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RESEARCH ARTICLE

Characterization of NDM-1-producing carbapenemase in *Acinetobacter* spp. and *E. coli* isolates from diseased pigs

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Abstract In recent years, the mobile metallo-β-lactamase (MBL) genes have been found to correspond to one of the most important resistance characters identified in Gram-negative bacteria, severely affecting clinical chemotherapy and threatening public health. The prevalence of mobile MBL genes and their flanking regions in Gram-negative bacteria from diseased pigs in China was investigated. A total of 334 lung samples from diseased pigs were screened for Gram-negative bacteria classified as non-susceptible to meropenem (MIC $\geqslant 4\ mg \cdot L^{-1}$). Six isolates, including three *Escherichia coli*, two *Acinetobacter baumanii* and one *A. calcoaeticus*, exhibited MBL production and carried the bla_{NDM-1} gene. S1-PFGE and Southern blot analysis showed that the bla_{NDM-1} gene was located on the chromosome of one *A. baumanii* isolate and on plasmids of various sizes in the other five isolates. MIC testing using broth microdilution revealed that all bla_{NDM-1}-carrying isolates and some of their transconjugants exhibited resistance to almost all β-lactams tested. Whole genome sequencing revealed that the flanking region of the bla_{NDM-1} gene from all porcine isolates had high levels of similarity with the corresponding regions in human isolates. One porcine *E. coli* isolate carrying bla_{NDM-1} was typed as ST48, a common sequence type in human *E. coli* isolates. These results suggest the possibility of human-to-food animal transfer of bla_{NDM-1}-producing *E. coli*, highlighting the need for surveillance of carbapenemase producers among bacteria from food animals. In addition, the prudent use of antimicrobial agents to decrease the opportunities for co-selection of carbapenemase genes in food animals is also urgently needed.

Keywords carbapenemase, NDM-1, IS*Aba125*, Enterobacteriaceae, food safety

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1 Introduction

The emergence of metallo-β-lactamases (MBL; also known as class B carbapenemases), which hydrolyze all classes of β-lactams except monobactams, in Gram-negative human pathogens represents a major threat for clinical chemotherapy and public health[1]. The NDM-type carbapenemases, first identified in a strain of *Klebsiella pneumoniae* isolated in 2008 in New Delhi, India[2], are one of the most important MBL types because of their clinical relevance and international dissemination across all continents except Antarctica[3]. To date, 16 variants of NDM (NDM-1 to NDM-16; no information on NDM-12, NDM-15 and NDM-16 is available in the GenBank database), which differ by one, two, or five amino acid substitutions at 14 positions (www.lahey.org/studies), have been identified mainly from Enterobacteriaceae isolated from nosocomial infections. In these NDM variants, the bla_{NDM-1} gene is mostly located on conjugative plasmids, which facilitates its rapid dissemination[4]. Detailed analysis of the genetic environment of the bla_{NDM-1} gene in the chromosome or on plasmids of various Gram-negative bacterial species revealed the presence of a conserved structure comprising the complete or truncated insertion sequence element IS*Aba125* and the bleomycin (ble_{MBL}) resistance gene in the up- and down-stream regions of the bla_{NDM-1} genes, respectively[4].

Compared with the high prevalence of NDM in Gram-negative bacteria of human origin, there are only limited and sporadic reports of this type of carbapenemase in bacteria from non-human origins. In the environment, the bla_{NDM-1} gene was first identified in multiple genera in 2011, including the opportunistic Enterobacteriaceae and non-fermentative Gram-negative bacteria, in the samples of both tap water and seepage water in New Deli, India[5]. The bla_{NDM-1} gene was then found in *K. pneumoniae* isolated from a river in Vietnam[6], as well as in

Acinetobacter baumannii isolated from sewage of hospitals in Beijing, China[7]. Recently, bla_{NDM-1}-carrying *A. calcoaceticus* and *A. junii* were identified in environmental samples from livestock farms in China[8]. In animals, a single isolate of NDM-1-producing *A. lwoffii* and *A. baumannii* has been reported from a chicken and a pig, respectively[9,10], and the bla_{NDM-1} gene was also observed in clinical *Escherichia coli* isolates recovered from cats and dogs in the USA[11].

As there have only been a few reports on the carbapenemases in bacteria from food animals, we screened the clinical samples of diseased pigs from an animal diagnostic laboratory in China to investigate the presence of the carbapenem resistance gene in Gram-negative bacteria, and further characterized the genetic environment of the carbapenem resistance gene.

