Characterization of NDM-5-producing Enterobacteriaceae isolates from retail grass carp (Ctenopharyngodon idella) and evidence of blaNDM-5-bearing IncHI2 plasmid transfer between ducks and fish

Lu-Chao Lv¹,², Yao-Yao Lu¹,², Xun Gao¹, Wan-Yun He¹, Ming-Yi Gao¹, Kai-Bin Mo¹, Jian-Hua Liu¹,²,*,¹

¹ College of Veterinary Medicine, Key Laboratory of Zoonosis of Ministry of Agricultural and Rural Affairs, National Risk Assessment Laboratory for Antimicrobial Resistant of Microorganisms in Animals, Guangdong Provincial Key Laboratory of Veterinary Pharmaceutics Development and Safety Evaluation, South China Agricultural University, Guangzhou, Guangdong 510642, China
² Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou, Guangdong 510642, China

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 carbanemase genes. Characterization of blaNDM-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 blaNDM-5 carriers (3.57%, 7/196). The blaNDM-5 genes were located on the IncX3 (n=5), IncHI2 (n=1), or IncHI2-IncF (n=1) plasmids. All blaNDM-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 blaNDM-5-bearing IncHI2 plasmids deposited in GenBank from Guangdong ducks. In all IncHI2 plasmids, blaNDM-5 was embedded in a novel transposon, Tn7051 (IS3000-ΔISaba125-IS5-ΔISaba125-blaNDM-5-βlæMBL-trpF-tat-Δdct-IS26-ΔumuD-ΔISKox3-IS3000), which was identical to the genetic structure surrounding blaNDM-5 found in some IncX3 plasmids. The IncHI2-IncF hybrid plasmid pHNTH9F11-1 was formed by homologous recombination of the blaNDM-5-carrying IncHI2 plasmid and a heavy-metal-resistant IncF plasmid through ΔTn1721. To the best of our knowledge, this is the first report on the characterization of blaNDM-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. Tn7051 is likely responsible for the transfer of blaNDM-5 from IncX3 to IncHI2 plasmids in Enterobacteriaceae, resulting in the expansion of transmission vectors of blaNDM-5.

Received: 10 January 2022; Accepted: 21 February 2022; Online: 22 February 2022

Foundation items: This study was supported by the National Natural Science Foundation of China (31625026, 32141002) and Innovation Team Project of Guangdong University (2019KXTD001)

*Authors contributed equally to this work

*Corresponding author, E-mail: jhliu@scau.edu.cn
Keywords: blaNDM-5; Enterobacteriaceae; Plasmid; Fish; Carbapenemase

INTRODUCTION

Carbapenem-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 blaNDM-1 and blaNDM-5 genes being the most prevalent (Wu et al., 2019).

In 2011, blaNDM-5 was first reported in an E. coli strain isolated from a patient in a hospital (Hormsey et al., 2011). Since then, blaNDM-5 has been detected in more than 40 countries (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#NDM-5). NDM-5 is the most common NDM variant in E. coli, especially in China and Southeast Asia. Although blaNDM-5 is widespread due to diverse self-transferable plasmids such as IncX3 and IncF (Fil, FIA, and FIB) (Wu et al., 2019), it is rarely reported in the IncHII 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 blaNDM-positive isolates and plasmids to understand the transmission mechanism of blaNDM in aquatic products.

MATERIALS AND METHODS

Sample collection, bacterial isolation, and detection of blaNDM

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 blaNDM-, blaKPC-, and blaOXA-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 blaNDM-positive isolate was used as the donor for conjugation by broth mating at 37 °C 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 blaNDM-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 blaNDM-carrying plasmids were further compared and analyzed using the BLAST ring image generator (Alikhan et al., 2011). The genetic context of blaNDM was analyzed by GalileoTM AMR (http://galileoamr.arcbio.com/mara/), Gene Construction Kit v4.5 software (Textco BioSoftware, USA), and Easyfig v2.2.5 (http://coli.iig Advance.com/Easyfig/files.html).

Nucleotide sequence accession numbers

The complete genome sequences of seven blaNDM-positive
Enterobacteriaceae were deposited in GenBank under BioProject No. PRJNA636005.

