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ORIGINAL RESEARCH

Prevalence and molecular epidemiology characteristics of carbapenem-resistant *Escherichia coli* in Heilongjiang Province, China

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Objective: This retrospective study was conducted to determine the prevalence and molecular epidemiology characteristics of carbapenem-resistant *Escherichia coli* (CRE).

Methods: A total of 593 *Escherichia coli* (*E. coli*) isolates were recovered from pigs and urban river from 2009 to 2014 in Heilongjiang Province of China. Forty CRE including 22 strains isolated from fecal samples of pigs and 18 strains isolated from water samples were selected. PCR detection of resistance determinants, multi-locus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and phylogenetic groups were performed to characterize CRE isolates. Conjugation experiments, plasmid stability testing, PCR-based replicon typing (PBRT), and PCR mapping were conducted to analyze bla_{NDM}-carrying plasmids. In vitro time–growth studies and competition experiments were carried out to assess the fitness impact of NDM carriage.

Results: Five NDM-1-positive *E. coli* isolates were identified from water samples. Genetic environment analysis revealed that a cluster of genes (*ISAba125*-bla_{NDM-1}-ble_{MBL}-Δ*trpF*) was detected in all of the NDM-1-positive isolates. Conjugation assays showed that bla_{NDM-1} could be successfully transferred to *E. coli* J53 from 5 donor strains at frequencies of 4.6×10^{-5} to 2.6×10^{-2}. The plasmids from all transconjugants belonged to different plasmid replicon types including IncA/C (n=2), IncFII (n=1) and IncX3 (n=2). In vitro time–growth studies revealed that bla_{NDM-1} did not have a significant impact on cell proliferation. Meanwhile, competition experiments showed that the acquisition of bla_{NDM-1} can place an energy burden on the bacterial host and incur fitness cost. However, plasmid stability testing showed that bla_{NDM-1}-carrying plasmid remained stable in the hosts after seven passages without antimicrobial selection.

Conclusion: The study revealed the early molecular epidemiology and dissemination characteristics of CRE. In addition, the overall antimicrobial resistance in *E. coli* recovered from water samples is higher than the strains isolated from fecal samples of pigs. Furthermore, we isolated and identified five NDM-1-producing *E. coli* strains from water samples.

Keywords: carbapenem-resistant, *Escherichia coli*, NDM-1, fitness cost

Introduction

The rapid emergence and dissemination of multiple antimicrobial-resistant (MDR) bacteria have posed a significant threat to global public health, which is commonly acknowledged to be caused by the indiscriminate, widespread and increasing use of antibiotics.[1,2] The rising patient mortality and morbidity caused by MDR bacterial infections, and the growing antibiotic resistance even pose a challenge to the vast

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medical advancements made by antibiotics. Moreover, due to the dearth of novel classes of antibiotics entering the clinic, the phenomenon has been exacerbated over the past 40 years.[1]

The appearance of MDR bacteria from animals is a growing area of concern due to the potential possibility for transfer of resistant pathogens and commensal bacteria to the human population.[3] Many studies demonstrated that the use of antibiotics can not only increase the level of resistance of pathogenic bacteria but also increase the level of resistance of commensal bacteria. Thus, the commensal bacteria are regarded as a reservoir of resistance genes for potential pathogenic bacteria. Some resistant commensal bacteria from food animals, such as zoonotic bacteria can reach the intestinal tract of humans through the contaminated meat, milk, or eggs.[3] *E. coli* is the most prevalent commensal bacteria in the intestinal tract of animals and humans, and many studies had shown that the animal and human infectious diseases may be also implicated with *E. coli*.[4] Therefore, the level of resistance of *E. coli* is usually recognized as a good indicator for monitoring selection pressure by antibiotic use, prevalence of resistance, and for detecting the transfer of resistant bacteria or resistance genes from animals to humans and vice versa.[3]

Carbapenems, a kind of β-lactams that have long served as reliable and potent agents against Gram-negative bacteria, and have been regarded as the last line for treatment of infections caused by MDR bacteria in clinics.[5] Due to the proliferation of MDR bacterial pathogens, the use of carbapenems such as imipenem, ertapenem, and meropenem in clinics has become more usually during the past two decades. Due to the increasing consumption of carbapenem, the carbapenem-resistant Gram-negative pathogens have been isolated worldwide.[6] A common mechanism mediating carbapenem resistance in Gram-negative bacteria is the presence of carbapenemases, and many studies have reported various types of carbapenemases, among which Class A (bla_{KPC}), Class B (bla_{NDM}, bla_{VIM}, bla_{IMP}), and Class D (bla_{OXA-48}) types are the most common in *Enterobacteriaceae*.[7]

Most of the genetic determinants for carbapenemases are commonly located on mobile genetic elements, such as plasmid, which make it feasible to transfer among various species of bacterial pathogens. The worldwide continuous emergence of carbapenemases in recent years has posed an increasing threat to the effectiveness of carbapenem. As an emerging carbapenemase, New Delhi metallo-lactamase (NDM) was identified in a broad-spectrum antibiotic-resistant strain of *Klebsiella pneumonia* isolated from a Swedish patient with a hospitalization history in India, which can mediate resistance to all β-lactams except for monobactams, and has been considered to have great potential to cause global health crisis.[8]

Since 2009, the cases of MDR bacteria harboring bla_{NDM} have been reported in almost all continents except Antarctica.[9] In China, since the first report of bla_{NDM} in *Acinetobacter baumannii* isolates, with an increasing number of *Enterobacteriaceae* have been identified as carriers of the bla_{NDM}.[10] Though the prevalence of MDR bacteria harboring bla_{NDM} is low, numerous cases of clinical infection caused by NDM-producing isolates have been reported in several regions of China, which suggests the high transferability of the bla_{NDM} and the severity of infections caused by NDM producer.[11] Worryingly, many reports regarding the detection and occurrence of bla_{NDM} in some environmental compartments, containing hospital sewage, municipal wastewater, seepage, and tap water, indicate the risk that NDM-positive strains could widely disseminate the NDM-1 gene from medical origins.[2,12]

In the present study, we examined the susceptibility of *E. coli* isolates recovered from fecal samples of pigs and water samples in the Heilongjiang province during 2009–2014. Additionally, the molecular characteristics of CREwere evaluated. Overall, our results demonstrated that the retrospective study on CRE was necessary to gain a better understanding of their molecular epidemiology characteristics and the rule of dissemination.