2 Materials and methods

2.1 Bacterial isolation and identification, and phenotypic and molecular detection of MBL in Gram-negative bacteria

A total of 334 lung samples were collected from the animal diagnostic laboratory of Foshan University in Foshan City, Guangdong Province, China, between July and August 2013. Each sample was isolated from an individual animal and spread on a brain heart infusion (BHI) agar plate containing 2 mg·L^{-1} meropenem (Ouhe Technology Company, Beijing, China) and 30 mg·L^{-1} vancomycin, then incubated for 18 h at 37°C. All colonies were selected and identified using Gram staining and sequence analysis of the 16S rDNA gene, using previously described primers[12]. Species identification of Gram-negative bacteria was further conducted by MALDI-TOF MS (BrukerDaltonik GmbH, Bremen, Germany). The imipenem-EDTA double-disc synergy test and E-test using MBL strips (bioMérieux, Craponne, France) were performed to screen for MBL production in these isolates. The MBL-producing isolates were screened for known mobile MBL-encoding genes, NDM-, VIM-, SIM-, GIM-, AIM- and DIM-type β-lactamases were investigated using previously described PCR assays[10].

2.2 Conjugation assay

Filter mating was performed with each of the original isolates using *E. coli* strain EC600 (a rifampicin-resistant strain) as the recipient. The original isolates and the recipient strains of *E. coli* EC600, both incubated overnight were adjusted to 0.5 McFarland standard in BHI broth, and 5 μL of parental and recipient strains were added to 1 mL of BHI broth and incubated for an additional 4 h at 37°C, respectively. Then, 20 μL of the parental strain and 60 μL of the recipient strains were incubated together on a microporous membrane (Millipore, Bedford, MA, USA) overnight for conjugation. The concentrations of antibiotics in the BHI agar plates used for the selection of transconjugants were 500 mg·L^{-1} for rifampin and 1 mg·L^{-1} for meropenem. Transconjugants were confirmed as MBL-producing isolates by PCR analysis, and pulsed-field gel electrophoresis (PFGE) was also performed to confirm that transconjugants were derivatives of the recipient strain EC600.

2.3 Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MIC) of a range of antibiotics against the MBL-producing isolates, as well as their transconjugants, were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute document M100-S25 (2015) and the MIC were interpreted according to these standard[13]. The *E. coli* isolate ATCC25922 was used for quality control.

2.4 Molecular analysis of MBL-producing isolates

The genetic relatedness of MBL-producing isolates was investigated by *Xba*I-PFGE for *E. coli* and *Sma*I-PFGE for *A. baumanii*. The location of MBL genes in the original isolates was determined by S1-nuclease PFGE mapping and Southern blot analysis as described previously[14]. Multilocus sequence typing (MLST) of the MBL-producing isolates was performed according to published protocols (mlst.warwick.ac.uk/mlst/dbs/Ecoli).

2.5 Sequence, assembly, and annotation of the flanking region of the MBL gene

The total DNA of MBL-producing isolates was extracted using the Wizard® Genomic DNA Purification kit (Promega, WI, USA), then subjected to whole genome sequencing. The library was constructed using the NEXT® Ultra™ DNA Library Prep kit (New England Biolabs, Ipswich, UK), and paired-end sequencing was conducted on an Illumina Hiseq2500 (Berry Genomics Company, Beijing, China). A draft assembly of the sequences was generated using CLC Genomics Workbench 5 (CLC Bio, Aarhus, Denmark). All contigs were searched for β-lactamase genes using standalone Blast analysis[15]. Gap closure of the flanking regions of MBL genes was performed by PCR using a modified random primer walking strategy[16]. The putative coding sequences of the flanking region of MBL genes were obtained using ORF Finder programs (www.ncbi.nlm.nih.gov/gorf/orfig.cgi) and the Vector NTI program (Invitrogen, CA, USA).