RESULTS

Characterization of bla<sub>NDM-5</sub>-carrying isolates
A total of seven (3.57%) unduplicated carbapenem-resistant isolates, including six <i>E. coli</i> and one <i>C. freundii</i>, were obtained from the seven intestinal samples of grass carp (Table 1). All seven isolates were identified as bla<sub>NDM-5</sub>-positive by PCR and sequencing, while bla<sub>PC</sub> and bla<sub>OXA-48</sub> were not detected.

The seven bla<sub>NDM-5</sub>-carrying isolates showed multidrug-resistant phenotypes and harbored multiple resistance genes (Tables 1, 2). Molecular typing results showed that the <i>C. freundii</i> strain belonged to ST557. Six of the NDM-5-positive <i>E. coli</i> strains belonged to five different STs, namely ST48, ST57, ST101, ST155, and ST9124. The two ST48 <i>E. coli</i> 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<sub>NDM-5</sub>-bearing plasmids
The conjugation experiments indicated that the seven bla<sub>NDM-5</sub>-bearing plasmids 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).

Table 1 Characterization of bla<sub>NDM-5</sub>-carrying <i>Escherichia coli</i> and <i>Citrobacter freundii</i> isolates

| Isolates  | Species | MLST | Farmers market (FM) | Other resistance genes | Heavy metal-resistant genes | Chromosomal mutations | Location of bla<sub>NDM-5</sub> | Plasmid size (bp) | Conjugation frequency<sup>a</sup> |
|----------|---------|------|---------------------|------------------------|-----------------------------|-----------------------|--------------------------|-----------------|------------------------|
| BY9F33M  | <i>E. coli</i> | ST57 | FM4                 | aac(3)-IV, aadA2b, aph(3’)-Ib, aph(3’)-Ia, aph(6)-ld, aph(4)-Ia, bla<sub>TEM</sub>-1b, floR, sul3, cmlA1 aadA1, aadA5, aph(3’)-Ia, mdf(A), mph(A), qnrS1, arr-2, sul1, tet(A), dfrA14, dfrA27 | terDZW, merPR, aminoglycoside resistance | N.D. | IncHI2, IncFIB | 238 926 | (4.93±0.91)x 10<sup>6</sup> |
| BY9F36M  | <i>C. freundii</i> | ST557 | FM4                | aac(3)-III Id, aadA16, aph(6)-Ib-cr, aph(3’)-Ib, aph(6)-ld, bla<sub>CMY</sub>-128, floR, qnrB18, qnrB26, qnrB6, arr-3, sul1, sul2, tet(A), dfrA27 | aminoglycoside resistance | N.D. | IncX3, IncFIB (K) | 46 161 | (2.05±0.21)x 10<sup>4</sup> |
| PY9F04M  | <i>E. coli</i> | ST48 | FM21               | aadA1, aadA2, aph(3’)-Ia, bla<sub>OXA-10</sub>, mdf(A), mph(A), erm(42), cmlA1, sulABCEFPRS floR, oxyAB, arr-2, sul2, tet(A), tet(M), dfrA12, dfrA14 | pcoABCDSR, iucABC, sulABCEFPRS | N.D. | IncX3, IncFIB, IncFI | 46 161 | (3.75±0.80)x 10<sup>5</sup> |
| PY9F07M  | <i>E. coli</i> | ST48 | FM21               | arr(3’)-Ia, aadA1, aadA2, aph(3’)-Ia, bla<sub>OXA-10</sub>, mdf(A), mph(A), erm(42), cmlA1, floR, oxyAB, arr-2, sul2, tet(A), tet(M), dfrA12, dfrA14 | pcoABCDSR, iucABC, sulABCEFPRS | N.D. | IncX3, IncFIB, IncFI | 46 161 | (2.88±1.41)x 10<sup>5</sup> |
| PY9F09M  | <i>E. coli</i> | ST155 | FM21              | aadA1, bla<sub>OXA-10</sub>, mdf(A), cmlA1, floR, qnrS1, arr-2, tet(A), dfrA14 | N.D. | N.D. | IncX3, IncFIB | 46 161 | (4.87±0.25)x 10<sup>5</sup> |
| TH9F11M  | <i>E. coli</i> | ST101 | FM8                | aac(3)-IV, aadA2b, aph(3’)-Ib, aph(3’)-Ia, aph(6)-ld, aph(4)-Ia, bla<sub>TEM</sub>-1b, bla<sub>CTX</sub>-M-45, fosA3, cmlA1, floR, qnrS1, sul3, tet(A), dfrA1, dfrA14, arr-3, mdf(A), arr-2, sul2 | iroBCDEN, aminoglycoside resistance | N.D. | IncHI2-incF, IncHi2, IncFIB, p0111 | 407 456 | (2.79±0.35)x 10<sup>5</sup> |
| HZ9F01M  | <i>E. coli</i> | ST9124 | FM12             | aadA22, aph(3’)-Ia, aph(6)-ld, bla<sub>TEM</sub>-1b, mdf(A), mph(A), dfrA27, floR, qnrS1, arr-2, sul3, tet(A), dfrA14 | terDZW | N.D. | IncX3, IncFIB, IncHI2, IncI | 46 161 | (2.35±0.31)x 10<sup>5</sup> |

