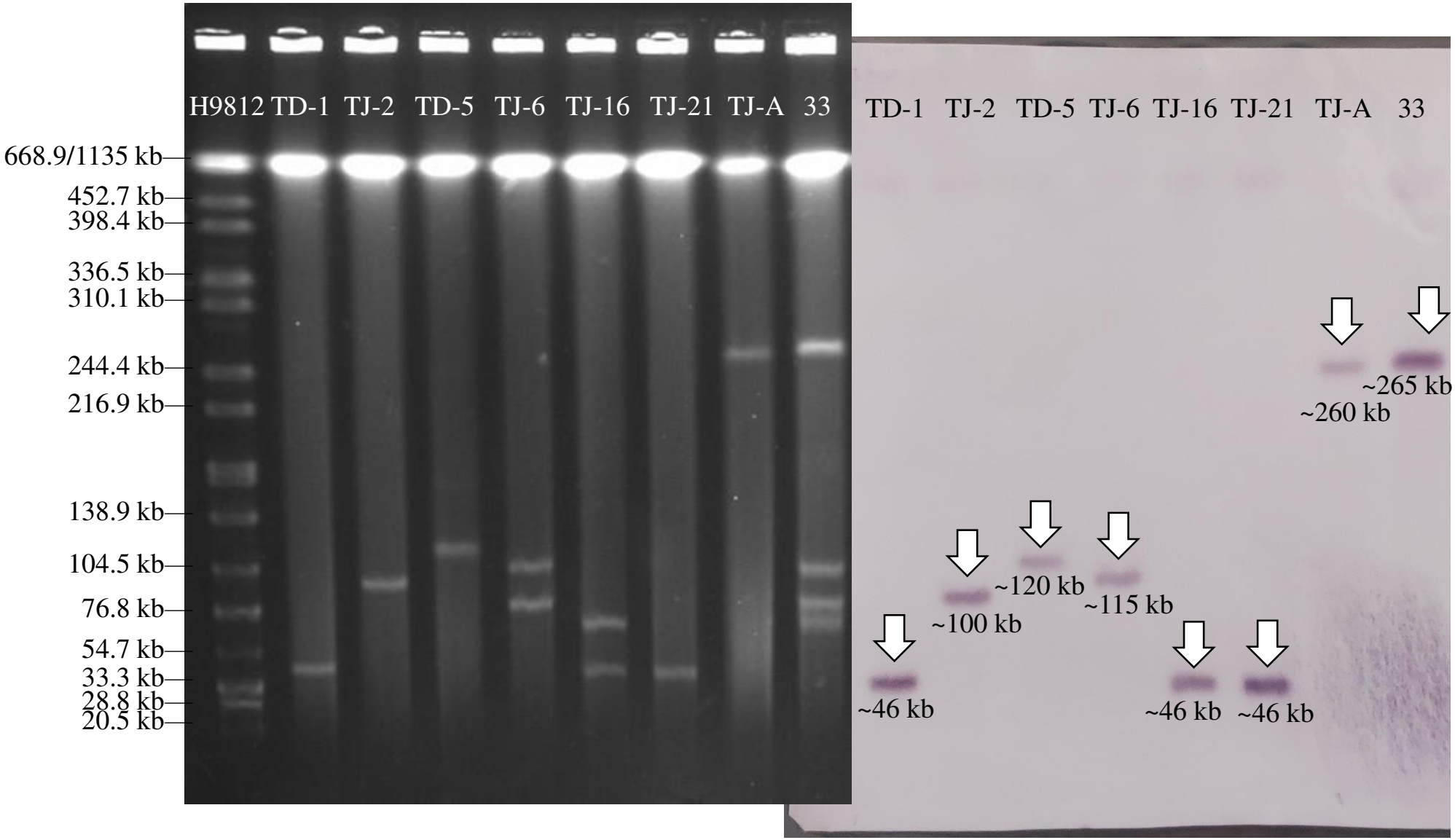

H9812
TD-1
TJ-2
TD-5
TJ-6
TJ-16
TJ-21
TJ-A
33
668.9/1135 kb
452.7 kb
398.4 kb
336.5 kb
310.1 kb
244.4 kb
216.9 kb
138.9 kb
104.5 kb
76.8 kb
54.7 kb
33.3 kb
28.8 kb
20.5 kb
TD-1
TJ-2
TD-5
TJ-6
TJ-16
TJ-21
TJ-A
33
~46 kb
~100 kb
~120 kb
~115 kb
~46 kb
~46 kb
~260 kb
~265 kb

