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Characterization of NDM-5-producing *Enterobacteriaceae* isolates from retail grass carp (*Ctenopharyngodon idella*) and evidence of *bla*$_{NDM-5}$-bearing IncHI2 plasmid transfer between ducks and fish

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ABSTRACT

We aimed to characterize NDM-5-producing *Enterobacteriaceae* from aquatic products in Guangzhou, China. A total of 196 intestinal samples of grass carp collected in 2019 were screened for carbapenemase genes. Characterization of *bla*$_{NDM-5}$ positive isolates and plasmids was determined by antimicrobial susceptibility testing, conjugation experiments, Illumina HiSeq, and Nanopore sequencing. One *Citrobacter freundii* and six *Escherichia coli* strains recovered from seven intestinal samples were verified as *bla*$_{NDM-5}$ carriers (3.57%, 7/196). The *bla*$_{NDM-5}$ genes were located on the IncX3 (*n*=5), IncHI2 (*n*=1), or IncHI2-IncF (*n*=1) plasmids. All *bla*$_{NDM-5}$-bearing plasmids were transferred by conjugation at frequencies of ~10^{-4}–10^{-6}. Based on sequence analysis, the IncHI2 plasmid pHNBYF33-1 was similar to other *bla*$_{NDM-5}$-carrying IncHI2 plasmids deposited in GenBank from Guangdong ducks. In all IncHI2 plasmids, *bla*$_{NDM-5}$ was embedded in a novel transposon, Tn*7051* (IS*3000* -ΔIS*Aba125*-IS*5*-ΔIS*Aba125*-*bla*$_{NDM-5}$-*ble*$_{MBL}$-*trpF*-*tat*-Δ*dct*-IS*26*-Δ*umuD*-ΔIS*Kox3*-IS*3000*), which was identical to the genetic structure surrounding *bla*$_{NDM-5}$ found in some IncX3 plasmids. The IncHI2-IncF hybrid plasmid pHNTH9F11-1 was formed by homologous recombination of the *bla*$_{NDM-5}$-carrying IncHI2 plasmid and a heavy-metal-resistant IncF plasmid through ΔTn*1721*. To the best of our knowledge, this is the first report on the characterization of *bla*$_{NDM-5}$-bearing plasmids in fish in China. The IncHI2 plasmid pHNBYF33-1 may be transmitted from ducks, considering the common duck-fish freshwater aquaculture system in Guangdong. Tn*7051* is likely responsible for the transfer of *bla*$_{NDM-5}$ from IncX3 to IncHI2 plasmids in *Enterobacteriaceae*, resulting in the expansion of transmission vectors of *bla*$_{NDM-5}$.

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INTRODUCTION

Carbapenemase-producing *Enterobacteriaceae* (CPE), especially New Delhi metal-β-lactamase (NDM) producers, have been increasingly reported worldwide and pose a significant challenge to public health (Wu et al., 2019). Since the discovery of NDM-1 in 2008 (Yong et al., 2009), NDM-producing *Enterobacteriaceae* have spread globally. To date, 41 NDM enzyme variants (NDM-1–NDM-41) (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#NDM) have been identified, with the *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$ genes being the most prevalent (Wu et al., 2019).

In 2011, *bla*$_{NDM-5}$ was first reported in an *E. coli* strain isolated from a patient in the UK (Hornsey et al., 2011). Since then, *bla*$_{NDM-5}$ has been detected in more than 40 countries (https://www.ncbi.nlm.nih.gov/pathogens/microbigge/#*bla*$_{NDM-5}$). NDM-5 is the most common NDM variant in *E. coli*, especially in China and Southeast Asia. Although *bla*$_{NDM-5}$ is widespread due to diverse self-transferable plasmids such as IncX3 and IncF (FII, FIA, and FIB) (Wu et al., 2019), it is rarely reported in the IncHI2 plasmid, except in swine- and duck-origin *E. coli* from Guangdong Province, China (Ma et al., 2021; Zhao et al., 2021b).

In China, NDM-5-producing *Enterobacteriaceae* have been widely detected in humans (Tian et al., 2020), farm animals (Ma et al., 2021), companion animals (Wang et al., 2021a), wild animals (Wang et al., 2017), retail meats (Zhang et al., 2019), and the environment (Zhao et al., 2021a), but rarely in aquatic products. Aquatic products are also considered important reservoirs and transmission vectors of resistant bacteria (Xu et al., 2020). Of note, the integrated duck-fish freshwater aquaculture system is very common in Guangdong, and antimicrobial resistant bacteria can be transmitted between ducks and fish (Shen et al., 2020). Grass carp (*Ctenopharyngodon idella*) is the most popular freshwater fish in aquaculture and is cultivated in 32 provinces in China. According to the China Fishery Statistical Yearbook 2020 (https://data.cnki.net/trade/Yearbook/Single/N2021020168?z=Z009), the production of grass carp reached 5.5 million tons in 2019, accounting for 21.7% of the maximum annual production in freshwater fish. However, the occurrence of clinically important resistant bacteria, such as CPE, in grass carp has rarely been studied. Hence, we investigated the prevalence of CPE in intestinal samples of grass carp from wet markets in Guangzhou and characterized *bla*$_{NDM}$-positive isolates and plasmids to understand the transmission mechanism of *bla*$_{NDM5}$ in aquatic products.

MATERIALS AND METHODS

Sample collection, bacterial isolation, and detection of *bla*$_{NDM}$

In January 2019, a total of 196 intestinal samples from grass carp were randomly collected from 24 wet markets located in seven districts of Guangzhou, China. We collected fish samples from different stalls, with three intestinal samples randomly collected from each sampling booth. Each sample was placed in a separate sterile sample bag and transported to the laboratory in a freezer box for processing within 12 h. The fish intestines were dissected with sterile surgical scissors, and 1 g of intestinal content was enriched in 2 mL of Luria-Bertani (LB) broth at 37 °C overnight with shaking. The overnight cultures were streaked onto MacConkey agar plates supplemented with 1 mg/L meropenem and incubated at 37 °C for 18–24 h. *Enterobacteriaceae* colonies with different morphologies were selected from the plates to screen for *bla*$_{NDM}$-, *bla*$_{KPC}$-, and *bla*$_{OXA-48}$-positive isolates using polymerase chain reaction (PCR) with specific primers as described previously (Poirel et al., 2011).