*: Average±Standard error (SE). N.D.: Not detected. Bold: bla<sub>NDM-5</sub>-carrying plasmids and other resistance genes.
carrying plasmids could be successfully transferred to the recipient \textit{E. coli} C600 strain, and replication results revealed that the \textit{bla}_{NDM-5} genes were located on IncX3 (\(n=5\)), IncH2 (\(n=1\)), and IncH2-IncF (\(n=1\)). The conjugation frequencies of the \textit{IncX3}-type plasmids varied from \(10^{-6}\) to \(10^{-5}\) cells/donor, while the conjugation frequencies of the IncH2-type and IncH2-IncF-type plasmids were \(10^{-5}\) and \(10^{-4}\) cells/donor, respectively (Table 1).

The complete sequences of all seven \textit{bla}_{NDM-5}-bearing plasmids were obtained using Illumina and Nanopore sequencing. The sequences of five IncX3 plasmids were similar to previously reported \textit{bla}_{NDM-5}-bearing IncX3 plasmids, including plasmids pGDK87112M-NDM (GenBank Accession No. MK628734, duck, China), pNDM5_IncX3 (KU761328.1, \textit{Homo sapiens}, China), pHNX638-1 (MK033577, pork, China), and pHND7608 (MN276080, dog, China) (Figure 1A).

Plasmid pHNYBF3-31, which belonged to IncH2-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 pHNYBF3-31 exhibited high similarity (\(\geq99.9\%\) identity and \(\geq93.4\%\) coverage) to four \textit{bla}_{NDM-5}-carrying IncH2 plasmids deposited in GenBank, i.e., pNDM3-1 (MN915011) (Zhao et al., 2021b), GFQ9D68 Contig5 (JAGFYC01000005), GDQ8D151 plasmid1 (JAGFYD010000002), and GDQ2D15 plasmid1 (JAGFYB010000003) (Figure 1B). Interestingly, all four plasmids were carried by \textit{E. coli} strains recovered from ducks in Guangdong, China.

Plasmid pHNT9F11-1 (IncH2-IncF) was 407,456 bp in size and had an average GC content of 48.03%. pHNT9F11-1 harbored three different replicons, including IncH2, IncFII, and IncFIB. BLASTn analysis showed that pHNT9F11-1 was a cointegrate plasmid comprised of sequences of IncH2 (designated as pHNT9F11-1_IncH2), harboring \textit{bla}_{NDM-5}, and IncF24-A-B1 (designated as pHNT9F11-1_IncF) (Figure 1C). In addition, the hybrid plasmid pHNT9F11-1 had 87% nucleotide sequence coverage of the IncH2-IncFII plasmid pP2-3T (MG014722, swine, China). The sequence of plasmid pHNT9F11-1_IncH2 harboring \textit{bla}_{NDM-5} was similar (\(\geq99.9\%\) identity and 100% coverage) to that of the \textit{bla}_{NDM-5}-carrying plasmid pHNG64-NDM (MW296099) from a swine \textit{E. coli} strain (Ma et al., 2021). Plasmid pHNT9F11-1_IncF exhibited similarity (\(\geq99.9\%\) identity and \(\geq90\%\) coverage) to IncF24-A-B1: plasmid pKKB658-165kb (CP080127, chicken, Pakistan), carrying multiple heavy metal resistance genes (\textit{arsABCD} and \textit{sitABCD}) and a phage resistance system (BREX, bacteriophage exclusion system) (Goldfarb et al., 2015). Further analysis revealed that pHNT9F11-1_IncF and pHNT9F11-1_IncH2 were bound by two identical \(\Delta\text{Tn721}\) transposons (\(\text{hp-tetR-tet}(A)-\text{eamA}\)) with a length of 5,492 bp, suggesting that the cointegrate plasmid pHNT9F11-1 was formed by homologous recombination of these two plasmids through \(\Delta\text{Tn721}\) (Figure 1C).