Figure S2

pNDM-HN380
JX104760, IncX3
K. Pneumoniae, human
Hong Kong

pCRENT-193_2
CP024814, IncX3
Enterobacter sp, human
South Korea

pNDM-T16
MN335922, IncX3
E. Coli, Chicken
Guangdong

pNDM-HK3774
MH234502, IncX3
E. Coli, human
HongKong

pNDM-T21
MN307121, IncX3
E. Coli, Chicken
Guangdong

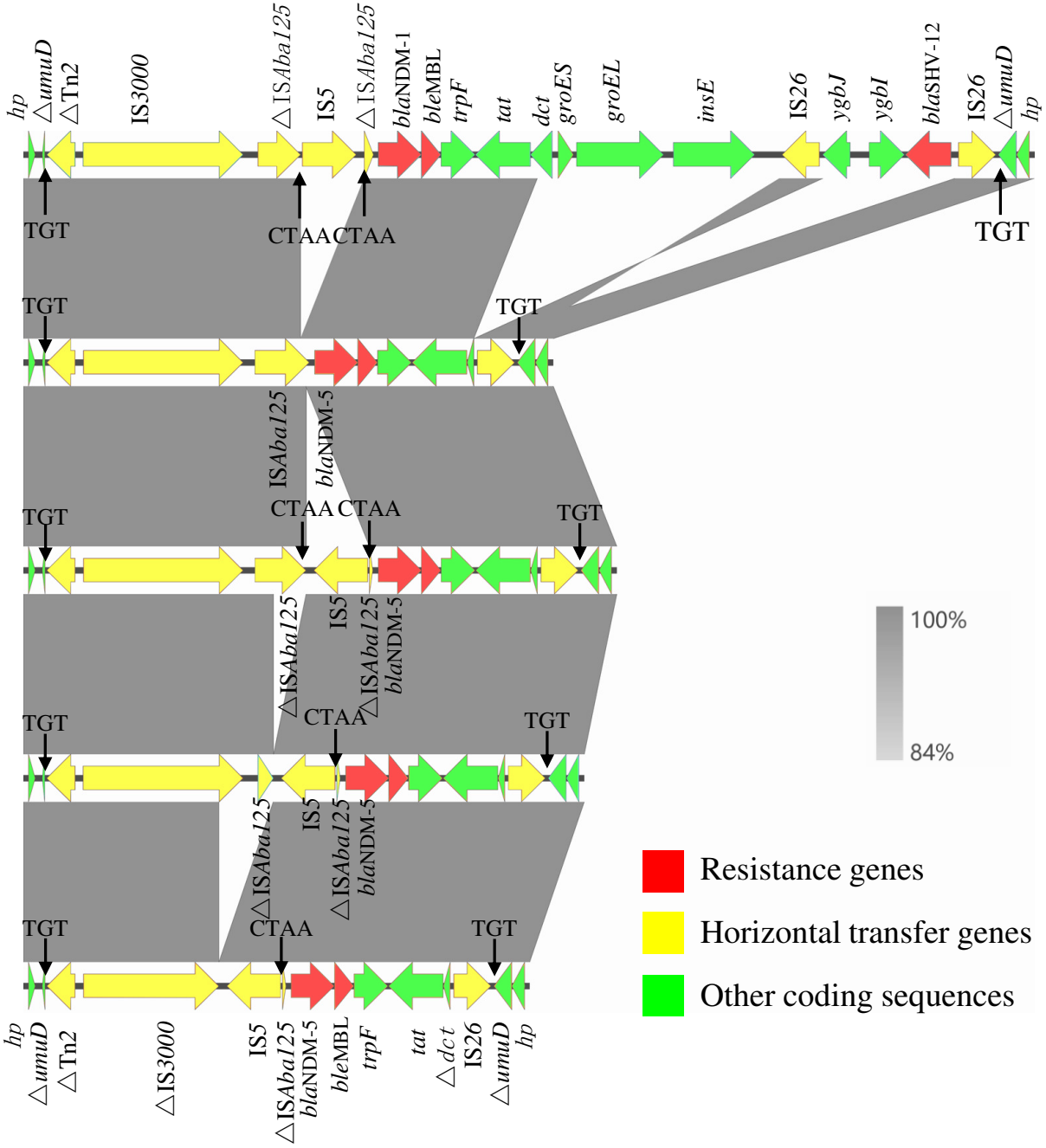
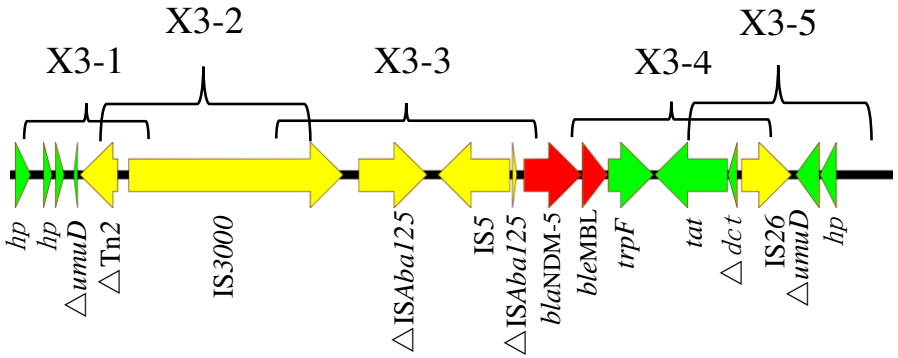


Figure S3

(a) pNDM-T16



(b) pNDM-T6