3 Results and discussion

In this study, six isolates classified as non-susceptible to

meropenem (MIC ⩾ 4 mg·L^{-1}), obtained from six of the 334 lung samples from the diseased pigs, were confirmed as Gram-negative bacteria. These isolates were further identified by 16S rRNA gene sequencing and the Bruker Daltonik MALDI Biotyper Classification system as *E. coli* (n = 3, FSEC38, FSEC39, and FSEC69), *A. baumanii* (n = 2, FSAB08 and FSAB62) and *A. calcoaceticus* (n = 1, FSABC15). Imipenem-EDTA double-disc synergy tests and E-test MBL strip tests confirmed that the six isolates were MBL-positive, and PCR analysis using primers specific for mobile MBL genes revealed that all of the isolates were positive for bla_{NDM}. The 813 bp nucleotide sequence of bla_{NDM} in the six isolates showed 100% identity to that of the bla_{NDM-1} gene on plasmid pKpANDM-1 of *K. pneumoniae* 05-506 (GenBank accession no. FN396876). The detection rate (1.8%, 6/334) of the mobile MBL gene (bla_{NDM-1}) in the lung samples of diseased pigs in the current study was considerably higher than both that in the lung samples of pigs from commercial farms in Guangdong province during 2011–2012 (0.3%, 1/313), and that in various samples (cloaca, rectum, nasal cavity, and lymph nodes) from commercial farms in Shandong province in 2012 (0.3%, 1/396)[9,10]. To our knowledge, this is the first time that NDM-producing Enterobacteriaceae have been reported from food animals.

PFGE analysis of the three bla_{NDM-1}-carrying *E. coli* isolates and two bla_{NDM-1}-carrying *A. baumanii* isolates revealed distinct genomic heterogeneity in their *Xba*I and *Sma*I patterns, respectively. Comparison with the allelic profiles available in the aforementioned MSLT website identified *E. coli* isolate FSEC69 as sequence type (ST) 48. ST48 has been associated with hospital-acquired extended-spectrum β-lactamase (ESBL)-producing *E. coli* isolates from patients in Belgium[17], and ESBL-producing *E. coli* isolates in fecal samples of healthy humans in Tunisia[18]. The sequence types of another two *E. coli* isolates FSEC38 and FSEC39 were novel and were designated as ST5084 and ST5069, respectively.

The six isolates exhibited resistance and high MIC to almost all β-lactam antibiotics tested, and only one *E. coli* isolate, FSEC39, showed borderline susceptibility to aztreonam (4 mg·L^{-1}). As the bla_{NDM-1} gene cannot confer resistance to monobactams, a search for β-lactamase genes in the contigs of the two aztreonam-resistant *E. coli* isolates revealed that the AmpC β-lactamases genes bla_{CMY-2} + bla_{DHA-1}, and bla_{ampC} were observed in FSEC38 and FSEC69, respectively. Three genes had previously been identified as being responsible for plasmid-mediated aztreonam resistance in the Enterobacteriaceae[19]. It is noteworthy that no aztreonam-resistance gene was detected in the contigs of the three *Acinetobacter* isolates, suggesting that an unknown monobactam resistance gene may exist, a possibility which needs further investigation. Moreover, all isolates exhibited MICs of 2 to 256 mg·L^{-1} to ciprofloxacin, which classified the isolates as resistant or intermediate. All isolates, except FSEC39, exhibited resistance to tetracycline, and had MIC for colistin of ⩾ 4 mg·L^{-1}, while all isolates except FSAB62 exhibited resistance to gentamicin (Table 1).

S1-nuclease PFGE mapping and Southern blot analysis revealed that the gene bla_{NDM-1} was located on various-sized plasmids, ranging from approximately 47–200 kb, in five isolates and on the chromosomal DNA of *A. baumanii* FSAB08 (Table 1, Fig. 1). However, only the bla_{NDM-1}-carrying plasmids pEC39 and pEC69 were successfully transferred from FSEC39 and FSEC69, respectively, to *E. coli* EC600 using filter mating. Susceptibility tests revealed that both of the transconjugants presented resistance to all tested β-lactams except aztreonam and to gentamicin compared with recipient *E. coli* EC600, but remained susceptible to ciprofloxacin and colistin. In addition, one transconjugant EC600-69 also exhibited resistance to tetracycline and gentamicin and an elevated MIC for

Table 1 Antimicrobial susceptibility profiles of *Acinetobacter. baumannii* FSAB08 and FSAB62, *A. calcoaceticus* FSABC15, *E. coli* FSEC38, FSEC39, FSEC69, transconjugants EC600-39 and EC600-69, and recipient strains EC600