**Table 2** Antibiotic susceptibility of \textit{bla}_{NDM-5}-carrying isolates and their transconjugants

| Isolates          | Species     |AMP (mg/L) |FOX |CTX |CAZ |IPM |APR |STR |CIP |DOX |TET |TIM |AMG |GEN |NEO |CL |SXT |FLR |FOS |
|-------------------|-------------|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|-----|-----|-----|
| BY9F33M           | \textit{E. coli} |>128 |>128 |>128 |8 |128 |32 |64 |>128 |0.5 |1 |32 |128 |0.25 |32 |>128 |16 |
| BY9F33M-1T        | \textit{E. coli} |>128 |128 |32 |>128 |>256 |0.25 |4 |16 |0.25 |1 |8 |128 |0.125 |16 |64 |16 |
| BY9F33M           | \textit{C. freundii} |>128 |>128 |>128 |8 |128 |8 |32 |128 |0.5 |1 |32 |1 |0.25 |32 |128 |16 |
| BY9F33M-1T        | \textit{C. freundii} |>128 |128 |64 |>128 |8 |>256 |0.008 |1 |1 |0.25 |1 |0.25 |0.25 |2 |16 |
| PY9F04M           | \textit{E. coli} |>128 |>128 |>128 |8 |64 |32 |64 |128 |0.5 |2 |0.5 |128 |0.25 |32 |128 |16 |
| PY9F04M-1T        | \textit{E. coli} |>128 |128 |64 |>128 |8 |>256 |0.008 |1 |1 |0.25 |1 |0.25 |1 |0.25 |2 |16 |
| PY9F07M           | \textit{E. coli} |>128 |>128 |>128 |8 |64 |32 |64 |>128 |0.5 |2 |0.5 |128 |0.25 |32 |128 |16 |
| PY9F07M-1T        | \textit{E. coli} |>128 |128 |64 |>128 |8 |>256 |0.004 |1 |1 |0.25 |2 |0.5 |1 |0.25 |2 |16 |
| TH9F11-1M         | \textit{E. coli} |>128 |>128 |>128 |8 |>256 |0.004 |1 |1 |0.25 |2 |0.5 |1 |0.25 |2 |16 |
| TH9F11-1M-1T      | \textit{E. coli} |>128 |128 |>128 |8 |>256 |0.25 |16 |16 |0.5 |1 |8 |>128 |0.125 |16 |64 |>256 |
| HZ9F010M          | \textit{E. coli} |>128 |>128 |>128 |8 |256 |32 |64 |>128 |1 |2 |0.5 |64 |0.25 |32 |128 |16 |
| HZ9F010M-1T       | \textit{E. coli} |>128 |128 |64 |>128 |8 |>256 |0.25 |1 |1 |0.25 |2 |0.5 |1 |0.25 |2 |16 |

MIC, minimal inhibitory concentration. AMP, ampicillin; FOX, cefoxitin; CTX, cefotaxime; CAZ, cefazidime; IPM, imipenem; APR, apramycin; STR, streptomycin; CIP, ciprofloxacin; DOX, doxycycline; TET, tetracycline; TIM, tigecycline; AMG, amikacin; GEN, gentamicin; NEO, neomycin; CL, colistin; SXT, sulfamethoxazole/trimethoprim; FLR, florfenicol; FOS, fosfomycin.
transposon as Tn7051 (https://transposon.lstmed.ac.uk/). Comparative analysis demonstrated that Tn7051 shared 99.98% nucleotide sequence similarity (two SNP differences) with the genetic context of blaNDM-5 in the IncX3 plasmid pHNYX638-1 (MK033577), except that ISKox3 in Tn7051 was truncated by a copy of IS3000, creating only 545 bp remains (∆ISKox3) (Figure 2). Interestingly, when comparing the Tn7051 sequence, we found a Tn7051-like structure (14,482 bp) in three hybrid plasmids obtained from swine E. coli isolates in China (Yao et al., 2020), namely pMCR1_025943 (CP027202), pHNGD64-NDM (MW296099), and pPK8568-156kb (CP080127), respectively. The genetic context of Tn7051-like structure exhibited 99.97%–100.00% nucleotide sequence identity (0–3 SNP differences) to the genetic context of blaNDM-5 in the IncX3 plasmid pHNYX638-1. The genetic context of blaNDM-5 in the IncX3 plasmid pHNYX638-1, we speculated that both Tn7051 and Tn7051-like transposons were likely derived from the IncX3 plasmid.

