AAC Accepted Manuscript Posted Online 8 January 2018
Antimicrob. Agents Chemother. doi:10.1128/AAC.02295-17
Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Accepted Manuscript Posted Online
Antimicrobial Agents and Chemotherapy
AAC
Antimicrobial Agents and Chemotherapy

Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

1 **AAC02295-17 Revised**

2 **Short-form paper**

3 **An IncX3 epidemic plasmid carrying *bla*$_{NDM-5}$ in *Escherichia coli* from swine in multiple**
4 **geographic areas in China**

5

6 **Pak-Leung Ho*, Ya Wang, Melissa Chun-Jiao Liu, Eileen Ling-Yi Lai, Pierra Ying-Tung Law,**
7 **Huiluo Cao, Kin-Hung Chow**

8 *Carol Yu Center for Infection and Department of Microbiology, University of Hong Kong, Hong*
9 *Kong, People's Republic of China;*

10

11 Running title: Epidemic IncX3 plasmid carrying *bla*$_{NDM-5}$

12 Keywords: carbapenems, antimicrobial resistance epidemiology, molecular epidemiology,
13 Enterobacteriaceae

14 Address for correspondence:

15 Pak-Leung Ho, Department of Microbiology, Queen Mary Hospital, The University of Hong
16 Kong, Pokfulam Road, Pokfulam, Hong Kong, People's Republic of China

17 Fax: 852–2855-1241; Tel: 852-2255-4892; E-mail: plho@hku.hk

18

Accepted Manuscript Posted Online

Six imported pigs originating from Guangdong, Henan, and Hunan provinces in China during October 2015-February 2017 were culture positive for meropenem-resistant *Escherichia coli*. The samples yielded 9 *E. coli* isolates of diverse sequence types carrying bla_{NDM-5} on IncX3 (8 isolates from 5 farms) or IncFII (1 isolate from 1 farm) plasmids. The *mcr-1* gene was co-harboured by four isolates. The IncX3 plasmids (~46 kb) carrying bla_{NDM-5} were identical or nearly identical to each other.

(74 words)

Antimicrobial Agents and Chemotherapy

AAC

Antimicrobial Agents and Chemotherapy

Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

Accepted Manuscript Posted Online

Antimicrobial Agents and Chemotherapy

AAC

Antimicrobial Agents and Chemotherapy

Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

The presence of carbapenemase-producing *Enterobacteriaceae* (CPE) in livestock animals is concerning as this may facilitate expansion of the gene pool from which pathogenic bacteria can pick up the resistance genes and consumers may be subsequently exposed through the food chain (1,2). For this reason, there is a need to enhance the monitoring of carbapenem resistance in the food supply (1). In Hong Kong, 80% of the food animals are imported from mainland China and involved farm suppliers from multiple provinces in the country (3).

From September 2008 to February 2017, rectal swabs were obtained from randomly selected fresh pig carcasses at a centralized slaughterhouse in Hong Kong by trained veterinary staff, as part of an ongoing surveillance (3). Each swab was collected from a single animal and was inoculated into nutrient broth with 10 mg/L vancomycin and 0.5 mg/L meropenem (4), followed by subculture on MacConkey agar plate supplemented with 2 mg/L meropenem. Five to ten colonies from each selective plate were picked. MALDI-TOF MS was used for bacterial identification. The agar dilution (for colistin) and disc diffusion (for other antibiotics) methods were used to determine antimicrobial susceptibility (5,6). Isolates from the same animal were considered to be unique if the resistance profiles for meropenem and colistin were different.

In total, 856 pigs were cultured over 263 sampling dates (Table S1). Six pigs originating from six different farms were culture positive for meropenem-resistant *E. coli* (Table 1). According to the susceptibility patterns, a total of nine isolates were considered to be unique and investigated further. All isolates exhibited positive CarbaNP test result and were resistant to ertapenem, imipenem and meropenem. The presence of carbapenemase genes and *mcr-1* were investigated by PCR and sequencing (7-9). The $bla_{\text{NDM-5}}$ gene was identified in all nine isolates, of which four were colistin-resistant and co-harbored *mcr-1*. The isolates were further investigated by MLST and replicon typing (7,9,10). Plasmids carrying $bla_{\text{NDM-5}}$ were of X3 (n=8, size ~45 kb) or F36 (n=1, size ~100 kb). Of the four *mcr-*