Materials and methods

Bacterial isolates and antimicrobial susceptibility testing

This retrospective study was conducted to examine the prevalence and molecular epidemiology characteristics of CRE isolates. From June 2009 to July 2014, a total of 593 *E. coli* including 488 isolates collected from 21 porcine farms in Heilongjiang Province of China, and 105 *E. coli* were recovered from water samples of an urban river in the city of Harbin, China from 2013 to 2014.

The minimum inhibitory concentrations (MICs) for 22 antimicrobial agents were determined by the broth microdilution method following Clinical and Laboratory Standards Institute (CLSI) guidelines. The results for various β-lactams, aminoglycosides, carbapenems, fluoroquinolones, and tetracyclines were interpreted according to CLSI guidelines; colistin and tigecycline were interpreted following European

Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. *E. coli* strain ATCC 25922 was used as a quality control strain. The CRE isolates were screened for carbapenemase using the modified Hodge test (MHT). In addition, metallo-β-lactamase (MBL) production was detected by double-disk synergy tests (DDST), which were performed using the imipenem–EDTA as previously described.[13]

Molecular detection of resistance genes

Total DNA was extracted from all CRE isolates by the kit following the manufacturer's instruction. Carbapenemase genes (bla_{BIC}, bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{GIM}, bla_{SPM}, bla_{SIM}, bla_{AIM}, bla_{DIM}, and bla_{OXA-48}),[14] extended spectrumβ-lactamase genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA}), plasmid-mediated AmpC genes (bla_{MOX}, bla_{CMY}, bla_{DHA}, bla_{ACC}, bla_{EBC}, and bla_{FOX}),[15] 16S rRNA methyltransferases (*armA, rmtA, rmtB, rmtC, rmtD, rmtE,* and *npmA*),[16] and quinolone resistance (*qnrA, qnrB, qnrC, qnrD, qnrS, qepA, oqxAB*, and *aac(6′)-Ibcr*) were examined by PCR.[17] *E. coli* ATCC25922 was used for the quality control. Positive amplifications were subjected to Sangon sequencing (Sangon Company, Shanghai, China).

PFGE and MLST

Molecular typing of CRE isolates was performed by PFGE. Genomic DNA of the CRE was prepared in agarose blocks and was digested with restriction enzyme *XbaI* (TaKaRa Biotechnology, Dalian, China). DNA fragments were separated using a CHEF II D-Mapper XA PFGE system (Bio-Rad, Hercules, CA) with running conditions as described previously.[18] MLST of CRE was conducted by PCR as previously described.[19] The allelic profiles and sequence types were identified by amplifying and sequencing the seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA*) according to the reference website (https://enterobase.warwick.ac.uk/species/index/ecoli). The phylogenetic groups of CRE were determined by multiplex PCR analysis.[20]

Conjugation assay and genetic environment analysis of the bla_{NDM-1} gene

The NDM-positive isolates were selected for conjugation experiment which was implemented by mix broth mating.[5] Enterobacterial repetitive intergenic consensus (ERIC)-PCR was used to further distinguish transconjugants from the donor strains. Antimicrobial susceptibility of transconjugants were determined using the broth microdilution method. Incompatibility groups of plasmids extracted from transconjugants were determined by PCR-based replicon typing as described previously.[21,22] The transfer frequency of carbapenem resistance was determined as described in a previous report.[23] Plasmid stability was assessed in daily serial passages of culture without antibiotic and the culture daily analyzed for carbapenem resistance and confirmed the presence of bla_{NDM-1} by PCR.[24]

PCR mapping and sequencing were applied to analyze the bla_{NDM-1} genetic structure. The plasmid of pNDM-BJ01 (accession no. JQ001791) from *Acinetobacter lwoffii* and *E. coli* plasmid of pBJ01 (GenBank accession no. JX296013) were used as the references. The primers were used in this study as described previously.[25]

Cloning of bla_{NDM-1}

To study the acquisition of plasmid with bla_{NDM-1} may have several effects on bacterial fitness. The bla_{NDM-1} with native promoter (NP) was amplified by PCR using primers NP-NDM-F (5′-CGGGATCCCACCTCATGTTTGAATTC GC-3′) and NP-NDM-R (5′-CCCAAGCTTCTCTGTCAC ATCGAAATCGC-3′), then cloned into the pMD18-T vector. The resulting plasmids were named pMD18-T/NP-NDM-1, which were transformed into *E. coli* DH5α by electrotransformation and confirmed by PCR and DNA sequencing, subsequently.

In vitro time–growth studies

The bacterial strains pMD18-T/DH5α and pMD18-T/NP-NDM-1/DH5α were grown in LB and LB containing 4 μg/mL imipenem at 37°C and 200 rpm overnight, respectively. The bacterial suspension was then diluted based on absorbance at 600 nm and transferred to flasks containing 10 mL LB. The final concentration of inoculum was approximately 1×10^5 CFU/mL. The bacterial strains with LB were incubated at 37°C and 200 rpm for 12 hrs. Serial samples (at 30 mins, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 11, and 12 hrs) were obtained in triplicate, and bacteria growth was monitored by measuring the OD600.

In vitro growth competition experiments

In vitro competition experiments between pMD18-T/DH5α and pMD18-T/NP-NDM-1/DH5α were performed as described previously.[24] Exponentially growing cells of the corresponding pMD18-T/DH5α and pMD18-T/NP-NDM-1/DH5α were adjusted to a 1.0 McFarland standard, diluted $1:10^4$ and then mixed in LB with a 1:1 ratio (time point zero). The mixture was inoculated into flasks

containing 10 mL LB and grown at 37°C and 180 rpm for 24 hrs. Serial 10-fold dilutions were plated in duplicate onto LBA alone and LBA with 4 μg/mL imipenem in order to determine the total colony forming unit (CFU) and the CFU of the mutant at 0 and 24 hrs, respectively. The number of CFU growing on antibiotic-supplemented LBA was subtracted from the number of CFU growing on antibiotic-free LBA to determine the number of susceptible cells in the mixed population. The relative fitness is calculated using the ratio of the growth rate of the resistant cells to that of the susceptible.