Although the IncH2 plasmids pHNYB533-1 and pNDM33-1 shared the same Tn7051 insertion site (Figure 2), compared with pNDM33-1, the plasmid pHNYB533-1 lacked the ∆IS26-∆umuD-∆ISKox3-∆IS10-IS26-IsuM-IsuM-F-aadA2-23S rRNA-16S rRNA segment, which could be readily explained by a deletion event mediated by two copies of IS26 located in the same orientation (Harmer & Hall, 2016). The genetic context of blaNDM-5 in the hybrid plasmid pHNT9H11-1 was the same as that in plasmid pHNGD4-NDM, and was very similar to pNDM33-1, except for the lack of the IS26-umuD-∆ISKox3-∆IS3000-∆IS10-IS26-IsuM-F-aadA2-23S rRNA-16S rRNA segment (Figure 2). This may be due to the deletion of genes mediated by homologous recombination between two copies of IS26 in the same direction (i.e., IS26 in Tn7051 and IS26 upstream of ∆TnEc1), as IS26 located upstream of ∆TnEc1 had only an 8 bp TSD (CTCTG) on one side (Figure 2).
Proposed formation model of genetic contexts of \textit{bla}_{NDM-5} in IncHI2 plasmids

Based on detailed sequence analysis, the co-integration mechanism of IS\textsubscript{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 \textit{bla}_{NDM-5} in plasmids pHNBYF33-1 and pHNTH9F11-1, as shown in Figure 3. The assumed plasmid evolution process was as follows: IS\textsubscript{3000} was inserted into IS\textsubscript{Kox3} of the IncX3 plasmid (Figure 3A), thus forming the IS\textsubscript{3000}-ΔIS\textsubscript{Aba125}-IS\textsubscript{5}-ΔIS\textsubscript{Aba125}-bla\textsubscript{NDM-5}-bla\textsubscript{MBL}-trpF-tat-dct-IS\textsubscript{26}-\textit{IS}u\textit{mUD-}\textit{ISkox3-IS3000-ΔISkox3} unit (Figure 3B), hypothesized due to the absence of this unit in GenBank. The two same-orientated copies of IS\textsubscript{3000}, surrounding \textit{bla}_{NDM-5}, generated the circular intermediate Tn\textsubscript{7051} (Figure 3C), which was further inserted into the region between IS\textsubscript{1} and IS\textsubscript{10} of the IncH2 plasmid (Figure 3D) with 5 bp TSDs (ACTTT), resulting in the formation of the IS\textsubscript{26}-\textit{IS}u\textit{mUD-ISkox3-IS3000-ΔISkox3} segment. As a result, the structure of the hypothetical plasmids (Figure 3F) entered the bla\textsubscript{NDM-5}-carrying plasmid pHNBYF33-1 (Figure 3G). Additionally, the two IS\textsubscript{26} elements in Figure 3F and Figure 3G (light green frame) integrated, resulting in the formation of the bla\textsubscript{NDM-5}-carrying plasmid pHNTH9F11-1 (Figure 3H). Therefore, in summary, we speculated that Tn\textsubscript{7051} may contribute to the transfer of \textit{bla}_{NDM-5} from the IncX3 plasmids to the IncH2 plasmids, and the genetic contexts of \textit{bla}_{NDM-5} on the IncH2 plasmids in fish were likely derived from plasmids carried by ducks in Guangdong, China.