Antimicrobial susceptibility testing

According to the recommendations of the Clinical and Laboratory Standards Institute (2017), the minimal inhibitory concentrations (MICs) of 18 antimicrobials against NDM-positive *Enterobacteriaceae* isolates were determined using the agar dilution or broth microdilution (colistin and tigecycline) methods. *Escherichia coli* ATCC 25922 was used as the control. The MICs were interpreted according to the criteria of CLSI (M100-S30) and EUCAST (http://www.eucast.org).

Conjugation experiments

In this study, streptomycin-resistant *E. coli* C600 was used as the recipient, and each *bla*$_{NDM}$-positive isolate was used as the donor for conjugation by broth mating at 37 ℃ for 16–20 h. Transconjugants were selected on MacConkey agar plates supplemented with 3 000 mg/L streptomycin and 1 mg/L meropenem. Conjugation frequency was calculated following previously reported methods (Chen et al., 2007).

Whole-genome sequencing and bioinformatics analysis

Whole-genomic DNA of NDM-positive isolates was sequenced using the Illumina Hiseq X Ten and Oxford Nanopore MinIon platforms, and complete genomes were obtained by hybrid assembly using Unicycler v0.4.7 (Wick et al., 2017). MLST v2.19 (https://github.com/tseemann/mlst) was used to identify the sequence type (ST) of the *bla*$_{NDM}$-positive strains. Plasmid replicons, antimicrobial resistance genes, and heavy metal resistance genes were analyzed using ABRicate v1.0 (https://github.com/tseemann/abricate) with the PlasmidFinder (Carattoli et al., 2014), ResFinder (Zankari et al., 2012), and AMRFinderPlus databases (https://github.com/ncbi/amr), respectively. Plasmid double-locus sequence typing (pDLST) for IncHI2 plasmids was identified using pMLST v2.0 (https://cge.cbs.dtu.dk/services/pMLST/). Insertion sequence (IS) elements were identified using ISfinder (https://isfinder.biotoul.fr/). Single nucleotide polymorphism (SNP) calling was performed using Snippy (https://github.com/tseemann/snippy). The *bla*$_{NDM}$-carrying plasmids were further compared and analyzed using the BLAST ring image generator (Alikhan et al., 2011). The genetic context of *bla*$_{NDM}$ was analyzed by Galileo™ AMR (http://galileoamr.arcbio.com/mara/), Gene Construction Kit v4.5 software (Textco BioSoftware, USA), and Easyfig v2.2.5 (http://mjsull.github.io/Easyfig/files.html).

Nucleotide sequence accession numbers

The complete genome sequences of seven *bla*$_{NDM-5}$-positive

Enterobacteriaceae were deposited in GenBank under BioProject No. PRJNA636005.

RESULTS

Characterization of $bla_{\text{NDM-5}}$-carrying isolates

A total of seven (3.57%) unduplicated carbapenem-resistant isolates, including six *E. coli* and one *C. freundii*, were obtained from the seven intestinal samples of grass carp (Table 1). All seven isolates were identified as $bla_{\text{NDM-5}}$-positive by PCR and sequencing, while bla_{KPC} and $bla_{\text{OXA-48}}$ were not detected.

The seven $bla_{\text{NDM-5}}$-carrying isolates showed multidrug-resistant phenotypes and harbored multiple resistance genes (Tables 1, 2). Molecular typing results showed that the *C. freundii* strain belonged to ST557. Six of the NDM-5-positive *E. coli* strains belonged to five different STs, namely ST48, ST57, ST101, ST155, and ST9124. The two ST48 *E. coli* isolates (PY9F04M and PY9F07M) were recovered from the same market but from different sample booths (Table 1) and were related as they showed only 10 core-genome SNP (cgSNP) differences from each other (Schürch et al., 2018), although the resistance genes they carried were not the same (Table 1).

Characterization of $bla_{\text{NDM-5}}$-bearing plasmids

The conjugation experiments indicated that the seven $bla_{\text{NDM-5}}$-

Table 1 Characterization of $bla_{\text{NDM-5}}$-carrying *Escherichia coli* and *Citrobacter freundii* isolates

Isolates	Species	MLST	Farmers market (FM)	Other resistance genes	Heavy metal-resistant genes	Chromosomal mutations	Location of $bla_{\text{NDM-5}}$	Plasmid size (bp)	Conjugation frequency[a]
BY9F33M	*E. coli*	ST57	FM4	***aac(3)-IV*, *aadA2b*, *aph(3'')-Ib*, *aph(3')-Ia*, *aph(6)-Id*, *aph(4)-Ia*, *bla*$_{\text{TEM-1B}}$, *floR*, *sul3*, *cmlA1*** *aadA1*, *aadA5*, *aph(3')-IIa*, *mdf(A)*, *mph(A)*, *qnrS1*, *arr-2*, *sul1*, *tet(A)*, *dfrA14*, *dfrA27*	***terDZW***, *merPRT*, *arsADR*	GyrA (p.S83L, p.D87N)ParC (p.S80I)	**IncHI2,** IncFIB, IncX1, IncY	238 926	(4.93±0.91)×10^{-6}
BY9F36M	*C. freundii*	ST557	FM4	*aac(3)-IId*, *aadA16*, *aac(6')-Ib-cr*, *aph(3'')-Ib*, *aph(6)-Id*, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CMY-129}}$, *mph(A)*, *floR*, *qnrB18*, *qnrB26*, *qnrB6*, *arr-3*, *sul1*, *sul2*, *tet(A)*, *dfrA27*	*arsADR*, *merCPTR*	N.D.	**IncX3**, IncFIB (K)	46 161	(2.05±0.21)×10^{-4}
PY9F04M	*E. coli*	ST48	FM21	*aadA1*, *aadA2*, *aph(3')-Ia*, *bla*$_{\text{OXA-10}}$, *mdf(A)*, *mph(A)*, *erm(42)*, *cmlA1*, *floR*, *oqxAB*, *arr-2*, *sul2*, *tet(A)*, *tet(M)*, *dfrA12*, *dfrA14*	*pcoABCDESR*, *iucABCD*, *silABCEFPRS*	GyrA (p.S83L, p.D87N)ParC (p.S80I)	**IncX3**, IncFII, IncFIB, IncFIA	46 161	(3.75±0.80)×10^{-5}
PY9F07M	*E. coli*	ST48	FM21	*ant(3'')-Ia*, *aadA1*, *aadA2*, *aph(3')-Ia*, *bla*$_{\text{OXA-10}}$, *mdf(A)*, *mph(A)*, *erm(42)*, *cmlA1*, *floR*, *oqxAB*, *arr-2*, *sul2*, *tet(A)*, *tet(M)*, *dfrA12*, *dfrA14*	*pcoABCDESR*, *iucABCD*, *silABCEFPRS*	GyrA (p.S83L, p.D87N)ParC (p.S80I)	**IncX3,** IncFII, IncFIB, IncFIA	46 161	(2.88±1.41)×10^{-5}
PY9F09M	*E. coli*	ST155	FM21	*aadA1*, *bla*$_{\text{OXA-10}}$, *mdf(A)*, *cmlA1*, *floR*, *qnrS1*, *arr-2*, *tet(A)*, *dfrA14*	–	N.D.	**IncX3**, IncFIB	46 161	(4.87±0.25)×10^{-5}
TH9F11M	*E. coli*	ST101	FM8	***aac(3)-IV*, *aadA2b*, *aph(3'')-Ib*, *aph(3')-Ia*, *aph(6)-Id*, *aph(4)-Ia*, *bla*$_{\text{OXA-10}}$, *bla*$_{\text{CTX-M-55}}$, *fosA3*, *cmlA1*, *floR*, *qnrS1*, *sul3*, *tet(A)*, *dfrA1*, *dfrA14*, *arr-3***, *mdf(A)*, *arr-2*, *sul2*	***iroBCDEN*, *arsADR*, *terDZW***	GyrA (p.S83L, p.D87N)ParC (p.S80I)ParE (p.S458A)	**IncHI2-IncF**, IncFII, p0111	407 456	(2.79±0.35)×10^{-5}
HZ9F01M	*E. coli*	ST9124	FM12	*aadA22*, *aph(3')-Ia*, *aph(6)-Id*, *bla*$_{\text{TEM-1B}}$, *mdf(A)*, *mph(A)*, *lnu(F)*, *floR*, *qnrS1*, *arr-2*, *sul3*, *tet(A)*, *dfrA14*	*terDZW*	GyrA (p.S83L, p.D87N)ParC (p.S80I)	**IncX3**, IncFII, IncHI2, IncI1	46 161	(2.35±0.31)×10^{-5}