In vitro time–kill studies

For the in vitro time–kill assay, five NDM-positive *E. coli* isolated from water samples were used as representative strains, *E.coil* ATCC25922 were chosen as control strain. The initial inoculum of 10^6 CFU/mL was incubated in antibiotic-supplemented imipenem at a concentration of 4 μg/mL and antibiotic-free MH broth, respectively. In addition, 100 μL of co-culture were collected at 0, 1, 2, 3, 4, 6, 8, 12, and 24 hrs post-inoculation for bacterial counts. The 100 μL of co-culture were serially diluted with 0.9 mL MH broth and plated onto MH agar plates then incubated at 37°C for 18–24 hrs. The CFU/mL was determined, and counts were performed in duplicate.

Statistical analysis

Frequency of antimicrobial resistance profiles between *E. coli* recovered from fecal samples of pigs and *E. coli* isolated from water samples was compared using the Pearson Chi-square test with the software SPSS 17.0, values of $P<0.05$ were considered significant, and values of $P<0.01$ were considered markedly significant.

Results

Bacterial strains and antimicrobial susceptibility testing

In this study, a total of 593 *E. coli* were collected including 488 *E. coli* recovered from fecal samples of pigs, 105 strains isolated from water samples of an urban river in the city of Harbin, China (Figure 1). The results of the in vitro antimicrobial susceptibility testing are shown in Tables 1 and S1. There was a high frequency (27.6–97.1%) of tigecycline, cefoxitin, cefotaxime, ceftazidime, gentamicin, aztreonam, ceftiofur, amoxicillin/clavulanic acid, doxycycline, florfenicol, chloramphenicol, and amoxicillin resistance in *E. coli* isolated from water samples, with only imipenem, meropenem, ertapenem, and colistin displaying low resistance rates (11.4–22.9%). *E. coli* isolated from fecal samples of pigs showed high resistance rates (63.5–96.7%) to gentamicin, ciprofloxacin, enrofloxacin, doxycycline, chloramphenicol, amoxicillin, and sulfamethoxazole/trimethoprim; moderate resistance rates (25.4–45.9%) to amikacin, cefoxitin, ceftazidime, aztreonam, cefotaxime, ceftiofur, ceftriaxone, and amoxicillin/clavulanic acid; and also showed low rates of resistance (1.2–2.1%) to imipenem, meropenem, ertapenem, tigecycline, and colistin. Moreover, among all *E. coli*, isolates from pigs showed significantly higher resistance to florfenicol, enrofloxacin, and ciprofloxacin ($P<0.01$), while isolates from water samples showed higher resistance to the rest of the tested antimicrobial agents except tetracycline, doxycycline, chloramphenicol, and sulfamethoxazole/trimethoprim.

Antimicrobial susceptible patterns of CRE

A total of 40 (6.74%, 40/593) non-duplicate *E. coli* isolates including 22 strains isolated from fecal samples of pigs and 18 strains isolated from water samples, which exhibited resistance to imipenem, ertapenem, or meropenem were selected. All CRE isolates were resistant to amoxicillin but remained susceptible to tigecycline, the resistance rate to colistin is only 10%. High resistance rates (>80%) were discovered among 40 CRE isolates for amoxicillin/clavulanic acid, ceftiofur, ceftriaxone, cefotaxime, ceftazidime, gentamicin, tetracycline, chloramphenicol, florfenicol, and sulfamethoxazole/trimethoprim. Relative low frequencies of resistance (55–72.5%) to cefoxitin, aztreonam, amikacin, doxycycline, enrofloxacin, and ciprofloxacin (as shown in Table 2).

Prevalence of carbapenemase genes among CRE

The results of MHT showed that 12 *E. coli* isolates including 5 strains isolated from fecal samples of pigs and 7 strains isolated from water samples were positive or weak positive. The DDST using EDTA as an inhibitor was positive for 5 strains isolated from water samples. Among the 40 CRE isolates, 5 (12.5%, 5/40) isolates were found to be bla_{KPC-2}-positive including 3 strains isolated from water samples and 2 strains isolated from fecal samples of pigs. Interestingly, 5 bla_{NDM-1}-positive were all isolated from water samples. Other carbapenemase genes were not detected in all of the CRE isolates.