Accepted Manuscript Posted Online

Antimicrobial Agents and Chemotherapy

AAC

Antimicrobial Agents and Chemotherapy

Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

1 gene identified, three were harbored on plasmids of different replicon types (X4, FIB and Y). In conjugation experiments (9,10), there was no co-transfer of carbapenem and colistin co-resistance but the plasmids carrying *bla*$_{NDM}$ or *mcr-1* could be separately transferred at frequencies of 10^{-4} to 10^{-5} and 10^{-1} to 10^{-6} transconjugants per donor cell, respectively.

Six isolates (one from each animal, Table 1) were sequenced by an Illumina MiSeq platform at >150-fold coverage. The plasmids were assembled de novo using a CLC Genomics Workbench (Qiagen, Redwood City, United States) and gaps were closed by additional PCRs and Sanger sequencing (7,9,10). ISfinder (https://www-is.biotoul.fr/about.php) was used to identify and annotate insertion sequences. In strain P748, *bla*$_{NDM-5}$ was found in a contig (size ~32 kb) with 100% coverage and 98% identity to p28078-NDM (accession no. MF156713).

Complete sequences of the five IncX3 plasmids with sizes of ~46 kb were obtained (Table S2). They have a plasmid scaffold typical of IncX3 plasmids (Figure 1a). The genetic load regions in the five plasmids were compared with two reference IncX3 plasmids (pNDM-HN380 and pIncX-SHV) (Figure 1b). In the *bla*$_{NDM}$-carrying plasmids, an IS*L3* with 8-bp flanking direct repeats (ATATGCAT) was found downstream of the resolvase gene. The *umuD* gene was split into two fragments (*umuDΔ*1 and *umuDΔ2*) at the same position as in pIncX-SHV, resulting in a pair of 3-bp direct repeats (TGT). In pNDM-HN380, *bla*$_{NDM}$ was inserted as an IS*26*-IS*Aba125* transposon-like structure (Figure 1b). Subsequently, the upstream IS*Aba125* was disrupted by IS*5* (10). In four plasmids with link to Guangdong and Henan provinces, the sequences inserted between the two *umuD* fragments were 100% identical (10117 bp in length). This inserted sequence differs from that in pNDM-HN380 by a deletion of 7874 bp (Figure 1b). The remaining plasmid with link to Henan has an additional deletion (616 bp) at the junction between the IS*5* and IS*Aba125*Δ1 remnant. In the five NDM-5 plasmids, IS*5* was inserted at the same position leading to the flanking 4-bp

Accepted Manuscript Posted Online

Antimicrobial Agents and Chemotherapy

AAC

Antimicrobial Agents and Chemotherapy

Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

77 direct repeats (CTAA). In pNDM-HN380, IS*5* was inserted at a different position in the
78 opposite orientation.

79 To explore the geographical distribution of potentially related *bla*$_{NDM}$-carrying IncX3
80 plasmids, the complete sequence of pP768-NDM5 (chosen as a representative) was used to
81 query the GenBank. Twenty-two plasmids related to pP768-NDM-5 were identified by a
82 query of the GenBank (Figure 1c). These include 14 plasmids from China, four from
83 Myanmar and one each from Canada, India, Kuwait and Oman (Table S3). The plasmids do
84 not carry resistance genes other than *bla*$_{NDM}$. Multiple NDM variants were carried by the
85 plasmids. These include NDM-1 and variants that differed by one to three amino acids,
86 including NDM-4 (M154L), NDM-5 (V88L, M154L), NDM-7 (D130N, M154L) and NDM-
87 17 (V88L, M154L, E170K).