[a]: Average±Standard error (*SE*). N.D.: Not detected. Bold: $bla_{\text{NDM-5}}$–carrying plasmids and other resistance genes.

Table 2 Antibiotic susceptibility of $bla_{\text{NDM-5}}$-carrying isolates and their transconjugants

Isolates	Species	MIC (mg/L)																	
		AMP	FOX	CTX	CAZ	IPM	APR	STR	CIP	DOX	TET	TIG	AMI	GEN	NEO	CL	SXT	FLR	FOS
BY9F33M	*E. coli*	>128	>128	>128	>128	8	>128	128	32	64	>128	0.5	1	32	128	0.25	32	>128	16
BY9F33M-1T		>128	128	32	>128	4	>128	>256	0.25	4	16	0.25	1	8	128	0.125	16	64	16
BY9F36M	*C. freundii*	>128	>128	>128	>128	8	8	128	8	32	128	0.5	1	32	1	0.25	32	128	16
BY9F36M-1T		>128	>128	64	>128	4	8	>256	0.008	1	1	0.25	1	0.5	1	0.125	0.25	2	16
PY9F04M	*E. coli*	>128	>128	128	>128	8	8	64	32	64	128	0.5	2	0.5	128	0.25	32	128	16
PY9F04M-1T		>128	>128	64	>128	4	8	>256	0.008	1	1	0.25	1	0.5	1	0.125	0.25	2	16
PY9F07M	*E. coli*	>128	>128	128	>128	8	8	64	32	64	128	0.5	2	0.5	128	0.25	32	128	16
PY9F07M-1T		>128	>128	128	>128	4	8	>256	0.004	8	1	0.25	2	0.5	1	0.125	0.25	2	16
PY9F09M	*E. coli*	>128	>128	128	>128	8	8	32	0.25	32	128	0.5	2	0.25	1	0.25	8	128	16
PY9F09M-1T		>128	>128	64	>128	4	8	>256	0.004	1	1	0.25	2	0.25	1	0.125	0.25	2	16
TH9F11-1M	*E. coli*	>128	>128	>128	>128	8	>128	256	128	64	>128	1	1	32	128	0.125	32	128	>256
TH9F11-1M-1T		>128	>128	128	>128	4	>128	>256	0.25	16	128	0.5	1	8	>128	0.125	16	64	>256
HZ9F01M	*E. coli*	>128	>128	128	>128	8	8	256	32	64	>128	1	2	0.5	64	0.25	32	128	16
HZ9F01M-1T		>128	>128	64	>128	4	8	>256	0.25	1	64	0.5	2	0.5	1	0.125	0.25	2	16

MIC, minimal inhibitory concentration. AMP, ampicillin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; APR, apramycin; STR, streptomycin; CIP, ciprofloxacin; DOX, doxycycline; TET, tetracycline; TIG, tigecycline; AMI, amikacin; GEN, gentamicin; NEO, neomycin; CL, colistin; SXT, sulfamethoxazole/trimethoprim; FLR, florfenicol; FOS, fosfomycin.

carrying plasmids could be successfully transferred to the recipient *E. coli* C600 strain, and replicon typing results revealed that the $bla_{\text{NDM-5}}$ genes were located on IncX3 (*n*=5), IncHI2 (*n*=1), and IncHI2-IncF (*n*=1). The conjugation frequencies of the IncX3-type plasmids varied from ~10^{-4} to 10^{-5} cells/donor, while the conjugation frequencies of the IncHI2-type and IncHI2-IncF-type plasmids were ~10^{-6} and ~10^{-5} cells/donor, respectively (Table 1).

