Occurrence and Transmission of *bla*_{NDM}-Carrying *Enterobacteriaceae* from Geese and the Surrounding Environment on a Commercial Goose Farm

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ABSTRACT We investigated the prevalence and transmission of NDM-producing *Enterobacteriaceae* in fecal samples of geese and environmental samples from a goose farm in southern China. The samples were cultivated on MacConkey agar plates supplemented with meropenem. Individual colonies were examined for *bla*_{NDM}, and *bla*_{NDM}-positive bacteria were characterized based on whole-genome sequencing (WGS) data from the Illumina and Oxford Nanopore Technologies (ONT) platforms. Of 117 samples analyzed, the carriage rates for New Delhi metallo-β-lactamase (NDM)-positive *Enterobacteriaceae* were 47.1, 18, and 50% in geese, inanimate environments (sewage, soil, fodder, and dust), and mouse samples, respectively. Two variants (*bla*_{NDM-1} and *bla*_{NDM-5}, in 4 and 40 isolates, respectively) were found among 44 *bla*_{NDM}-positive *Enterobacteriaceae*; these variants belonged to eight species, and *Escherichia coli* was the most prevalent (50%). WGS analysis revealed that *bla*_{NDM} coexisted with diverse antibiotic resistance genes (ARGs). Population structure analysis showed that most *E. coli* and *Enterobacter* sp. isolates were highly heterogeneous, while most *Citrobacter* sp. and *P. stuartii* isolates possessed extremely high genetic similarities. In addition, *bla*_{NDM-5}-positive ST4358/ST48 *E. coli* isolates were found to be clonally spread between geese and the environment and were highly genetically similar to those reported from ducks, farm environments, and humans in China. Plasmid analysis indicated that IncX3 pHNYX644-1-like (*n*=40) and untypeable pM2-1-like plasmids (*n*=4) mediated *bla*_{NDM} spread. pM2-1-like plasmids possessed diverse ARGs, including *bla*_{NDM-1}, the arsenical and mercury resistance operons, and the maltose operon. Our findings revealed that the goose farm is a reservoir for NDM-positive *Enterobacteriaceae*. The *bla*_{NDM} contamination of wild mice and the novel pM2-1-like plasmid described here likely adds to the risk for dissemination of *bla*_{NDM} and associated resistance genes.

IMPORTANCE Carbapenem-resistant bacteria, in particular NDM-producing *Enterobacteriaceae*, have become a great threat to global public. These bacteria have been found not only in hospital and community environments but also among food animal production chains, which are recognized as reservoirs for NDM-producing *Enterobacteriaceae*. However, the dissemination of NDM-producing bacteria in waterfowl farms has been less well explored. Our study demonstrates that the horizontal spread of *bla*_{NDM}-carrying plasmids and the partial clonal spread of *bla*_{NDM}-positive *Enterobacteriaceae* contribute to the widespread contamination of *bla*_{NDM} in the goose farm ecosystem, including mice. Furthermore, we found a novel and transferable *bla*_{NDM-1}-carrying multidrug resistance (MDR) plasmid that possessed multiple environmental adaptation-related genes.

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The outcomes of this study contribute to a better understanding of the prevalence and transmission of *bla*_{NDM}-carrying *Enterobacteriaceae* among diverse niches in the farm ecosystem.

KEYWORDS carbapenem resistant, *Enterobacteriaceae*, New Delhi metallo- β -lactamases, transmission, goose farm, farm environment, WGS

Carbapenemase-producing multidrug-resistant (MDR) Gram-negative bacteria are a current threat to global public. In particular, the New Delhi metallo- β -lactamases (NDMs) confer resistance to almost all β -lactam antibiotics, including the carbapenems. Since the initial discovery of NDM-1 in 2008 (1), 29 NDM variants have been discovered and now have a global presence across a variety of bacterial species from diverse ecological niches (2, 3). Even though carbapenems are not approved for use in animals, there has been an increase in the incidence of NDM-producing *Enterobacteriaceae* on Chinese chicken farms (4–6). In addition to chickens, the transmission of these bacteria was also linked to flies, dogs, and wild birds (4), as well as building surfaces, soil, sediment, air, and sewage in the farm environment (5). These locations might serve as reservoirs for the transmission and evolution of *bla*_{NDM} genes and carbapenem-resistant Gram-negative bacteria, especially *Enterobacteriaceae*.

In addition to the poultry industry in China, the waterfowl industry supplied 76% of the global demand for duck and goose meat (<https://www.euromeatnews.com/Article-The-global-duck-and-goose-meat-consumption-will-grow-to-8-million-tonnes/3267>). However, in contrast to the confined methods mostly used for chicken farming, domestic duck and geese are reared using a traditional free-range system, which allows contact with wild waterfowl, poultry, livestock, and humans (7). This feeding mode most likely facilitates dissemination of resistant bacteria and antibiotic resistance genes (ARGs).