DISCUSSION

As the most common CPE, \textit{bla}_{NDM-5}-positive Enterobacteriaceae have been isolated from seafood and aquatic environments in several countries (Das et al., 2019; Köck et al., 2018). Moreover, \textit{bla}_{NDM-5}-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, the genetic contexts of \textit{bla}_{NDM-5} in fish have not been reported.
this is the first report of blaNDM 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 blaNDM-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 blaNDM-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), blaCTX-M (Long et al., 2019; Zhi et al., 2016), as well as various NDM-type carbapenemase genes, such as blaNDM-1, blaNDM-9, and blaNDM-4 (Liu et al., 2017; Oueslati et al., 2021). However, there are very few reports of blaNDM-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 blaNDM-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 blaNDM-5-positive Enterobacteriaceae, it is worth noting that the detection rate of the blaNDM 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 blaNDM-5-bearing IncHI2 plasmids in the fish and ducks, and that the blaNDM-5-bearing IncHI2 plasmid is currently only found in Guangdong, we speculate that the blaNDM-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 ΔTn1721. In gram-negative bacteria, the fusion of plasmids mediated by insertion sequences, such as IS26, 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 IS26 between the blaNDM-5-bearing IncHI2 plasmid and blaCMY-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 IS26, forming co-integrate plasmid pD72C with a conjugation frequency of 8×10^{-5} cells/donor (He et al., 2019). Hence, the co-integrate plasmid pHNTHF11-1 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 Tn7051 and Tn7051-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 blaNDM-5 gene and expanding its transmission vectors. It has been widely reported that blaNDM-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 blaNDM-5 to the IncHI2 plasmid mediated by Tn7051 and to the IncH1-IncY-IncFIA-IncFIB plasmid mediated by Tn7051-like suggested that these transposons may further accelerate the horizontal spread of the blaNDM-5 gene to various strains and plasmids, like Tn3000 and Tn125, which mediate the between-plasmid jumps of blaNDM-1 and accelerate the transfer of blaNDM-1 in different strains (Acman et al., 2021).

CONCLUSIONS

This study revealed the emergence of blaNDM-5 in Enterobacteriaceae of fish origin in China. To the best of our knowledge, this is the first report of the blaNDM-5 gene, as well as blaNDM-5-bearing plasmids, in isolates from fish products in China. Our findings indicated that blaNDM-5 in the IncHI2 plasmids may originate from the IncX3 plasmid, transferred by the novel composite transposon Tn7051. Furthermore, the blaNDM-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://mgdc.cnbc.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.

REFERENCES

Acman M, Wang RB, van Dorp L, Shaw LP, Wang Q, Luhmann N, et al. 2021. Role of the mobiome in the global dissemination of the carbapenem resistance gene blaNDM. bioRxiv. doi:10.1101/2021.01.14.426698.

Alikhan NF, Petty NK, Ben Zakour NL, Beattson SA. 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics, 12: 402.

Cao YP, Lin QQ, He WY, Wang J, Yi MY, Lv LC, et al. 2020. Co-selection may explain the unexpectedly high prevalence of plasmid-mediated colistin resistance gene mcr-1 in a Chinese broiler farm. Zoological Research, 41(5): 569–575.

Caratoli A, Zankari E, Garcia-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrobial Agents and Chemotherapy, 58(7): 3895–3903.

Chen L, Chen ZL, Liu JH, Zeng ZL, Ma JY, Jiang HK. 2007. Emergence of RmtB metallo-β-lactamase-producing Enterobacteria coli and Enterobacter cloacae isolates from pigs in China. Journal of Antimicrobial Chemotherapy, 59(5): 880–885.

Clinical and Laboratory Standards Institute. 2017. Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. Wayne: Clinical and Laboratory Standards Institute.

Das UN, Singh AS, Lekshmi M, Nayak BB, Kumar S. 2019. Characterization of blaNDM-harbouring, multidrug-resistant Enterobacteriaceae isolated from seafood. Environmental Science and Pollution Research, 26(3): 2455–2463.

Dolejška M, Villa L, Minoia M, Guardabassi L, Caratoli A. 2014. Complete sequences of IncH1 plasmids carrying blaCTX-M-1 and qnrS1 in equine Escherichia coli provide new insights into plasmid evolution. Journal of Antimicrobial Chemotherapy, 69(9): 2388–2393.

Goldfarb T, Sberro H, Weinstock E, Cohen O, Doron S, Charpak-Amikam Y, et al. 2015. BREX is a novel phage resistance system widespread in microbial genomes. The EMBO Journal, 34(2): 169–183.