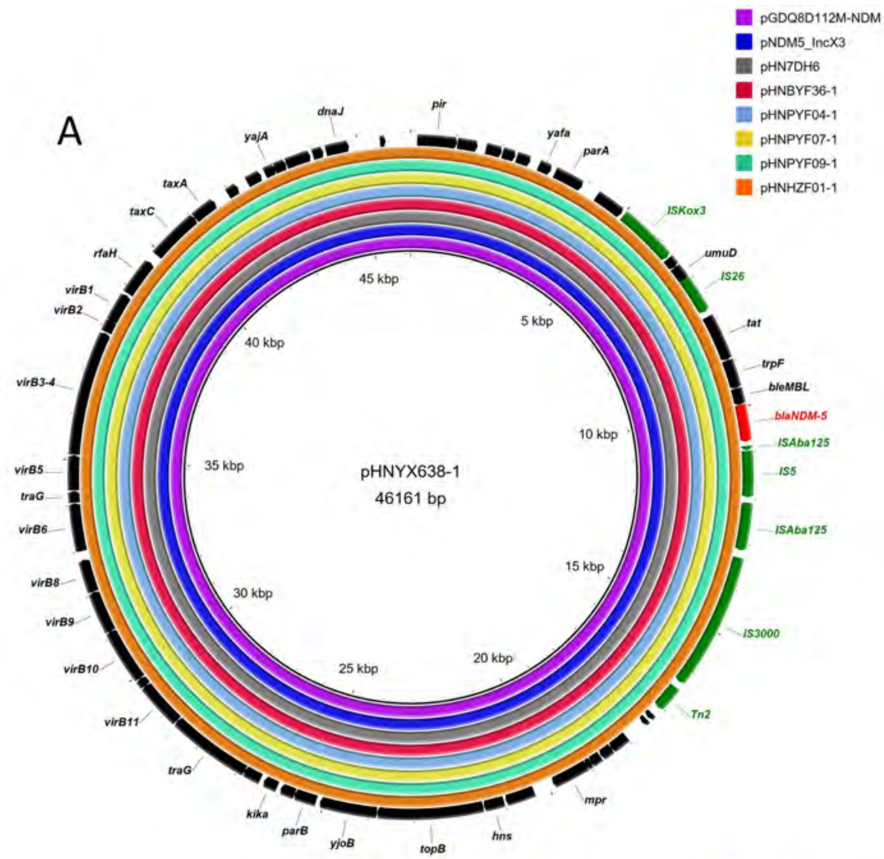
The complete sequences of all seven $bla_{\text{NDM-5}}$-bearing plasmids were obtained using Illumina and Nanopore sequencing. The sequences of five IncX3 plasmids were similar to previously reported $bla_{\text{NDM-5}}$-bearing IncX3 plasmids, including plasmids pGDQ8D112M-NDM (GenBank Accession No. MK628734, duck, China), pNDM5_IncX3 (KU761328.1, *Homo sapiens*, China), pHNYX638-1 (MK033577, pork, China), and pHN7DH6 (MN276080, dog, China) (Figure 1A).

Plasmid pHNBYF33-1, which belonged to IncHI2-ST3, was 238 926 bp in length with a GC content of 46.30% and carried 12 resistance genes. The BLASTn results indicated that plasmid pHNBYF33-1 exhibited high similarity (≥99.9% identity and ≥93.4% coverage) to four $bla_{\text{NDM-5}}$-carrying IncHI2 plasmids deposited in GenBank, i.e., pNDM33-1 (MN915011) (Zhao et al., 2021b), GFQ9D68 Contig5 (JAGFYC010000005), GDQ8D151 plasmid1 (JAGFYD010000002), and GDQ20D15 plasmid1 (JAGFYB010000003) (Figure 1B). Interestingly, all four plasmids were carried by *E. coli* strains recovered from ducks in Guangdong, China.

Plasmid pHNTH9F11-1 (IncHI2-IncF) was 407 456 bp in size and had an average GC content of 48.03%. pHNTH9F11-1 harbored three different replicons, including IncHI2, IncFII, and IncFIB. BLASTn analysis showed that pHNTH9F11-1 was a cointegrate plasmid comprised of sequences of IncHI2 (designated as pHNTH9F11-1_IncHI2), harboring $bla_{\text{NDM-5}}$, and IncF24:A-:B1 (designated as pHNTH9F11-1_IncF) (Figure 1C). In addition, the hybrid plasmid pHNTH9F11-1 had 87% nucleotide sequence coverage of the IncHI2-IncFII plasmid pP2-3T (MG014722, swine, China). The sequence of plasmid pHNTH9F11-1_IncHI2 harboring $bla_{\text{NDM-5}}$ was similar (≥99.99% identity and 100% coverage) to that of the $bla_{\text{NDM-5}}$-carrying plasmid pHNGD64-NDM (MW296099) from a swine *E. coli* strain (Ma et al., 2021). Plasmid pHNTH9F11-1_IncF exhibited similarity (≥99.99% identity and ≥90% coverage) to IncF24:A-:B- plasmid pPK8568-156kb (CP080127, chicken, Pakistan), carrying multiple heavy metal resistance genes (*arsABCD* and *sitABCD*) and a phage resistance system (BREX, bacteriophage exclusion system) (Goldfarb et al., 2015). Further analysis revealed that pHNTH9F11-1_IncF and pHNTH9F11-1_IncHI2 were bound by two identical ΔTn*1721* transposons (*hp-tetR-tet*(A)-*eamA*) with a length of 5 492 bp, suggesting that the cointegrate plasmid pHNTH9F11-1 was formed by homologous recombination of these two plasmids through ΔTn*1721* (Figure 1C).

Genetic contexts of $bla_{\text{NDM-5}}$ genes in IncX3 and IncHI2

All five $bla_{\text{NDM-5}}$-carrying IncX3 plasmids showed identical genetic contexts (i.e., IS*3000*-ΔIS*Aba125*-IS*5*-ΔIS*Aba125*-$bla_{\text{NDM-5}}$-ble_{MBL}-*trpF-tat*-IS*26*-Δ*umuD*-IS*Kox3*) (Figure 2), similar to that of the classical IncX3 plasmid pHNYX658-1 (Zhang et al., 2019). The genetic contexts of $bla_{\text{NDM-5}}$ in pHNTH9F11-1_IncHI2 and pHNBYF33-1 (IncHI2) were similar to other $bla_{\text{NDM-5}}$-carrying IncHI2 plasmids in GenBank, including pNDM33-1, GDQ8D151 plasmid1, GFQ9D68 Contig5, and GDQ20D15 plasmid1 (Figure 2). In these four IncHI2-type plasmids, the $bla_{\text{NDM-5}}$ gene was identically embedded in a novel composite transposon (IS*3000*-ΔIS*Aba125*-IS*5*-ΔIS*Aba125*-$bla_{\text{NDM-5}}$-ble_{MBL}-*trpF-tat*-Δ*dct*-IS*26*-Δ*umuD*-ΔIS*Kox3*-IS*3000*) inserted between IS*1* and IS*10R* of the multidrug resistance region of the IncHI2 plasmid with 5 bp target site duplications (TSDs) (ACTTT). Previous research has found that excision of this transposon from the plasmid pNDM33-1 forms a circular intermediate (Zhao et al., 2021b). Here, we renamed this novel 13 918 bp long