88 We identified the occurrence of similar IncX3 plasmids carrying *bla*$_{NDM-5}$ in pigs
89 originating from multiple farms across three different Chinese provinces. The involvement of
90 IncX3 plasmids (represented by pNDM-HN380) in the dissemination of NDM in multiple
91 geographic areas in China was initially reported by our group in 2012 (7). Subsequently,
92 sporadic reports of pNDM-HN380-like plasmids carrying different NDM variants have also
93 been reported in India, Arabian Pennisula, Europe and Australia (11-14). Recently, a Chinese
94 national survey and several provincial studies revealed that IncX3 plasmids harbouring
95 different *bla*$_{NDM}$ variants were frequently found among clinical isolates of different
96 multilocus sequence types and species, suggesting that it is an important vector responsible
97 for the wide dissemination of NDM in China (15-17). IncX3 plasmids have a narrow host
98 range and have mainly been found in *Enterobacteriaceae* (18). Our finding from analysis of
99 complete plasmid sequences indicated that the five IncX3 plasmids originating from pigs are
100 related to pNDM-HN380 as well as plasmids originating from many other geographic areas,
101 thus confirming that this mobile NDM vector is widespread in the ecosystem.

Accepted Manuscript Posted Online

As carbapenems have never been licensed for use in food animals in China, the NDM-producing pig isolates detected in the present study may have been introduced to the farms via human activity or contaminated feeds. It is worrisome that some of the NDM-producing isolates in the present study were found to co-harbour *mcr-1* in another plasmid or the chromosome. Nonethelesss, our isolates co-harbouring *mcr-1* were recovered before the ban of colistin in animal feeds was implemented in November 2016 in China.

In conclusion, this study identified an epidemic IncX3 plasmid carrying $bla_{\text{NDM-5}}$ disseminated among *E. coli* originating from pigs with epidemiological links to geographically segregated areas in China.

Accession number(s). The complete sequence of the five IncX3 plasmids have been deposited into the GenBank database under accession numbers MF547511 (pP744-NDM5), MF547510 (pP768-NDM5), MF547509 (pP785-NDM5), MF547507 (pP788A-NDM5), and MF547508 (pP855-NDM5).

ACKNOWLEDGEMENTS

We thank the staff at the Food and Environmental Hygiene Department (FEHD) of the Hong Kong Special Administrative Region (HKSAR) for assistance with specimen collection.

This work was supported by grants from the Health and Medical Research Fund of the Food and Health Bureau of the HKSAR, and the Consultancy Service for Enhancing Laboratory Surveillance of Emerging Infectious Disease for the HKSAR Department of Health.

Antimicrobial Agents and Chemotherapy
AAC
Antimicrobial Agents and Chemotherapy
Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

Accepted Manuscript Posted Online

Antimicrobial Agents and Chemotherapy

AAC

Antimicrobial Agents and Chemotherapy

Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

REFERENCES

1. **Woodford, N., Wareham, D. W., Guerra, B., and Teale, C.** 2014. Carbapenemase-producing Enterobacteriaceae and non-Enterobacteriaceae from animals and the environment: an emerging public health risk of our own making? J. Antimicrob. Chemother. **69**:287-291.

2. **Mollenkopf, D. F., Stull, J. W., Mathys, D. A., Bowman, A. S., Feicht, S. M., Grooters, S. V., Daniels, J. B., and Wittum, T. E.** 2017. Carbapenemase-producing Enterobacteriaceae recovered from the environment of a swine farrow-to-finish operation in the United States. Antimicrob. Agents Chemother. **61**.

3. **Ho, P. L., Chow, K. H., Lai, E. L., Lo, W. U., Yeung, M. K., Chan, J., Chan, P. Y., and Yuen, K. Y.** 2011. Extensive dissemination of CTX-M-producing *Escherichia coli* with multidrug resistance to 'critically important' antibiotics among food animals in Hong Kong, 2008-10. J. Antimicrob. Chemother. **66**:765-768.

4. **Darling, L. A., Evans, A. M., Stellrecht, K. A., Nattanmai, S. M., and Montero, C. I.** 2017. A Triple-Disk Enrichment Method for Carbapenem-Resistant Enterobacteriaceae (CRE) Screening. J. Clin. Microbiol. **55**:3557-3559.

5. **Clinical and Laboratory Standards Institute**. 2017. Performance standards for antimicrobial susceptibility testing, 27th ed. CLSI supplement M100S. CLSI, Wayne, PA.