Isolate	Species	PFGE type	MLST type	MIC/(mg·L^{-1})										Location of bla_{NDM-1}
				IMP	MERO	CTZ	CAZ	AZT	CIP	GEN	COL	FFC	TET	
FSAB08	*A. baumanii*	1	–	512	512	>1024	256	>512	2	64	128	8	32	C
FSAB62	*A. baumanii*	2	–	256	256	1024	>1024	64	128	0.125	4	256	512	P/pAB62(~47 kb)
FSABC15	*A. calcoaceticus*	–	–	256	512	1024	>1024	128	256	128	4	512	>512	P/pABC15 (~47 kb)
FSEC38	*E. coli*	a	5084	64	32	1024	>1024	512	2	128	8	512	256	P/pEC38 (~50 kb)
FSEC39	*E. coli*	b	5069	128	128	512	>1024	4	8	>512	2	32	4	P/pEC39 (~70 kb)
EC600-39	*E. coli*	–	–	32	32	128	1024	0.25	0.125	512	0.5	4	4	P/pEC39 (~70 kb)
FSEC69	*E. coli*	c	48	128	256	>1024	>1024	16	128	256	4	512	256	P/pEC69 (~200 kb)
EC600-69	*E. coli*	–	–	64	64	256	>1024	0.125	0.25	64	0.5	512	64	P/pEC69 (~200 kb)
EC600	*E. coli*	–	–	0.25	0.03	2	0.5	0.125	0.015	1	0.25	4	2	–

Note: IMP, imipenem; MERO, meropenem; CTZ, ceftizoxime; CAZ, ceftazidime; AZT, aztreonam; CIP, ciprofloxacin; GEN, gentamicin; COL, colistin; FFC, florfenicol; TET, tetracycline.

florfenicol. These results indicate that the determinants conferring resistance to three antimicrobials other than β-lactams can be co-transferred with the bla_{NDM-1} gene from the donor to the recipient *E. coli* EC600 (Table 1).

Analysis of the flanking regions of the bla_{NDM-1} gene on two plasmids and in one chromosome of three *Acinetobacter* isolates revealed that the bla_{NDM-1} gene was located in an identical 9923 bp fragment, which exhibited 100% nucleotide sequence identity to the corresponding region of plasmid pNDM-BJ01 (JQ001791) isolated from human clinical *A. lwoffii* strain WJ10621 from Beijing, China[20]. In addition, a 5029 bp region including the bla_{NDM-1} gene, its upstream IS*Aba125* and aminoglycoside resistance gene *aphA6*, and its downstream gene ble_{MBL} also had 99.9% (5017/5029) nucleotide sequence identity to that in plasmid pAL-01 (JN616388) in chicken *A. lwoffii* strain SGC-HZ9 (Fig. 2)[10]. Further downstream of the ble_{MBL} gene, a truncated putative phosphoribosylanthranilate isomerase gene *trpF* was identified, followed by *dsbC*, *cutA1*, *groES* and *groEL*, and *insE*, encoding the oxidoreductase protein, then tolerance protein, heat chaperonin protein and transposase, respectively.

In the bla_{NDM-1}-carrying regions in plasmids from porcine *E. coli* isolates, the nucleotide sequences of a 15914 bp segment of pEC38 (GenBank accession no. KT164808), a 10384 bp fragment of pEC39 and a 6406 bp fragment of pEC69 (GenBank accession no. KT164809) were obtained by whole genome sequencing and primer walking (Fig. 2). This 15.9 kb bla_{NDM-1}-harboring region in pEC38 had 100% nucleotide identity with the corresponding region of plasmid pKpn-SX04 (accession no. NG_041664) from *K. pneumoniae* derived from a patient from the program of Chinese Antimicrobial Resistance Surveillance of Nosocomial Infections in 2012[21]. In both cases, a disrupted IS*Aba125* (ΔIS*Aba125*) resulted from the insertion of another insertion sequence, the IS*5* element. Small differences between these two plasmids existed: a truncation of the left direct target site (5′-CTAA-3′) and the 234 bp IS*Aba125* sequence was observed in pEC38. However, it was reported that the 7830 bp fragment, namely the 3′ region of the ΔIS*Aba125*, bla_{NDM-1}, ble_{MBL}, *trpF*, *dsbC*, *cutA1*, *groES*, *groEL*, and *insE*, facilitated the horizontal mobilization of the bla_{NDM-1} gene among Enterobacteriaceae[21].