Hamza D, Dorgham S, Ismael E, El-Moez SIA, Elhariri M, Elhewel R, et al. 2020. Emergence of β-lactamase- and carbapenemase-producing Enterobacteriaceae at integrated fish farms. Antimicrobial Resistance & Infection Control, 9(1): 67.

Harmer CJ, Hall RM. 2016. IS26-mediated formation of transposons carrying antibiotic resistance genes. mSphere, 1(2): e00038–16.

He DD, Zou J, Li X, Liu XS, Li JH, Yuan L, et al. 2019. Emergence of a hybrid plasmid derived from IncN1-F33: A- bearing blaNDM-5 and Tn000 plasmids may originate from the IncX3 plasmid, transferred by IS11 and IS126 mediated by Tn000 plasmids. mSphere, 4: 1–12.

He DD, Zou J, Wang J, Yi MY, Li X, et al. 2019. Emergence of β-lactamase- and carbapenemase- producing Enterobacteriaceae isolated from seafood. Zoological Research, 40(2): 255−264, 2022.
et al. 2016. Carbenem-resistant Enterobacteriaceae in wildlife, food-producing, and companion animals: a systematic review. Clinical Microbiology and Infection, 24(12): 1241–1250.

Li RC, Xie MM, Liu LZ, Huang YL, Wu XY, Wang ZQ, et al. 2020. Characterisation of a coinserted plasmid harbouring blaNDM-5 in a clinical Salmonella Lomita strain. International Journal of Antimicrobial Agents, 55(1): 105817.

Liu BT, Song FJ, Zou M, Zhang QD, Shan H. 2017. High incidence of Escherichia coli strains harbouring mcr-1 and blaNDM from chickens. Antimicrobial Agents and Chemotherapy, 61(3): e02347–16.

Long HY, Feng Y, Ma K, Liu L, McNally A, Zong ZY. 2019. The co-transfer of plasmid-borne colistin-resistant genes mcr-1 and mcr-3.5, the carbapenemase gene blaNDM, and the 165 methylase gene mmiB from Escherichia coli. Scientific Reports, 9(1): 696.

Lü Y, Kang HQ, Fan JM. 2020. A novel blaCTX-M48s-Harboring IncHI2 plasmid pE648CTX-M-65 isolated from a clinically extensively-drug-resistant Escherichia coli ST648. Infection and Drug Resistance, 13: 3383–3391.

Ma TF, Fu JN, Xie N, Ma SZ, Lei L, Zhai WS, et al. 2020. Fitness cost of blaNDM-carrying p3R-IncX3 plasmids in wild-type NDM-free Enterobacteriaceae. Microorganisms, 8(3): 377.

Ma ZB, Zeng ZL, Liu J, Liu C, Pan Y, Zhang YA, et al. 2021. Emergence of IncHI plasmid-harboring blaNDM5 from porcine Escherichia coli isolates in Guangdong, China. Pathogens, 10(8): 954.

Nakayama T, Hoa TTT, Huyen HM, Yamaguchi T, Jinnai M, Minh DTN, et al. 2022. Isolation of carbapenem-resistant Enterobacteriaceae harbouring NDM-1, 4, 5, OXA48 and KPC from river fish in Vietnam. Food Control, 133: 108594.

Ouessal S, Emeraud C, Gosperrin V, Levy M, Cotellion G, Creton E, et al. 2021. Polyclonal dissemination of NDM-1- and NDM-9-producing Escherichia coli and Klebsiella pneumoniae in French Polynesia. Antimicrobial Agents and Chemotherapy, 65(4): e02437-20.

Piegu B, Bire S, Arenburger P, Bigot Y. 2015. A survey of transposable element classification systems—a call for a fundamental update to meet the challenge of their diversity and complexity. Molecular Phylogenetics and Evolution, 86: 90–109.

Poiriel L, Walsh TR, Cuvillier V, Nordmann P. 2011. Multiplex PCR for the detection of acquired carbapenemase genes. Diagnostic Microbiology and Infectious Disease, 70(1): 119–123.

Schürch AC, Arrendondo-Alonso S, Willems RJK, Goering RV. 2018. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene-based approaches. Clinical Microbiology and Infection, 24(4): 350–354.