6. **Wong, S. C., Tse, H., Chen, J. H., Cheng, V. C., Ho, P. L., and Yuen, K. Y.** 2016. Colistin-resistant Enterobacteriaceae carrying the *mcr-1* gene among patients in Hong Kong. Emerg Infect Dis **22**:1667-1669.

Accepted Manuscript Posted Online

7. **Ho, P. L., Li, Z., Lo, W. U., Cheung, Y. Y., Lin, C. H., Sham, P. C., Cheng, V. C., Ng, T. K., Que, T. L., and Chow, K. H.** 2012. 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. Emerg Microbes. Infect **1**:e39.

8. **Ho, P. L., Cheung, Y. Y., Wang, Y., Lo, W. U., Lai, E. L., Chow, K. H., and Cheng, V. C.** 2016. Characterization of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* from a healthcare region in Hong Kong. Eur. J. Clin. Microbiol. Infect Dis **35**:379-385.

9. **Wang, Y., Lo, W. U., Lai, R. W., Tse, C. W., Lee, R. A., Luk, W. K., Cheng, V. C., Que, T. L., Chow, K. H., and Ho, P. L.** 2017. IncN ST7 epidemic plasmid carrying *bla*$_{IMP-4}$ in Enterobacteriaceae isolates with epidemiological links to multiple geographical areas in China. J. Antimicrob. Chemother. **72**:99-103.

10. **Ho, P. L., Lo, W. U., Yeung, M. K., Lin, C. H., Chow, K. H., Ang, I., Tong, A. H., Bao, J. Y., Lok, S., and Lo, J. Y.** 2011. Complete sequencing of pNDM-HK encoding NDM-1 carbapenemase from a multidrug-resistant *Escherichia coli* strain isolated in Hong Kong. PLoS. One. **6**:e17989.

11. **Wailan, A. M., Paterson, D. L., Caffery, M., Sowden, D., and Sidjabat, H. E.** 2015. Draft genome sequence of NDM-5-producing *Escherichia coli* sequence type 648 and genetic context of *bla*$_{NDM-5}$ in Australia. Genome Announc. **3**.

12. **Petrosillo, N., Vranic-Ladavac, M., Feudi, C., Villa, L., Fortini, D., Barisic, N., Bedenic, B., Ladavac, R., D'Arezzo, S., Andrasevic, A. T., and Capone, A.** 2016. Spread of *Enterobacter cloacae* carrying *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$ and plasmid-

Antimicrobial Agents and Chemotherapy
AAC
Antimicrobial Agents and Chemotherapy
Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

Accepted Manuscript Posted Online

mediated quinolone resistance genes in a surgical intensive care unit in Croatia. J. Glob. Antimicrob. Resist. **4**:44-48.

13. **Choudhury, N. A., Paul, D., Chakravarty, A., Bhattacharjee, A., and Dhar, Chanda D.** 2017. IncX3 plasmid mediated occurrence of *bla*$_{NDM-4}$ within *Escherichia coli* ST448 from India. J. Infect Public Health.

14. **Riazzo, C., Lopez-Cerero, L., Rojo-Martin, M. D., Hoyos-Mallecot, Y., Fernandez-Cuenca, F., Martin-Ruiz, J. L., Pascual-Hernandez, A., Naas, T., and Navarro-Mari, J. M.** 2017. First report of NDM-1-producing clinical isolate of *Leclercia adecarboxylata* in Spain. Diagn. Microbiol. Infect Dis **88**:268-270.

15. **Zhang, R., Liu, L., Zhou, H., Chan, E. W., Li, J., Fang, Y., Li, Y., Liao, K., and Chen, S.** 2017. Nationwide surveillance of clinical carbapenem-resistant Enterobacteriaceae (CRE) strains in China. EBioMedicine. **19**:98-106.

16. **Yang, Q., Fang, L., Fu, Y., Du, X., Shen, Y., and Yu, Y.** 2015. Dissemination of NDM-1-producing Enterobacteriaceae mediated by the IncX3-Type Plasmid. PLoS. One. **10**:e0129454.

17. **Zhang, F., Xie, L., Wang, X., Han, L., Guo, X., Ni, Y., Qu, H., and Sun, J.** 2016. Further spread of *bla*$_{NDM-5}$ in Enterobacteriaceae via IncX3 plasmids in Shanghai, China. Front Microbiol. **7**:424.