Both the 9693 bp and 5340 bp bla_{NDM-1}-carrying fragments of pEC39 and pEC69 had > 99.9% nucleotide sequence identity with the corresponding region of plasmid pGUE-NDM (accession no. JQ364967) from *E. coli* isolate GUE, a community-acquired strain from India (Fig. 2)[22]. Downstream of the bla_{NDM-1} gene, the ble_{MBL}, *trpF*, and *dsbC* genes were observed in pEC39, followed by the insertion sequence IS*CR1*, and by a class 1 integron structure carrying three gene cassettes, including streptomycin/spectinomycin resistance gene *aadA2*, the gene *orfF* for a hypothetical protein, and the trimethoprim resistance gene *dfrA12*. The sequence further downstream of *int1* also had a high degree of nucleotide identity (99.9%) to IS*26* present in pGUE-NDM. Instead of the intact or truncated IS*Aba125* element observed immediately upstream of the bla_{NDM-1} gene in other cases in this study, a rifampicin resistance gene *aar-3* and chloramphenicol resistance gene *catB3* were observed in pEC69 (Fig. 2).

4 Conclusions

The results of this study confirm that bla_{NDM-1} is the only mobile MBL gene present in *Acinetobacter* spp. and *E. coli*

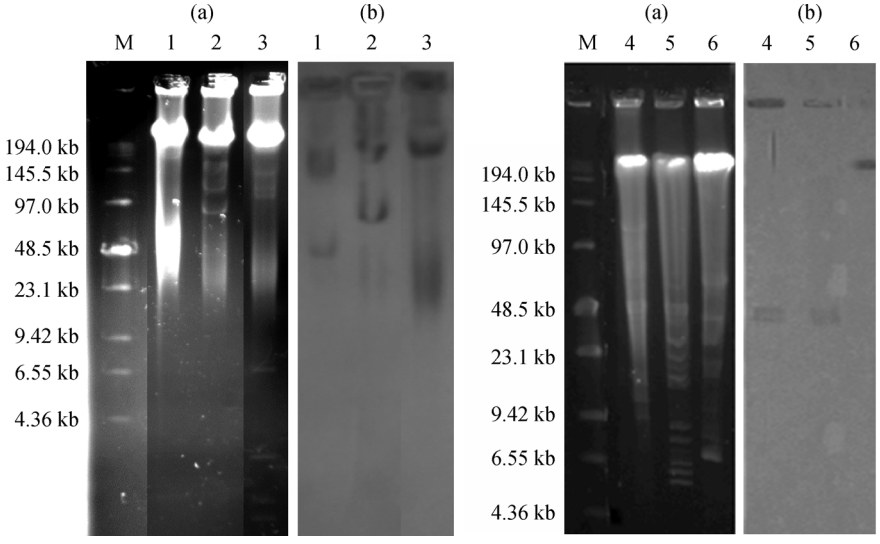

Fig. 1 Localization of bla_{NDM-1} in NDM-1-producing isolates by S1-PFGE (a) and Southern blot hybridization with bla_{NDM-1} probe (b). Lane M, low-range pulsed-field gel marker (New England BioLabs, Beverly, MA). Lane 1–3, *E. coli* isolates FSEC38, FSEC39 and FSEC69; lane 4, *Acinetobacter. calcoaceticus* isolate FSABC15; lane 5–6, *A. baumanii* isolates FSAB62 and FSAB08.