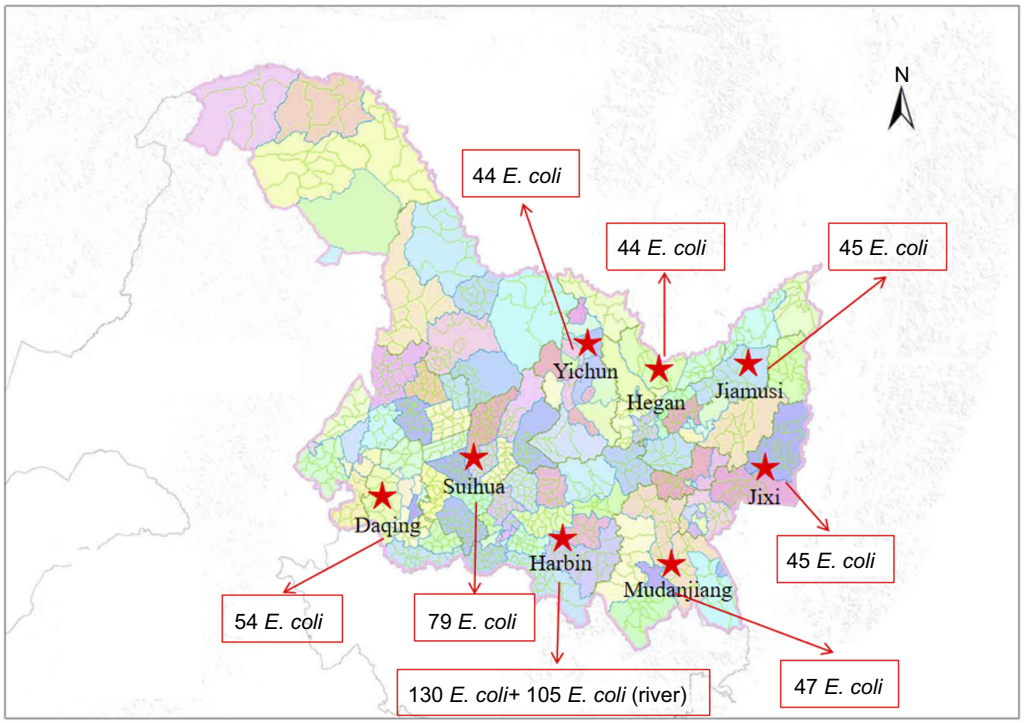

Figure 1 Sample collection sites in the map of Heilongjiang province. Numbers within pointers denote the number of *E. coli* isolated from each area.

Presence of additional antibiotic resistance genes in CRE

The frequency of the presence of additional antibiotic resistance genes in CRE isolates is shown in Figure 2. Overall, ESBL genes including $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and bla_{OXA} were identified in 38, 25, 9, and 12 isolates, respectively. $bla_{\text{CTX-M}}$ was the most common ESBL gene in this study including $bla_{\text{CTX-M-3}}$ (n=4), $bla_{\text{CTX-M-15}}$ (n=12), $bla_{\text{CTX-M-55}}$ (n=8), $bla_{\text{CTX-M-64}}$ (n=2), $bla_{\text{CTX-M-14}}$ (n=13), $bla_{\text{CTX-M-27}}$ (n=2), $bla_{\text{CTX-M-65}}$ (n=4), and $bla_{\text{CTX-M-125}}$ (n=1). All bla_{OXA} positive isolates were identified as $bla_{\text{OXA-1}}$ (n=9). Moreover, $bla_{\text{TEM-1}}$ (n=21), $bla_{\text{TEM-52}}$ (n=4), $bla_{\text{SHV-11}}$ (n=2), and $bla_{\text{SHV-12}}$ (n=7) were identified. Only $bla_{\text{CMY-2}}$ (n=7) and $bla_{\text{CMY-30}}$ (n=4) of AmpC gene were detected in 11 strains. The plasmid-encoded 16S rRNA methylases *armA* (n=9) and *rmtB* (n=7) were detected in 16 isolates. The plasmid-mediated quinolone resistance genes *oqxAB, qnrS, aac(6')-Ib-cr*, and *qepA* were detected in 22, 16, 15 and 9 isolates, respectively.

Molecular typing of CRE

PFGE and MLST for 40 CRE isolates were performed. Among 40 CRE isolates, 4 strains were untypable and the rest of 36 strains exhibited 19 different clusters as A~S clone type. The MLST analysis identified 23 different sequence types (STs) among 40 CRE isolates. The most commonly identified genotypes were ST131 and ST648 (n=5), followed by ST38 and ST72 (n=3), whereas the isolates belonging to ST4 (n=2), ST34 (n=2), ST43 (n=2), ST48 (n=2), ST167 (n=2), ST410 (n=2), ST10, ST23, ST315, ST405, ST25, ST69, ST169, ST251, ST252, ST745, ST1209, ST1454, and ST2324 were also identified. The NDM-1-producing *E. coli* isolates were divided into four STs, as ST72, ST131, ST167 (n=2), and ST410. The results of phylogenetic group for CRE revealed that 24 isolates were assigned to low virulence A type and B1 type, and the other 16 isolates were assigned to high virulence B2 and D type. Four NDM-1-producing CRE were divided to low virulence type, and only one belonged to high virulence type. (as shown in Figure 2)

Transfer of carbapenem resistance

All of the plasmids harboring bla_{NDM} gene from 5 selected CRE isolates were successfully transferred to *E. coli* J53 through conjugation at a frequency of 4.6×10^{-5} to 2.6×10^{-2}. As shown in Table 3, all of the transconjugants exhibited multidrug resistance phenotypes which are similar to those of the donor strains, and the susceptibility to the tested carbapenems reduced. PCR assays confirmed

Table 1 Resistance rates of *E. coli* of different origin against 22 antimicrobial agents

Antimicrobial agents	*E. coli* isolated from pigs (n=488)		*E. coli* isolated from river (n=105)		*P*-value
	Number of resistant isolates	Percentage (%)	Number of resistant isolates	Percentage (%)	
AMX	464	95.1	102	97.1	>0.05
AMC	224	45.9	74	70.5	<0.01**
CEF	212	43.4	72	68.6	<0.01**
CRO	220	45.1	79	75.2	<0.01**
CTX	188	38.5	53	50.5	<0.05*
CAZ	168	34.4	65	61.9	<0.01**
FOX	129	26.4	34	32.4	>0.05
ATM	193	39.5	72	68.6	<0.01**
IPM	6	1.2	11	10.5	<0.01**
MEM	10	2.0	12	11.4	<0.01**
ETP	13	2.7	15	14.3	<0.01**
GEN	310	63.5	70	66.7	>0.05
AKN	124	25.4	28	26.7	>0.05
TET	463	94.9	99	94.3	>0.05
DOX	402	82.3	86	81.9	>0.05
CHL	425	87.1	90	85.7	>0.05
FLO	465	95.3	87	82.9	<0.01**
ENR	397	81.4	62	59.1	<0.01**
CIP	380	77.9	56	53.3	<0.01**
SXT	472	96.7	100	95.2	>0.05
COL	10	2.1	24	22.9	<0.01**
TGC	7	1.4	29	27.6	<0.01**

Notes: The values of *$P<0.05$ were considered significant, and values of **$P<0.01$ were considered markedly significant.
Abbreviations: AMX, amocicillin; AMC, amocicillin/clavulanic acid; CEF, ceftiofur; CRO, ceftriaxone; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; ATM, aztreonam; IPM, imipenem; MEM, meropenem; ETP, ertapenem; GEN, gentamicin; AKN, amikacin; TET, tetracycline; DOX, doxycycline; CHL, chloramphenicol; FLO, florfenicol; ENR, enrofloxacin; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim; COL, colistin; TGC, tigecycline.

that bla_{NDM-1} was successfully transferred to *E. coli* J53 from CRE isolates along with other resistance genes, such as *blaTEM-1, armA*, and rmtB. The plasmids from all transconjugants belonged to different plasmid replicon types including IncA/C (n=2), IncFII (n=1), and IncX3 (n=2). The results of plasmid stability testing showed that the ratio of CFU growing on antibiotic-supplemented LBA to CFU on antibiotic-free LBA was not statistically significant ($P>0.05$) after seven passages.