A
pGDQ8D112M-NDM
pNDM5_IncX3
pHN7DH6
pHNBYF36-1
pHNPYF04-1
pHNPYF07-1
pHNPYF09-1
pHNHZF01-1
pHNYX638-1
46161 bp
45 kbp
5 kbp
10 kbp
15 kbp
20 kbp
25 kbp
30 kbp
35 kbp
40 kbp
pir
yafa
parA
ISKox3
umuD
IS26
tat
trpF
bleMBL
blaNDM-5
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IS5
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IS3000
Tn2
mpr
hns
topB
yjoB
parB
kika
traG
virB11
virB10
virB9
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virB2
virB1
rfaH
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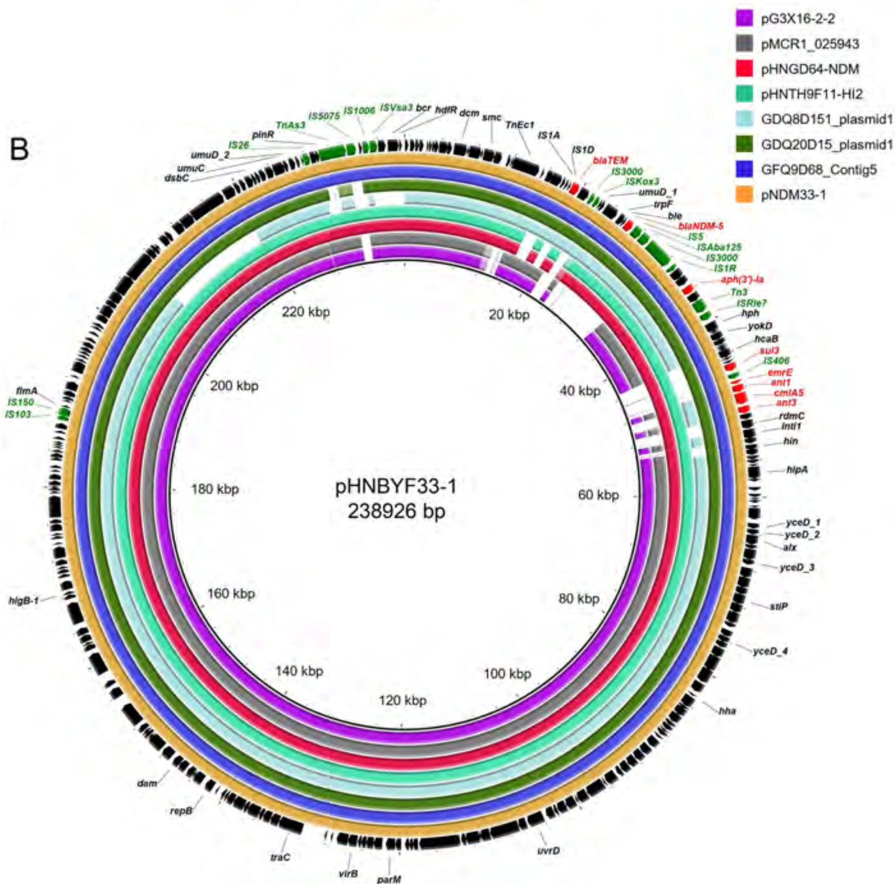
taxA
yajA
dnaJ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pMCR1_025943
pHNGD64-NDM
pHNTH9F11-HI2
GDQ8D151_plasmid1
GDQ20D15_plasmid1
GFQ9D68_Contig5
pNDM33-1
pHNBYF33-1
238926 bp
20 kbp
40 kbp
60 kbp
80 kbp
100 kbp
120 kbp
140 kbp
160 kbp
180 kbp
200 kbp
220 kbp
dsbC
umuC
umuD_2
IS26
pinR
TnAs3
IS5075
IS1006
ISVsa3
bcr
hdfR
dcm
smc
TnEc1
IS1A
IS1D
blaTEM
IS3000
ISKox3
umuD_1
trpF
ble
blaNDM-5
IS5
ISAba125
IS3000
IS1R
aph(3')-Ia
Tn3
ISRle7
hph
yokD
hcaB
sul3
IS406
emrE
ant1
cmlA5
ant3
rdmC
intI1
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IS103
IS150
fimA