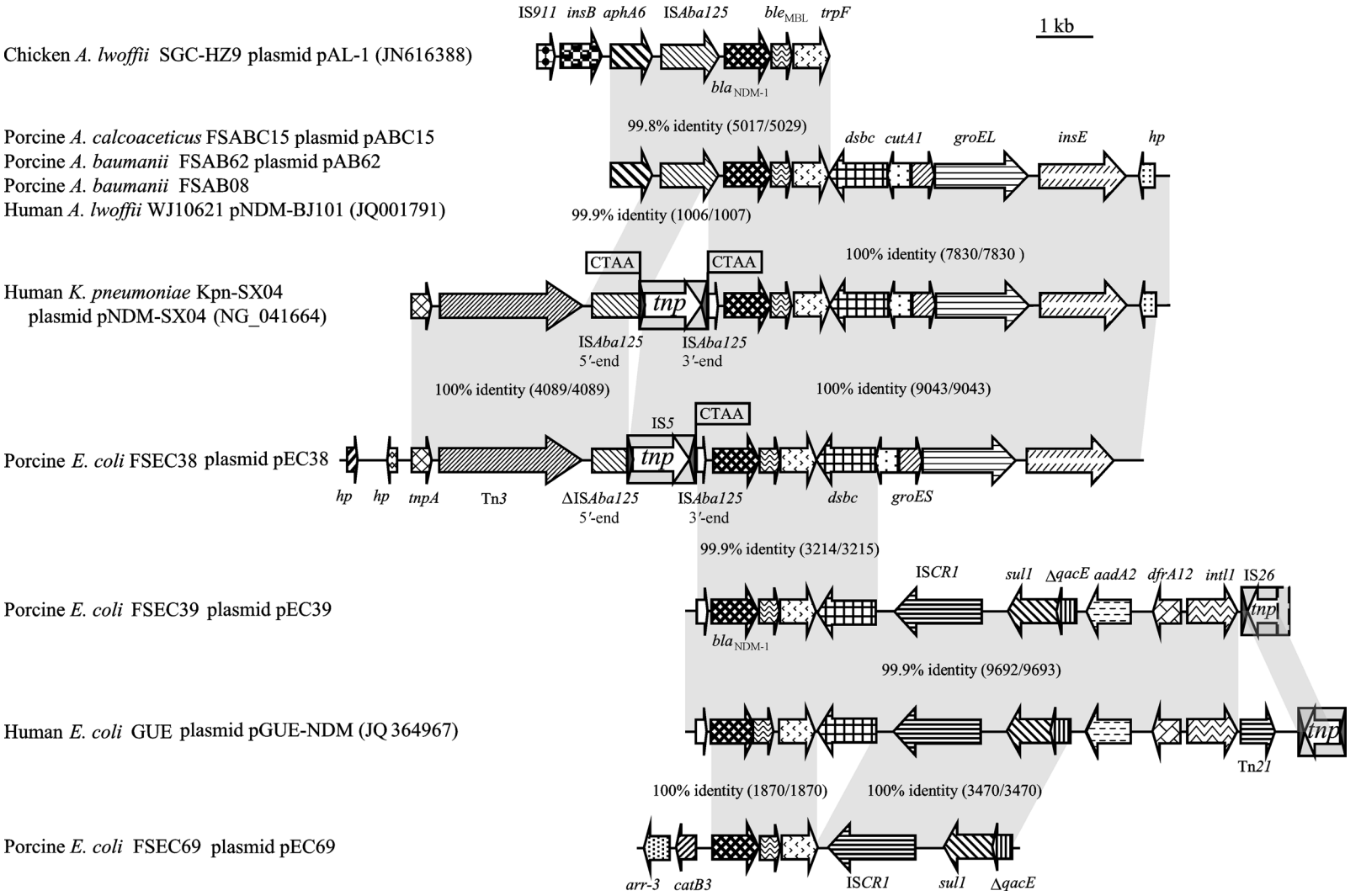

Fig. 2 Genetic environment of bla_{NDM-1} in *E. coli* and *Acinetobacter* spp. isolates of pig origin, and its structural comparison with the corresponding genetic regions in plasmids pAL-1, pNDM-SX04, and pGUE-NDM. The arrows indicate the positions and directions of transcription of the genes. Different genes are indicated by different types of shading. Regions of ⩾99.8% homology are marked by gray shading

isolates from food animals. Similar to the spread of bla_{NDM-1} in bacteria of clinical origin, both the plasmid and insertion sequence elements, such as IS*Aba125*, IS*5* and IS*26*, may be important in the dissemination of the bla_{NDM-1} gene and its conserved flanking regions in bacteria from food animals. Moreover, the frequency of identification of ESBL-carrying *E. coli* ST48 from both human fecal and clinical samples, as well as the high level of similarity of the genetic environment of the bla_{NDM-1} gene from the porcine *E. coli* isolate found in human Enterobacteriaceae, suggests the possibility of human-to-food animal transfer of this bla_{NDM-1}-producing isolate. Thus, enhanced and continued efforts are needed to monitor carbapenemase producers in bacteria from food animals. It should be noted that carbapenems are not approved for use in food animals, and the bla_{NDM-1} gene was found to coexist with other genes conferring resistance to aminoglycosides, phenicols, and tetracyclines in isolates in this study, the co-selection and co-transfer of carbapenemase genes under the selective pressure imposed by these genes seems be key to a major role in their dissemination. Therefore, the prudent use of antimicrobial agents to decrease the opportunities for co-selection of carbapenemase genes in food animals is urgently needed.

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Compliance with ethics guidelines Rongmin Zhang, Yang Wang, Zhihai Liu, Jiyun Li, Wenjuan Yin, Lei Lei, Congming Wu, and Jianzhong Shen declare that they have no conflict of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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