Genetic environments of bla_{NDM-1}

PCR mapping was performed to identify the genetic environment surrounding the *blaNDM-1* gene in all of the five NDM-1-producing isolates. Sequence analysis of PCR products revealed that a cluster of genes (*ISAba125*-bla_{NDM-1}-ble_{MBL}-Δ*trpF*) was detected in all of the five NDM-positive isolates. Two of the isolates contained a common genomic structure around the *blaNDM-1* (*ISAba125*-bla_{NDM-1}-ble_{MBL}-Δ*trpF-groES-groL-insE-ISAba125*), which was similar to the genetic environment of the *Acinetobacter lwoffii*.

In vitro growth curves

To determine the effects of bla_{NDM-1} on the *E. coli* growth, growth curve experiments were conducted to assess the growth rates under noncompetitive conditions. As shown in Figure 3, the results showed that the curve patterns and growth rate of pMD18-T/DH5α and pMD18-T/NP-NDM-1/DH5α were almost similar. Additionally, it was found that the time to reach non-exponential growth was also similar.

In vitro competition experiments

In antibiotic-free environment, pMD18-T/NP-NDM-1/DH5α competed with pMD18-T/DH5α. As shown in Figure 4, the results showed that the pMD18-T/NP-NDM-1/DH5α harboring bla_{NDM-1} originated from *E. coli* (MJ2, MI19, MJ22, MJ26, and MJ90) showed a relative fitness of 0.89±0.06, 0.88±0.07, 0.91±0.06, 0.86

Table 2 Percentages of antimicrobial resistance in CRE of different origins

Antimicrobial agents	CRE isolated from pigs (n=22)		CRE isolated from river (n=18)		*P*-value
	Number of resistant isolates	Percentage (%)	Number of resistant isolates	Percentage (%)	
AMX	22	100	18	100	>0.05
AMC	20	90.9	17	94.4	>0.05
CEF	21	95.5	17	94.4	>0.05
CRO	21	95.5	18	100	>0.05
CTX	20	90.9	16	88.9	>0.05
CAZ	19	86.4	14	77.8	<0.05*
FOX	13	59.1	16	88.9	<0.01**
ATM	9	40.9	14	77.8	<0.01**
IPM	6	27.3	11	61.1	<0.01**
MEM	10	45.5	12	66.7	<0.01**
ETP	13	59.1	15	83.3	<0.01**
GEN	18	81.8	16	88.9	<0.05*
AKN	11	50	9	50	>0.05
TET	21	95.5	16	88.9	<0.05*
DOX	19	86.4	10	55.6	<0.01**
CHL	21	95.5	17	94.4	>0.05
FLO	19	86.4	15	83.3	>0.05
ENR	19	86.4	8	44.4	<0.01**
CIP	15	68.2	7	38.9	<0.01**
SXT	21	95.5	18	100	>0.05
COL	2	9.1	2	11.1	>0.05
TGC	0	0	0	0	>0.05

Notes: The values of *$P<0.05$ were considered significant, and values of **$P<0.01$ were considered markedly significant.

Abbreviations: CRE, carbapenem-resistant *Escherichia coli;* AMX, amocicillin; AMC, amocicillin/clavulanic acid; CEF, ceftiofur; CRO, ceftriaxone; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; ATM, aztreonam; IPM, imipenem; MEM, meropenem; ETP, ertapenem; GEN, gentamicin; AKN, amikacin; TET, tetracycline; DOX, doxycycline; CHL, chloramphenicol; FLO, florfenicol; ENR, enrofloxacin; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim; COL, colistin; TGC, tigecycline.