There is a lack of information concerning the prevalence and transmission of NDM-producing *Enterobacteriaceae* in waterfowl farm ecosystems (8–10). Here, we selected a typical commercial goose farm and investigated the prevalence and transmission of *bla*_{NDM}-carrying bacteria isolated from the geese and the associated farm environment and characterized the *bla*_{NDM}-carrying isolates and plasmids using whole-genome sequencing (WGS).

RESULTS

Prevalence of NDM-producing *Enterobacteriaceae*. We recovered 57 meropenem-resistant isolates (≥ 1 mg/liter) from 117 samples, and 44 meropenem-resistant isolates were found to carry *bla*_{NDM}. No other tested carbapenemase resistance genes were found among these meropenem-resistant isolates. The presence of *bla*_{NDM}-carrying isolates was identified in 41 samples (35.0%, 41/117), including 24 geese (47.1%, 24/51), 8 mice (50%, 8/16), and 9 others (18%, 9/50) that included 4 sewage isolates, 3 soil isolates, 1 fodder isolate, and 1 dust isolate. No *bla*_{NDM}-carrying isolates were found in drinking water. The combined species identification and WGS analysis indicated that 44 *bla*_{NDM}-carrying isolates belonged to eight *Enterobacteriaceae* species, including *E. coli* ($n=22$), *Citrobacter braakii* ($n=6$), *Enterobacter hormaechei* ($n=5$), *Providencia stuartii* ($n=4$), *Enterobacter cloacae* ($n=2$), *Enterobacter asburiae* ($n=2$), *Citrobacter freundii* ($n=2$), and *Klebsiella pneumoniae* ($n=1$) (Table 1). NDM-producing *E. coli*, *Enterobacter* sp., and *Citrobacter* sp. isolates were obtained from three to four different sources, and the goose origin predominated (Fig. 1). In contrast, almost all of the four NDM-producing *P. stuartii* isolates were from mice except for one from a goose. In addition, *C. braakii* strain F2R and *E. asburiae* strain F2W were taken from the same single goose cloacal swab as were *C. freundii* strain F3R and *P. stuartii* strain F3W. We also identified two *bla*_{NDM} variants, and *bla*_{NDM-5} was the most prevalent (40/44, 90.9%), followed by *bla*_{NDM-1} (4/44, 9.1%). The latter were all present in *P. stuartii*.

TABLE 1 Characteristics of 44 NDM-producing *Enterobacteriaceae* from a geese farm in China, during July 2018 to February 2019