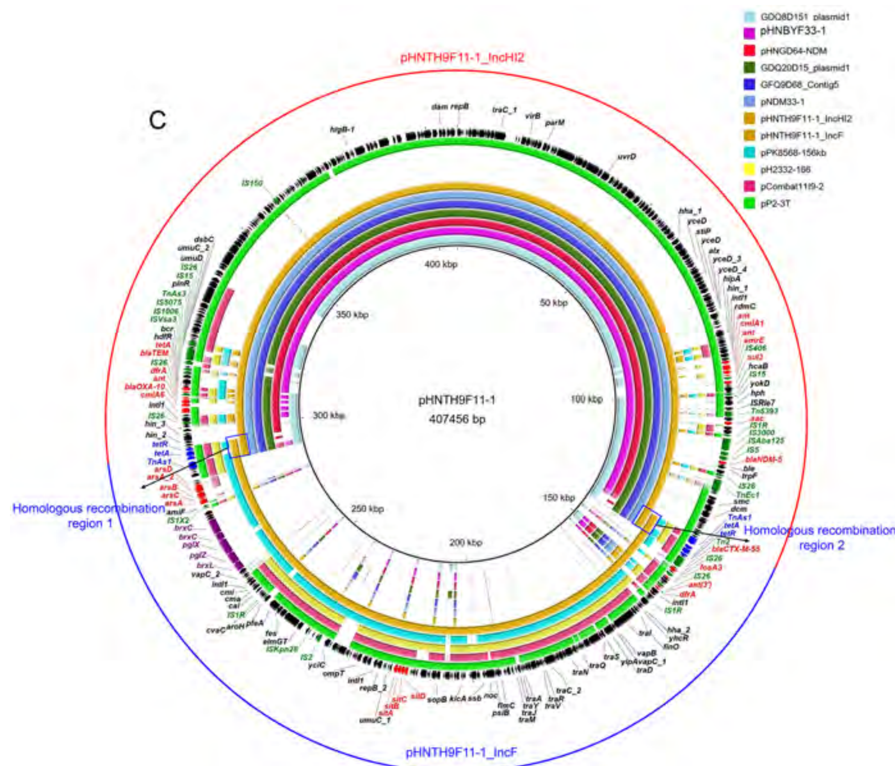

Figure 1 Comparison of *bla*$_{NDM-5}$-carrying plasmids

A: *bla*$_{NDM-5}$-harboring IncX3 plasmids in this study with other similar plasmids. Plasmid pHNYX638-1 (MK033577) is reference plasmid. pGDQ8D112M-NDM (MK628734), pNDM5_IncX3 (CP065346), pHN7DH6 (MN276080), pHNBYF36-1, pHNPYF04-1, pHNPYF07-1, pHNPYF09-1, and pHNZF01-1. B: *bla*$_{NDM-5}$-harboring IncHI2 plasmid pHNBYF33-1 in this study with other similar plasmids. pG3X16-2-2 (CP038139), pMCR1_025943 (CP027202), pHNGD64-NDM (MW296099), pHNTH9F11-HI2 (this study), GDQ8D151 plasmid1 (JAGFYD010000002), GDQ20D15 plasmid1 (JAGFYB010000003), GFQ9D68 Contig5 (JAGFYC010000005), and pNDM33-1 (MN915011). C: *bla*$_{NDM-5}$-harboring IncHI2-IncF24:A-:B1 plasmid pHNTH9F11-1 in this study with other plasmids. GDQ8D151 plasmid1 (JAGFYD010000002), pHNBYF33-1 (this study), pHNGD64-NDM (MW296099), GDQ20D15 plasmid1 (JAGFYB010000003), GFQ9D68 Contig5 (JAGFYC010000005), pNDM33-1 (MN915011), pHNTH9F11-1_IncHI2, pHNTH9F11-1_IncF, pPK8568-156kb (CP080127), pH2332-166 (NC_025175), pCombat11I9-2 (CP021728), and pP2-3T (MG014722). All rings are represented from inside to outside. Red arrows, resistance genes; green arrows, mobile genetic elements; purple arrows, phage resistance system; blue arrows, homologous recombination regions. Red and blue semicircles in C represent pHNTH9F11-1_IncHI2 and pHNTH9F11-1_IncF, respectively.

transposon as Tn*7051* (https://transposon.lstmed.ac.uk/). Comparative analysis demonstrated that Tn*7051* shared 99.98% nucleotide sequence similarity (two SNP differences) with the genetic context of *bla*$_{NDM-5}$ in the IncX3 plasmid pHNYX638-1 (MK033577, pork, China), except that IS*Kox3* in Tn*7051* was truncated by a copy of IS*3000*, creating only 545 bp remains (ΔIS*Kox3*) (Figure 2). Interestingly, when comparing the Tn*7051* sequence, we found a Tn*7051*-like structure (14 482 bp) in three hybrid plasmids obtained from swine *E. coli* isolates in China (Yao et al., 2020), namely p4M9F (IncFIA-IncHI1A-IncHI1B, MN256759), p4M8F (IncHI1-IncY-IncFIA-IncFIB, MN256758), and p4M18F (IncHI1-IncY-IncFIA-IncFIB, MN256757). In the Tn*7051*-like structure, IS*Kox3* had more residues (1 108 bp) than that in Tn*7051*. Furthermore, the Tn*7051*-like structure exhibited 99.97%–100.00% nucleotide sequence identity (0–3 SNP differences) to the genetic context of *bla*$_{NDM-5}$ in the IncX3 plasmid pHNYX638-1. Given that Tn*7051* and Tn*7051*-like transposons were similar to the genetic context of *bla*$_{NDM-5}$ in the IncX3 plasmid pHNYX638-1, we speculated that both Tn*7051* and Tn*7051*-like transposons were likely derived from the IncX3 plasmid.

Although the IncHI2 plasmids pHNBYF33-1 and pNDM33-1 shared the same Tn*7051* insertion site (Figure 2), compared with pNDM33-1, the plasmid pHNBYF33-1 lacked the ΔIS*3000*-ΔIS*10*-IS*26*-*lnu*(F)-*aadA2*-*hp*-IS*26* segment, which could be readily explained by a deletion event mediated by two copies of IS*26* located in the same orientation (Harmer & Hall, 2016). The genetic context of *bla*$_{NDM-5}$ in the hybrid plasmid pHNTH9F11-1 was the same as that in plasmid pHNGD64-NDM, and was very similar to pNDM33-1, except for the lack of the IS*26*-Δ*umuD*-ΔIS*Kox3*-IS*3000*-ΔIS*10*-IS*26*-*lnu*(F)-*aadA2*-*hp*-IS*26*-*hp*-IS*26*-*bla*$_{TEM}$-IS*1X* unit (Figure 2). This may be due to the deletion of genes mediated by homologous recombination between two copies of IS*26* in the same direction (i.e., IS*26* in Tn*7051* and IS*26* upstream of ΔTn*Ec1*), as IS*26* located upstream of ΔTn*Ec1* had only an 8 bp TSD (CTTCTGGT) on one side (Figure 2).

Figure 2 Genetic contexts of $bla_{\text{NDM-5}}$ gene in IncX3 and IncHI2 plasmids

Regions with ≥99.0% nucleotide sequence identity are shaded blue. Open reading frames (ORFs) are depicted by arrows. Red, green, purple, and gray arrows represent resistance gene, insertion sequence, IS*Kox3*, and hypothetical protein, respectively. Rods represent direct repeat sequences.

Proposed formation model of genetic contexts of $bla_{\text{NDM-5}}$ in IncHI2 plasmids