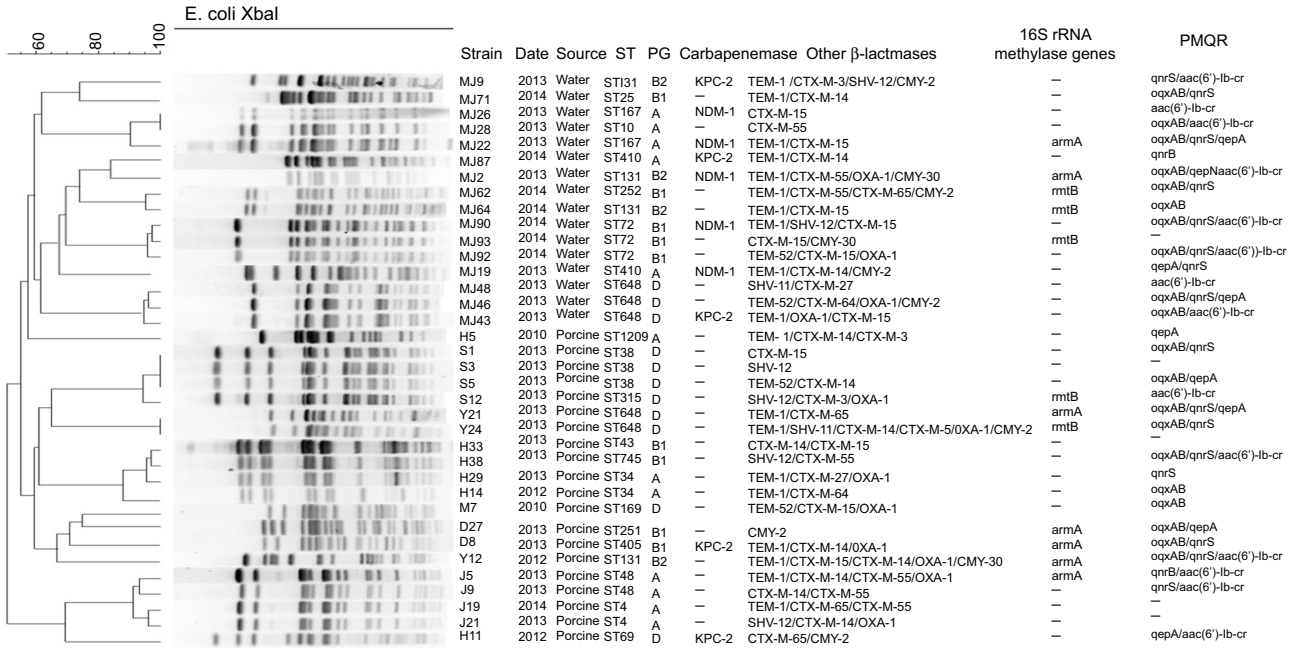

Figure 2 The molecular characteristics of CRE isolates.

Abbreviations: PG, phylogenetic group; CRE, carbapenem-resistant *Escherichia coli.*

Table 3 The characteristics of 5 NDM-1 positive *E. coli* and their transconjugants

E. coli/transconjugants	MIC(μg/ml)			Resistance genes	Plasmid type/transfer frequency
	ETP	IPM	MEM		
E. coli-MJ2	256	16	128	*NDM-1/TEM-1/CTX-M-55/OXA-1/CMY-30/armA/oqxAB/qepA/aac(6')-Ib-cr*	3.7×10^{-3}
E. coli-MJ2-J53	256	32	64	*NDM-1/TEM-1/armA*	IncA/C
E. coli-MJ19	128	64	128	*NDM-1/TEM-1/CTX-M-14/CMY-2/rmtB/qepA/qnrS*	4.6×10^{-5}
E. coli-MJ19-J53	128	32	64	*NDM-1/TEM-1/rmtB*	IncFII
E. coli-MJ22	128	16	64	*NDM-1/TEM-1/CTX-M-15/armA/oqxAB/qnrS/qepA*	4.2×10^{-4}
E. coli-MJ22-J53	64	32	32	*NDM-1/TEM-1/armA*	IncA/C
E. coli-MJ26	64	8	32	*NDM-1/CTX-M-15/aac(6')-Ib-cr*	8.6×10^{-5}
E. coli-MJ26-J53	32	8	16	*NDM-1*	IncX3
E. coli-MJ90	128	32	32	*NDM-1/TEM-1/SHV-12/CTX-M-15/oqxAB/qnrS/aac(6')-Ib-cr*	2.6×10^{-2}
E. coli-MJ90-J53	256	64	32	*NDM-1/TEM-1*	IncX3

Abbreviations: IPM, imipenem; MEM, meropenem; ETP, ertapenem.

±0.06, and 0.84±0.06 at 95% confidence intervals, respectively.

In vitro time–kill studies

As presented in Figure 5, the difference of growth curve patterns for all strains was not obvious in antibiotic-free MH broth, imipenem could hardly inhibit the growth of CRE isolates, but imipenem at 4 μg/mL could obviously inhibit the growth of control strain.

Discussion

Numerous retrospective and prospective studies revealed that the frequent use of antimicrobials as therapy and prophylaxis of infectious diseases or feed additives in animals could select high antimicrobial resistance in bacteria, which is becoming a serious issue in China.[26] In the present study, all *E. coli* isolates including 488 *E. coli* strains isolated from fecal samples of pigs and 105 *E. coli* isolated from water samples were tested for their susceptibility to 22 antimicrobial agents. The antimicrobial susceptibility test showed that most of the *E. coli* were multidrug resistant. Agreeing with previous studies,[27] the isolates showed high resistance to most of the conventional antimicrobial agents, such as gentamicin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole which are commonly used for the treatment of post-weaning diarrhea in pig farm.

Overall, as shown in Table S2, the prevalence of CRE in river water isolates was higher than that in pig isolates (17.14% vs 4.51%). The prevalence of CRE was continually increased in *E. coli* isolates of pig origins during the period of investigation (2009–2014), which increased from 1.61% during 2009–2010 to 7.73% during 2013–2014 (as shown in Figure S1). The *E. coli* isolates from different regions have different prevalence of CRE, as Yichun (6.82%), Jiamusi (8.89%), Mudanjiang (4.26%), Harbin (5.38%), Suihua (5.06%), and Daqing (3.70%). Specifically, the prevalence of CRE in isolates of swine origin from Hegan and Jixi was (0/89) (Table S2).

The remarkable finding in the present study was that there were significant difference between strains isolated from fecal samples of pigs and water samples on resistance rates to antimicrobial agents. Comparing the frequency of resistance to these 22 antimicrobial agents, higher resistance rates to most of the tested antimicrobial agents were present in *E. coli* strains isolated from water samples than isolates originated from pigs. It was noteworthy that our study revealed that 22.9% and 27.6% of the strains recovered from water samples were resistant to

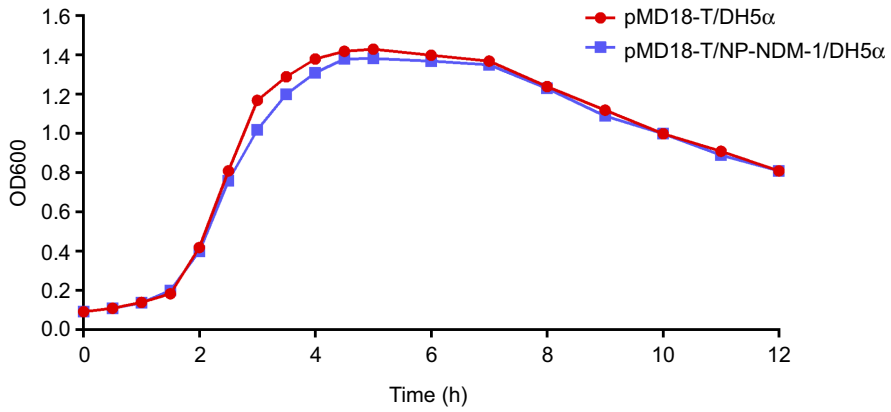

Figure 3 The growth curves of pMD18-T/DH5α and pMD18-T/NP-NDM-1/DH5α.