Shen YB, Zhang R, Schwarz S, Wu CM, Shen JZ, Walsh TR, et al. 2020. Farm animals and aquaculture: significant reservoirs of mobile colistin resistance genes. Environmental Microbiology, 22(7): 2469–2484.

Tian DX, Wang BJ, Zhang H, Pan F, Wang C, Shi YY, et al. 2020. Dissemination of the blaNDM gene via IncX3-type plasmid among Enterobacteriaceae in Children. mSphere, 5(1): e00699–19.

Wang J, Ma ZB, Zeng ZL, Yang XW, Huang Y, Liu JH. 2017. The role of wildlife (wild birds) in the global transmission of antimicrobial resistance genes. Zoological Research, 38(2): 55–80.

Wang J, Xia YB, Huang XY, Wang Y, Lv LC, Lin QO, et al. 2021a. Emergence of blaNDM5 in Enterobacteriaceae isolates from companion animals in Guangzhou, China. Microbial Drug Resistance, 27(8): 809–815.

Wang MG, Yu Y, Wang D, Yang RS, Jia L, Cai DT, et al. 2021b. The emergence and molecular characteristics of New Delhi Metallo β-lactamase-producing Escherichia coli from ducks in Guangdong, China. Frontiers in Microbiology, 12: 677633.

Wang ZY, Xu HY, Tang YY, Li QC, Jiao XA. 2020. A multidrug-resistant monosalicin Salmonella Typhimurium Co-harboring mcr-1, fosA3, blaCTX-M-1 in a transmissible IncHI2 plasmid from a healthy catering worker in China. Infection and Drug Resistance, 13: 3569–3574.

Wick RR, Judd LM, Goriie CL, Holt KE. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Computational Biology, 13(6): e1005595.

Wong MHY, Chan EWC, Chen S. 2017. IS26-mediated formation of a virulence and resistance plasmid in Salmonella Enteritidis. Journal of Antimicrobial Chemotherapy, 72(10): 2750–2754.

Wu WJ, Feng Y, Tang GM, Qiao F, McNally A, Zong ZY. 2019. NDM Metallo-β-lactamases and their bacterial producers in health care settings. Clinical Microbiology Reviews, 32(2): e00115-18. Xu CY, Lv ZQ, Shen YB, Liu DJ, Yu YL, Zhou L, et al. 2020. Metagenomic insights into differences in environmental resistome profiles between integrated and monoculture aquaculture farms in China. Environment International, 144: 106005.

Yao H, Li AJ, Yu RH, Schwarz S, Dong HY, Du XD. 2020. Multiple copies of blaNDM-5 located on conjugal megaplasmids from porcine Escherichia coli sequence type 216 isolates. Antimicrobial Agents and Chemotherapy, 64(5): e02134–19.

Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, et al. 2009. Characterization of a new metallo-β-lactamase gene, blaNDM-5, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrobial Agents and Chemotherapy, 53(12): 5046–5054.

Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. 2012. Identification of acquired antimicrobial resistance genes. Journal of Antimicrobial Chemotherapy, 67(11): 2640–2644.

Zhang QH, Lv LC, Huang XY, Huang Y, Zhuang ZL, Lu JX, et al. 2019. Rapid increase in carbenemase-producing Enterobacteriaceae in retail meat driven by the spread of the blaNDM-5-carrying IncX3 plasmid from 2016 to 2018. Antimicrobial Agents and Chemotherapy, 63(8): e00573–19.

Zhao Q, Berglund B, Zou HY, Zhou ZY, Xia HY, Zhao L, et al. 2021a. Dissemination of blaNDM5 via IncX3 plasmids in carbenem-resistant Enterobacteriaceae among humans and in the environment in an intensive vegetable cultivation area in eastern China. Environmental Pollution, 273: 116370.

Zhou QY, Zhu JH, Cai RM, Zheng XR, Zhang LJ, Chang MX, et al. 2021b. IS26 is responsible for the evolution and transmission of blaNDM5-harboring plasmids in Escherichia coli of poultry origin in China. mSystems, 6(4): e006421.

Zhi CP, Lv LC, Yu LF, Dii Y, Liu JH. 2016. Emergence of mcr-1 colistin resistance gene. The Lancet Infectious Diseases, 16(3): 292–293.