Based on detailed sequence analysis, the co-integration mechanism of IS*26* (Harmer & Hall, 2016), and the copy-out-paste-in mechanism of composite transposons (Piégu et al., 2015), we proposed a genetic environment formation model of $bla_{\text{NDM-5}}$ in plasmids pHNBYF33-1 and pHNTH9F11-1, as shown in Figure 3. The assumed plasmid evolution process was as follows: IS*3000* was inserted into IS*Kox3* of the IncX3 plasmid (Figure 3A), thus forming the IS*3000*-ΔIS*Aba125*-IS*5*-ΔIS*Aba125*-$bla_{\text{NDM-5}}$-bla_{MBL}-*trpF*-*tat*-Δ*dct*-IS*26*-Δ*umuD*-ΔIS*kox3*-IS*3000*-ΔIS*kox3* unit (Figure 3B), hypothesized due to the absence of this unit in GenBank. The two same-orientated copies of IS*3000*, surrounding $bla_{\text{NDM-5}}$, generated the circular intermediate Tn*7051* (Figure 3C), which was further inserted into the region between IS*1* and IS*10* of the IncHI2 plasmid (Figure 3D) with 5 bp TSDs (ACTTT), resulting in the formation of the $bla_{\text{NDM-5}}$-carrying IncHI2 plasmids (i.e., pNDM33-1, MN915011; GFQ9D68 Contig5, JAGFYC010000005; GDQ8D151 plasmid1, JAGFYD010000002; and GDQ20D15 plasmid1, JAGFYB010000003) (Figure 3E). IS*3000*, located downstream of IS*Kox3*, was truncated by IS*26* and, consequently, the IncHI2 plasmids evolved into the structure shown in Figure 3F (hypothesized due to the absence of a similar structure in GenBank). The IS*26* that previously truncated IS*3000* was recombined with the IS*26* adjacent to $bla_{\text{TEM-1}}$ (purple frame in Figure 3F), leading to the deletion of the ΔIS*3000*-ΔIS*10*-IS*26*-*lnu*(F)-*aadA2*-*hp*-IS*26* segment. As a result, the structure of the hypothetical plasmids (Figure 3F) entered the $bla_{\text{NDM-5}}$-carrying plasmid pHNBYF33-1 (Figure 3G). Additionally, the two IS*26* elements in Figure 3F and Figure 3G (light green frame) integrated, resulting in the formation of the $bla_{\text{NDM-5}}$-carrying plasmid pHNTH9F11-1 (Figure 3H). Therefore, in summary, we speculated that Tn*7051* may contribute to the transfer of $bla_{\text{NDM-5}}$ from the IncX3 plasmids to the IncHI2 plasmids, and the genetic contexts of $bla_{\text{NDM-5}}$ on the IncHI2 plasmids in fish were likely derived from plasmids carried by ducks in Guangdong, China.

DISCUSSION

As the most common CPE, bla_{NDM}-positive *Enterobacteriaceae* have been isolated from seafood and aquatic environments in several countries (Das et al., 2019; Köck et al., 2018). Moreover, bla_{NDM}-positive *Enterobacteriaceae* have been detected in freshwater fish in Vietnam (Nakayama et al., 2022) and farmed fish in Egypt (Hamza et al., 2020). To the best of our knowledge, however,

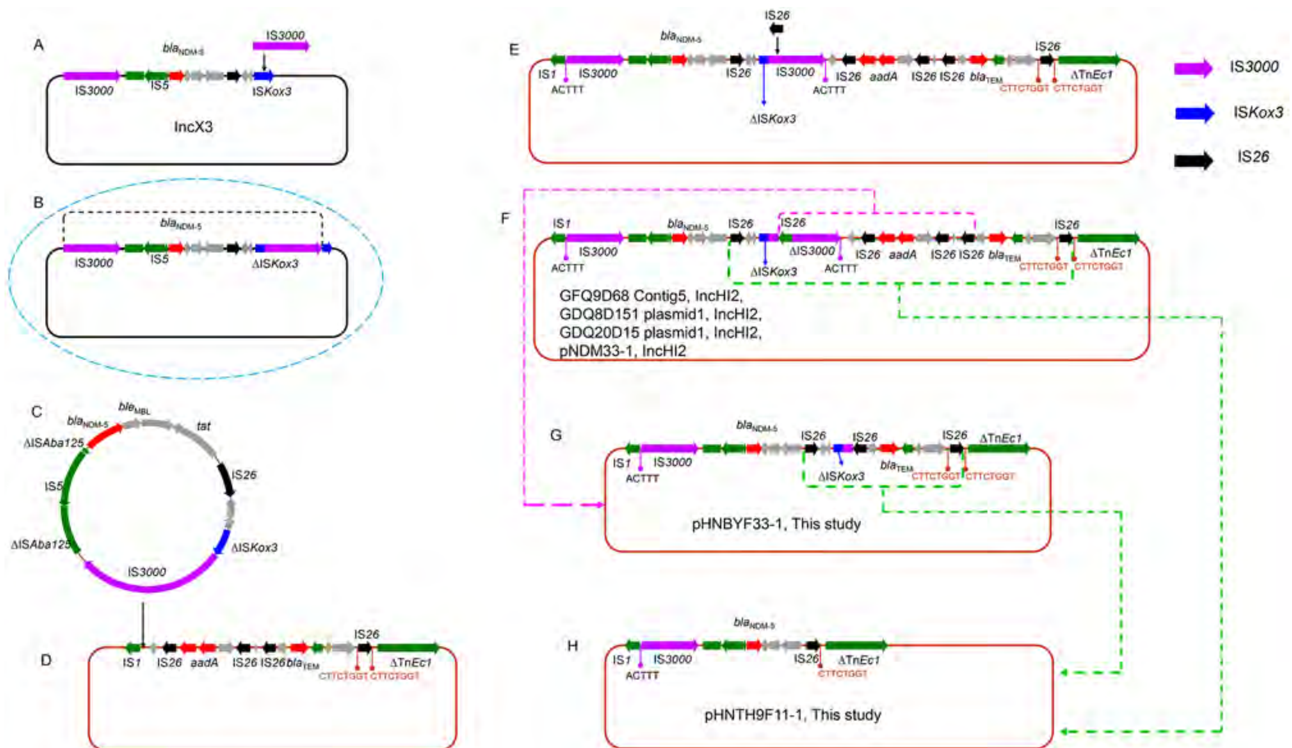

Figure 3 Proposed formation mechanism of genetic environment of *bla*$_{NDM-5}$ in plasmids pHNBYF33-1 and pHNTH9F11-1
Purple, blue, black, green, and red arrows represent IS*3000*, IS*Kox3*, IS*26*, other insertion sequence, and resistance gene, respectively.

this is the first report of *bla*$_{NDM}$ in freshwater fish from China. Of concern, as fish intestines are consumed in Guangdong, NDM-5-positive *Enterobacteriaceae* in the intestines of retail fish products could spread to humans via the food chain.