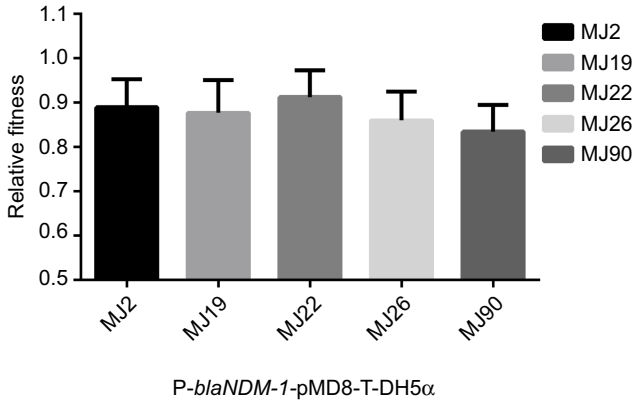

Figure 4 Relative fitness of pMD18-T/NP-NDM-1/DH5α. A relative fitness of 1 indicates that the harboring NDM-1 undergo no fitness cost, whereas a ratio of greater than or less than 1 indicates increased or decreased fitness. Data are means ±SD (error bars).

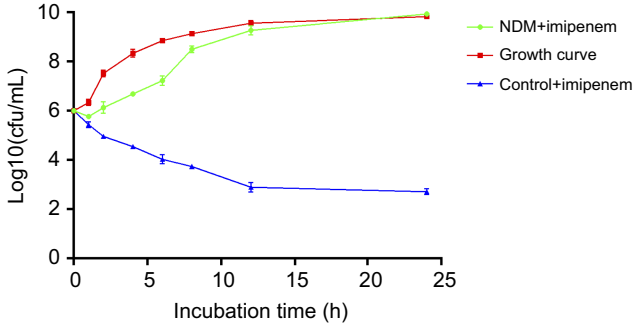

Figure 5 Average time–kill curve of imipenem against 5 isolates of CRE. Data are means±SD (error bars).
Abbreviations: CRE, carbapenem-resistant *Escherichia coli*; NDM, New Delhi metallo-lactamase.

colistin and tigecycline, respectively. Due to the widespread and excessive use of antibiotics, the aquatic environment has been contaminated by antibiotic, which is becoming a global problem.[28] The antibiotic contamination is associated with municipal sewage discharges and animal production wastewaters, which are constantly released into the aquatic environment along with various bacteria carrying antibiotic resistance genes and antibiotics.[29]

In the present study, we isolated and identified 5 *bla*$_{NDM}$-carrying strains which were all isolated from water samples among 40 CRE, and we also detected 5 *bla*$_{KPC}$-carrying strains including 3 strains isolated from water samples and 2 strains isolated from fecal samples of pigs, other carbapenemase genes were not detected in all of the CRE isolates. However, a meta surveillance performed in European countries demonstrated 71% of the CRE were carbapenemase-producing, together with a wide variety of carbapenemases were detected, which is contradicted with the findings of this work.[30] Generally, the emergence and dissemination of the *bla*$_{NDM}$ have continuously increased worldwide. Furthermore, a multicenter study of the China CRE network showed that 74.4% were NDM producer among 39 CRE isolates, indicating that combating infections caused by this "superbug" is becoming a serious issue.[31]

The genetic environments surrounding the *bla*$_{NDM-1}$ gene were determined to be IS*Aba125*- *bla*$_{NDM-1}$-*ble*$_{MBL}$-Δ*trpF* in all of the NDM-positive isolates. The gene cluster (*bla*$_{NDM-1}$-*ble*$_{MBL}$-Δ*trpF*) has been reported in *E. coli* plasmids (pNDM-HK, pBJ01, and pNDM_Dok01) and *Acinetobacter lwoffii* plasmids (pNDM-BJ01 and pNDM-BJ02), which was highly conserved.[32] Some study demonstrated that *bla*$_{NDM-1}$ and *ble*$_{MBL}$ were under the control of the same promoter, and this structure may facilitate the spread of *bla*$_{NDM-1}$ under the antibiotics selective pressure.[33] It has been showed that IS*Aba125* could provide the 35 regions of the promoter sequence for *bla*$_{NDM-1}$ in all reported cases.[32] Two of the isolates contained a common genomic structure around the *bla*$_{NDM-1}$ (*ISAba125*-

bla$_{NDM-1}$-*ble*$_{MBL}$-Δ*trpF-groES-groL-insE*-I*SAba125*), which was similar to the genetic environment of the *Acinetobacter lwoffii*,[13] it revealed that the resistant plasmid could be transferred between *Enterobacteriaceae* and *Acinetobacter*.

It is now commonly accepted that the main risk factors for the rapid increase in the prevalence of CRE are the mobile genetic elements mediated *bla*$_{NDM-1}$ transfer and clonal spread of strains which containing mobile resistance elements among the *Enterobacteriaceae* species.[34] Numerous studies showed that the *bla*$_{NDM-1}$-like genes are predominantly detected in the ST131 and ST101 of *E. coli*, and ST11 of *Klebsiella pneumonia*, which suggested that the transmission of mobile resistance elements in CRE was associated with the sequence types of bacterial strains.[35,36] In this study, four STs (ST131, ST167, ST410, and ST72) were identified among the five NDM-1-positive *E. coli* isolates. Two *E. coli* isolates (MJ22 and MJ26) recovered from two different locations of the river share the same ST (ST167), suggesting that they were clonally related. It has been demonstrated that ST131 was the most prevalent strain type of *E. coli* worldwide, ST167 has strong association with clinical infections in China.[37] Recently, some studies have reported several sporadic cases of clinical infections caused by NDM producers which were related to *E. coli* ST167 carrying *bla*$_{NDM-5}$ in various parts of China.[37]