18. **Johnson, T. J., Bielak, E. M., Fortini, D., Hansen, L. H., Hasman, H., Debroy, C., Nolan, L. K., and Carattoli, A.** 2012. Expansion of the IncX plasmid family for improved identification and typing of novel plasmids in drug-resistant Enterobacteriaceae. Plasmid **68**:43-50.

Antimicrobial Agents and Chemotherapy

AAC

Antimicrobial Agents and Chemotherapy

Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

Accepted Manuscript Posted Online

Antimicrobial Agents and Chemotherapy

AAC

Antimicrobial Agents and Chemotherapy

Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

193 **Table 1.** Sources and characteristics for nine NDM-positive *E. coli* isolates

Strain	Specimen	Source[b]	Date collected	MLST	bla_{NDM-5}	*mcr-1*	Replicon type of plasmid harboring		Resistance patterns[d]
							bla_{NDM}	*mcr-1*	
P744A	Pig 1	Henan (A1)	Oct 2015	ST 10	+	−	X3	none	Chl, Nit
P744T[a]	Pig 1	Henan (A1)	Oct 2015	ST 1602	+	+	X3	X4	Chl, Cip, Nit
P748[a]	Pig 2	Hunan (B)	Jan 2016	ST 167	+	−	F36	none	Gen, Chl, Cip, Nit
P768	Pig 3	Henan (A2)	May 2016	ST 117	+	−	X3	none	Gen, Chl, Cip
P768-11[a]	Pig 3	Henan (A2)	May 2016	ST 871	+	+	X3	FIB	Cip
P785[a]	Pig 4	Guangdong (C1)	Jun 2016	ST 7512	+	+	X3	Chromosomal[c]	Gen, Chl, Cip, Nit
P788A	Pig 5	Guangdong (C2)	Jun 2016	ST1286	+	−	X3	none	Nit
P788A-32[a]	Pig 5	Guangdong (C2)	Jun 2016	ST 7510	+	+	X3	Y	Gen, Chl, Nit
P855[a]	Pig 6	Guangdong (C3)	Feb 2017	ST 7511	+	−	X3	none	Gen, Chl, Cip, Nit

194 [a]The six isolates were investigated further by genome sequencing.

195 [b]Province (farm) origin of the pig

196 [c]Chromosomal location of *mcr-1* in the isolate was confirmed by genome sequencing.

197 [d]Resistance patterns for amikacin (Ak), chloramphenicol (Chl), ciprofloxacin (Cip), fosfomycion (Fos), gentamicin (Gen) and nitrofurantoin
198 (Nit)

199

200

Accepted Manuscript Posted Online

Figure 1. Comparisons of IncX plasmids in this study. (a) Circular map of the plasmid pP768-NDM5. This plasmid was used to illustrate the backbone shared by all the analyzed plasmids and the location of the genetic load region. (b) Comparison of the genetic load region in 5 plasmids harbouring *bla*$_{NDM-5}$ with two reference plasmids (pIncX-SHV and pNDM-HN380). (c) Alignment of pP768-NDM5 with 22 plasmids identified in the GenBank (last accessed 27 Oct 2017). The circular maps were generated with the BLAST Ring IMAGE Generator and each plasmid was colored by the geographical origin (China, blue; Myanmar, yellow; Oman, orange; India, red; Canada, pink and Kuwait, purple) in the following order (outer to inner circles): pP768-NDM5, pCREC-A6-NDM, pSCE516-2, pNDM5_IncX3, pEc1929, pECNDM101, pAD-19R, pNDM5_WCHEC0215, pK518_NDM5, pK516_NDM5, NUHL24835, pNDM-QD28, pNDM-QD29, pEC50-NDM7, pZHDC40,pJEG027, pM216_X3, pM213_X3 and pM110_X3, pOM26-1, pNDM-MGR194 and pKpN01-NDM7, pKW53T-NDM (full details of plasmids in Table S3).