In China, IncX3 plasmids are the most common type of plasmid carrying *bla*$_{NDM-5}$ (Ma et al., 2020). NDM-5-producing IncX3 plasmids are widespread in environmental, animal, and clinical isolates (Ma et al., 2020), but are rarely reported in *Enterobacteriaceae* of freshwater fish origin. The similar IncX3 plasmids found in this study further highlight the importance of the epidemic IncX3 plasmid in the spread of the *bla*$_{NDM-5}$ gene within the entire ecosystem. IncHI2/ST3 plasmids have been reported to mediate the transfer of various antibiotic resistance genes (ARGs), such as *fosA3* (Wang et al., 2020), *floR* (Cao et al., 2020), *bla*$_{CTX-M}$ (Lü et al., 2020), and *mcr* (Long et al., 2019; Zhi et al., 2016), as well as various NDM-type carbapenemase genes, such as *bla*$_{NDM-1}$, *bla*$_{NDM-9}$, and *bla*$_{NDM-4}$ (Liu et al., 2017; Oueslati et al., 2021). However, there are very few reports of *bla*$_{NDM-5}$-carrying IncHI2 (Ma et al., 2021; Zhao et al., 2021b). Consequently, we downloaded all available complete genomes (*n*=5 974; as of 1 September 2021) of *Enterobacteriaceae* submitted to the NCBI assembly database (https://www.ncbi.nlm.nih.gov/assembly/) and found only five *bla*$_{NDM-5}$-carrying IncHI2 plasmids (four from ducks and one from swine), all of which were from Guangdong, China. Although we could not trace the location of the grass carp farms and investigate the contamination source of the *bla*$_{NDM-5}$-positive *Enterobacteriaceae*, it is worth noting that the detection rate of the *bla*$_{NDM}$ gene in duck samples from Guangdong is high (>30%) (Wang et al., 2021b) and integrated duck-fish farming is very common in Guangdong (Shen et al., 2020). In the duck-fish farm model, duck feces are discharged without treatment, and a large number of ARGs or residual agents can directly contaminate the fish ponds, promoting the transmission of ARGs between ducks and fish. Thus, considering the high similarity of the *bla*$_{NDM-5}$-bearing IncHI2 plasmids in the fish and ducks, and that the *bla*$_{NDM-5}$-bearing IncHI2 plasmid is currently only found in Guangdong, we speculate that the *bla*$_{NDM-5}$-bearing IncHI2 plasmids found in *Enterobacteriaceae* from retail fish may have been derived from duck feces-contaminated fish ponds in Guangdong. As such, greater attention should be paid to the transfer risk of antimicrobial resistant bacteria in integrated duck-fish farming.

Here, pHNTH9F11-1 (IncHI2-IncF) was identified as a hybrid plasmid, formed by homologous recombination through ΔTn*1 721*. In gram-negative bacteria, the fusion of plasmids mediated by insertion sequences, such as IS*26*, is rather universal, leading to a plasmid that can encode multiple resistance and hypervirulence genes, thereby posing a considerable threat to human health; for example, the co-integration event mediated by IS*26* between the *bla*$_{NDM-5}$-bearing IncX3 plasmid and *bla*$_{CMY-2}$-bearing IncA/C plasmid (Li et al., 2020). Moreover, the fusion of plasmids can expand the number of replicons and host range of plasmids, accelerating the dissemination of ARGs among various bacterial species (Dolejska et al., 2014; Wong et al., 2017). Of note, this fusion can also enable a non-conjugative plasmid to acquire conjugation ability, thereby facilitating the transmission of resistance genes, e.g., the recombination of non-conjugative

mcr-1-carrying P7 phage-like plasmid pD72-mcr1 and conjugative F33:A-:B- plasmid pD72-F33 mediated by IS*26*, forming cointegrate plasmid pD72C with a conjugation frequency of 8×10^{-3} cells/donor (He et al., 2019). Hence, the cointegrate plasmid pHNTH9F11-1 with multidrug resistance, heavy metal resistance, and phage resistance system (ability to resist invasion of bacteriophages) may provide an advantage for the host to survive in the environment.

Composite transposons can mediate the jump of ARGs between different DNA molecules. The novel Tn*7051* and Tn*7051*-like transposons can both be moved by a copy-out-paste-in mechanism utilizing a double-stranded circular DNA intermediate (Yao et al., 2020; Zhao et al., 2021b), thereby contributing to the transfer of the *bla*$_{NDM-5}$ gene and expanding its transmission vectors. It has been widely reported that *bla*$_{NDM-5}$ genes are mainly located on narrow-host-range plasmids (e.g., IncX3, IncF, and IncB/O) (Wu et al., 2019). However, the transfer of *bla*$_{NDM-5}$ to the IncHI2 plasmid mediated by Tn*7051* and to the IncHI1-IncY-IncFIA-IncFIB plasmid mediated by Tn*7051*-like suggested that these transposons may further accelerate the horizontal spread of the *bla*$_{NDM-5}$ gene to various strains and plasmids, like Tn*3000* and Tn*125*, which mediate the between-plasmid jumps of *bla*$_{NDM-1}$ and accelerate the transfer of *bla*$_{NDM-1}$ in different strains (Acman et al., 2021).

CONCLUSIONS

This study revealed the emergence of *bla*$_{NDM-5}$ in *Enterobacteriaceae* of fish origin in China. To the best of our knowledge, this is the first report of the *bla*$_{NDM-5}$ gene, as well as *bla*$_{NDM-5}$-bearing plasmids, in isolates from fish products in China. Our findings indicated that *bla*$_{NDM-5}$ in the IncHI2 plasmids may originate from the IncX3 plasmid, transferred by the novel composite transposon Tn*7051*. Furthermore, the *bla*$_{NDM-5}$-bearing IncHI2 plasmid may be transmitted from ducks, considering the common duck-fish freshwater aquaculture system in Guangdong. Based on the concept of “One health”, the surveillance of antibiotic resistance in aquatic products should be strengthened, and more measures should be taken to reduce the transfer of clinically important resistant bacteria, such as CPE, between food-producing animals and animal products.

DATA AVAILABILITY

The datasets in this study can be found in NCBI under BioProjectID PRJNA636005. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in the National Genomics Data Center (Nucleic Acids Res 2021), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA005844), publicly accessible at https://ngdc.cncb.ac.cn/gsa.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS

J.H.L. and L.C.L. conceived the research. X.G., Y.Y.L., M.Y.G., K.B.M., W.Y.H., and L.C.L. collected the data. L.C.L., J.H.L., Y.Y.L., X.G., and W.Y.H. analyzed and interpreted the data. Y.Y.L. and L.C.L. drafted the manuscript, J.H.L., W.Y.H., and X.G. revised the report. All authors read and approved the final version of the manuscript.

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