The high efficiency of mobile resistance elements transfer facilitates the spread of *bla*$_{NDM}$ worldwide. In this study, conjugative assays revealed that all of the *bla*$_{NDM-1}$ plasmids were successfully transferred to *E. coli* J53 from the 5 donors by conjugation. Furthermore, the plasmids from all transconjugants belonged to different plasmid replicon types including IncA/C (n=2), IncFII (n=1), and IncX3 (n=2). It has been demonstrated that the widespread *bla*$_{NDM-1}$-carrying plasmid throughout the world was related to multiple replicon types, including IncX3, IncF, and IncA/C, etc. IncA/C belongs to broad-host range plasmid, which was predominantly reported for carrying *bla*$_{NDM-1}$ as well as other resistance genes, and widely disseminated among Gram-negative bacteria worldwide.[38] The identification of the IncX3-type plasmid carrying the *bla*$_{NDM-1}$ gene was first reported in 2012 in multiple cities in China.[39] Recently, numerous studies showed that IncX3 plasmids carrying different *bla*$_{NDM}$ variants were frequently found among clinical isolates, indicating that it is an important vector to facilitate the widespread of NDM in China.[39]

The impact of *bla*$_{NDM-1}$ on growth and fitness was assessed by comparing pMD18-T/DH5α and pMD18-T/NP-NDM-1/DH5α. No difference in growth between pMD18-T/DH5α and pMD18-T/NP-NDM-1/DH5α could be observed after 12 hrs, indicating that NDM-1 does not have a significant impact on cell proliferation, which is in line with the previous study.[40] The results of competition experiments demonstrated that the expression of *bla*$_{NDM-1}$ could bring energy burden on the host and cause fitness cost, which is in agreement with the previous study that acquisition of *bla*$_{NDM-1}$ plasmid could lead to a loss of fitness for *E. coli* J53 receipt.[40]

Conclusion

In summary, the overall antimicrobial resistance in *E. coli* recovered from water samples is higher than the strains isolated from fecal samples of pigs. Genes encoding five different groups of resistance enzymes (carbapenemase, ESBL, 16S rRNA methyltransferases, Ampc, and quinolone resistance) were detected among 40 CRE. Furthermore, we isolated and identified five NDM-1-producing *E. coli* strains isolated from water samples. All of the plasmids harboring *bla*$_{NDM-1}$ from five selected CRE isolates were successfully transferred to *E. coli* J53. The plasmids from all transconjugants belonged to different plasmid replicon types including IncA/C, IncFII, and IncX3. The carriage of *bla*$_{NDM-1}$ did not have a significant impact on cell proliferation but reduced fitness of bacterial hosts. Therefore, studies on early epidemic characteristics of NDM-positive *E. coli* are necessary to provide comprehensive, extensive, and clear information to optimize antibiotic policy in endemic areas.

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Author contributions

XZ and GX conceived and designed the experiments. PC FL, RL, TX, and YY performed the practical work, completed the experiments and analyzed the data. PC and MI wrote and revised the manuscript. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

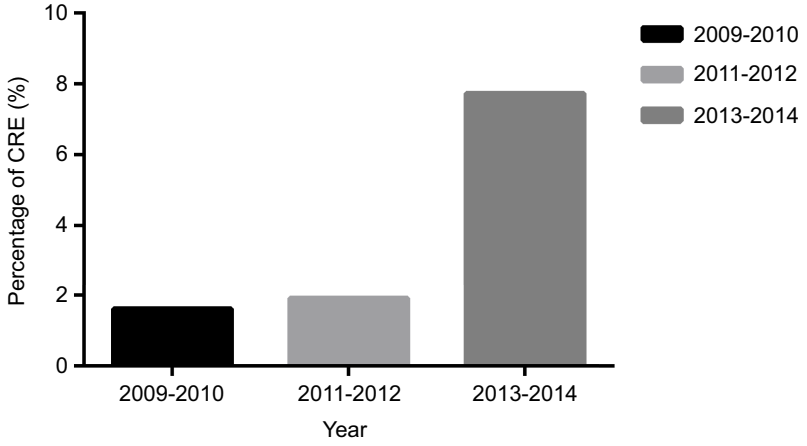

Figure S1 Percentage of CRE isolated from fecal samples of pigs in different years.
Abbreviation: CRE, carbapenem-resistant *Escherichia coli*.

Table S1 Antimicrobial susceptibility of CRE strains to carbapenems antibiotic

Antimicrobial agents	Number (%) of resistant isolates	CRE isolated from pigs (n=22)				CRE isolated from river (n=18)			
		MIC (μg/mL)			Number (%) of resistant isolates	MIC (μg/mL)			Number (%) of resistant isolates
		Range	MIC_{50}	MIC_{90}		Range	MIC_{50}	MIC_{90}	
IPM	17 (42.5)	0.5–16	1	16	6 (27.3)	0.5–64	4	32	11 (61.1)
MEM	22 (55.0)	0.5–16	2	16	10 (45.5)	0.5–128	8	128	12 (66.7)
ETP	28 (70.0)	0.5–32	2	32	13 (59.1)	1–256	8	128	15 (83.3)

Abbreviations: IPM, imipenem; MEM, meropenem; ETP, ertapenem.

Table S2 Prevalence of CRE isolates in fecal samples of pigs and water samples from different regions of Heilongjiang province

	Yichun	Hegan	Jiamusi	Jixi	Mudanjiang	Harbin	Suihua	Daqing	Total
Swine									
N collected samples	57	63	71	60	55	152	103	69	630
N isolated *E.coli*	44	44	45	45	47	130	79	54	488
N CRE (percentage)	3 (6.82)	0 (0)	4 (8.89)	0 (0)	2 (4.26)	7 (5.38)	4 (5.06)	2 (3.70)	22 (4.51)
N NDM-positive	0	0	0	0	0	0	0	0	0
Water	ND	ND	ND	ND	ND		ND	ND	ND
N collected samples						133			133
N isolated *E.coli*						105			105
N CRE (percentage)						18 (17.14)			18 (17.14)
N NDM-positive						5			

Abbreviations: N, number of; ND, not determined.