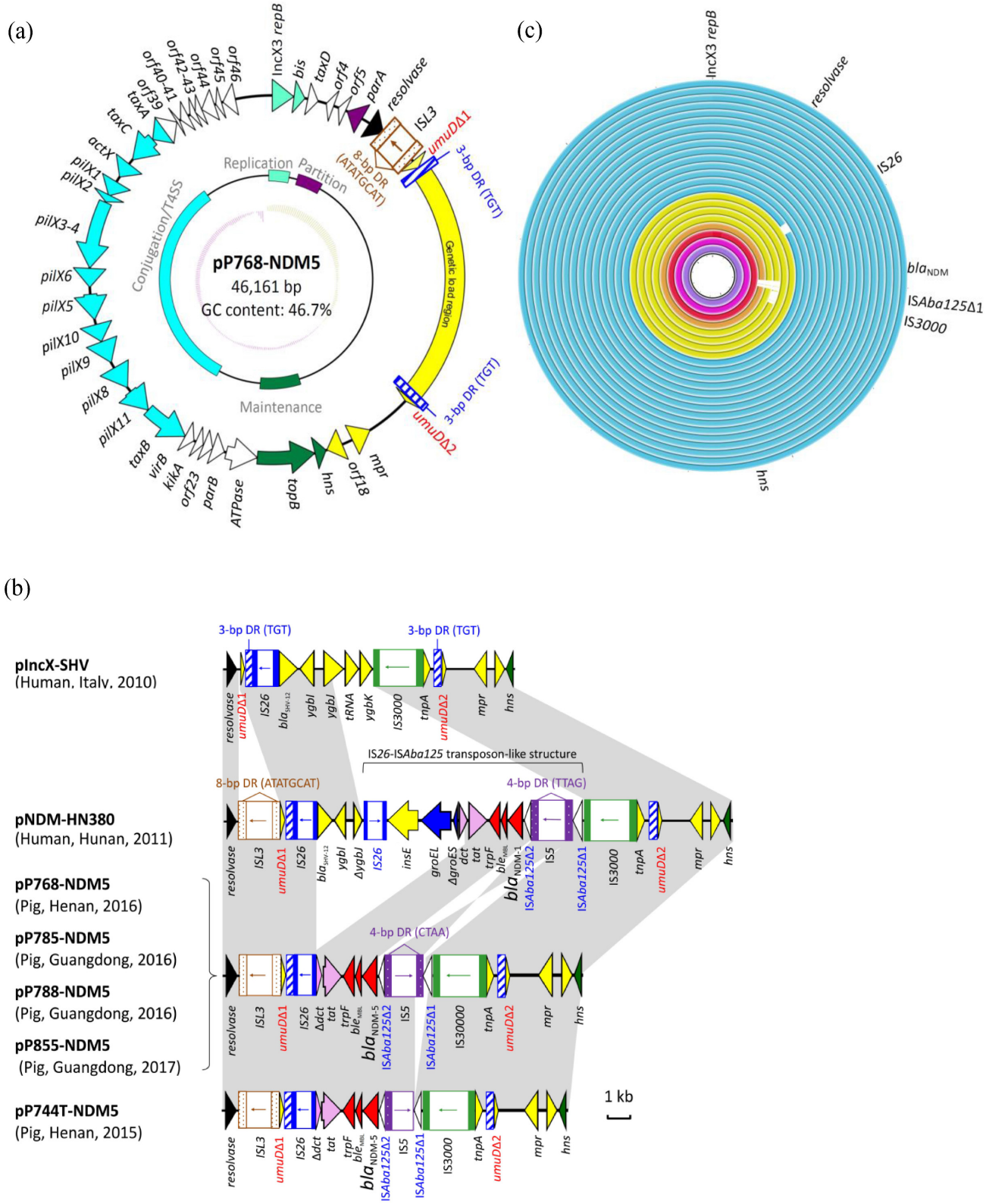
Antimicrobial Agents and Chemotherapy

AAC

Antimicrobial Agents and Chemotherapy

Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London

Accepted Manuscript Posted Online
Antimicrobial Agents and Chemotherapy
AAC
Antimicrobial Agents and Chemotherapy



Downloaded from http://aac.asm.org/ on January 8, 2018 by Queen Mary, University of London