Strain	Species ^a	Collection date	Source	Location ^b	NDM type	Other resistance phenotype ^c	NDM-carrying plasmids		
							Replicon type	Size (bp)	X3 plasmid type
H2F19R	<i>E. coli</i>	201807	Goose	House	NDM-5	AZT, MEM, ETP, IMP, S/T, FFC, STR, GEN, DOX, TET, FOS	IncX3	46,161	I
H2F5R	<i>E. coli</i>	201807	Goose	House	NDM-5	AZT, S/T, FFC, STR, DOX, TET, FOS	IncX3	46,161	I
H2F1W	<i>E. coli</i>	201807	Goose	House	NDM-5	AZT, S/T, FFC, STR, DOX, TET, FOS	IncX3	46,161	I
H2F2W	<i>E. coli</i>	201807	Goose	House	NDM-5	AZT, S/T, FFC, STR, DOX, TET, FOS	IncX3	45,212	III
H2F9R	<i>E. coli</i>	201807	Goose	House	NDM-5	AZT, S/T, FFC, STR, DOX, TET, FOS, CIP	IncX3	46,161	I
H2E1R	<i>E. coli</i>	201807	Dust	Drooping board	NDM-5	AZT, S/T, FFC, STR, DOX, TET, FOS, CIP	IncX3	46,161	I
H2F14W	<i>E. coli</i>	201807	Goose	House	NDM-5	AZT, S/T, FFC, STR, DOX, TET, CIP, GEN	IncX3	46,161	I
DF3W	<i>E. coli</i>	201811	Goose	House	NDM-5	AZT, S/T, FFC, STR, DOX, TET, CIP, TIG	IncX3	46,201	I
H2F18W	<i>E. coli</i>	201807	Goose	House	NDM-5	S/T, FFC, STR, DOX, TET, CIP	IncX3	46,161	I
CW1R	<i>E. coli</i>	201811	Sewage	Pool	NDM-5	S/T, FFC, STR, DOX, TET, CIP	IncX3	46,161	I
DF3R	<i>E. coli</i>	201811	Goose	House	NDM-5	S/T, FFC, STR, DOX, TET, CIP	IncX3	46,161	I
H2F12R	<i>E. coli</i>	201807	Goose	House	NDM-5	S/T, FFC, DOX, TET, CIP	IncX3	44,374	VI
H2F7R	<i>E. coli</i>	201807	Goose	House	NDM-5	AZT, S/T, FFC, STR, DOX, TET, CIP	IncX3	46,161	I
AG1	<i>E. coli</i>	201811	Goose	House	NDM-5	S/T, FFC	IncX3	46,161	I
BF1	<i>E. coli</i>	201811	Goose	House	NDM-5	S/T, FFC, DOX, TET, CIP	IncX3	45,760	IV
AF3	<i>E. coli</i>	201811	Goose	House	NDM-5	S/T, FFC, DOX, TET, CIP	IncX3	46,155	I
INS	<i>E. coli</i>	201811	Soil	Indoor	NDM-5	S/T, FFC, STR, DOX, TET, TIG, CIP	IncX3	45,408	II
HF1R	<i>E. coli</i>	201811	Mouse	Hatchery	NDM-5	S/T, FFC, DOX, TET, CIP	IncX3	46,161	I
M13	<i>E. coli</i>	201902	Mouse	Pharmacy	NDM-5	S/T, FFC, STR, DOX, TET	IncX3	48,744	VII
M9	<i>E. coli</i>	201902	Mouse	Pharmacy	NDM-5	S/T, FFC, STR, DOX, TET	IncX3	46,161	I
M10	<i>E. coli</i>	201902	Mouse	Pharmacy	NDM-5	AZT, S/T, FFC, STR, DOX, TET, CIP	IncX3	46,143	I
M11	<i>E. coli</i>	201902	Mouse	Pharmacy	NDM-5	AZT, S/T, FFC, STR, DOX, TET, CIP	IncX3	46,143	I
CF4	<i>E. hormaechei</i>	201811	Goose	House	NDM-5	S/T, FFC, STR, TET, DOX, TIG	IncX3	46,373	I
CF3W	<i>E. hormaechei</i>	201811	Goose	House	NDM-5	S/T, FFC, STR, GEN, DOX, TET, TIG, CIP, FOS	IncX3	46,199	I
DW1W	<i>E. cloacae</i>	201811	Sewage	Pool	NDM-5	S/T, FFC, STR, GEN, DOX, TET, CIP, FOS	IncX3	46,161	I
C53W	<i>E. cloacae</i>	201811	Soil	Outdoor	NDM-5	S/T, FFC, STR, DOX, TET, FOS	IncX3	46,161	I
F2W	<i>E. asburiae</i>	201902	Goose	House	NDM-5	AZM, S/T, FFC, STR, GEN, AMK, DOX, TET, CIP, FOS	IncX3	46,161	I
F1	<i>E. asburiae</i>	201902	Goose	House	NDM-5	AZM, S/T, FFC, STR, GEN, AMK, DOX, TET, CIP, FOS	IncX3	46,161	I
W2	<i>E. hormaechei</i>	201902	Sewage	Sewage trench	NDM-5	S/T, FFC, DOX, TET, TIG	IncX3	46,161	I
H2F4R	<i>E. hormaechei</i>	201807	Goose	House	NDM-5	AZT, S/T, FFC, GEN, DOX, TET, TIG, CIP, FOS	IncX3	46,161	I
H2F3W	<i>E. hormaechei</i>	201807	Goose	House	NDM-5	S/T, FFC, GEN, DOX, TET, TIG, CIP	IncX3	46,161	I
H2F15	<i>C. braakii</i>	201807	Goose	House	NDM-5	S/T, FFC, DOX, TET, GEN	IncX3	46,161	I
TS3W	<i>C. freundii</i>	201811	Soil	Outdoor	NDM-5	AZM, S/T, FFC, STR, GEN, DOX, TET, TIG, CIP	IncX3	46,162	I
F2R	<i>C. braakii</i>	201902	Goose	House	NDM-5	S/T, FFC, DOX, TET, CIP	IncX3	44,885	II
F5	<i>C. braakii</i>	201902	Goose	House	NDM-5	S/T, FFC, STR, DOX, TET, CIP	IncX3	45,122	II
F4	<i>C. braakii</i>	201902	Goose	House	NDM-5	S/T, FFC, DOX, TET, CIP	IncX3	45,134	II
F6	<i>C. braakii</i>	201902	Goose	House	NDM-5	S/T, FFC, DOX, TET, GEN, CIP	IncX3	45,122	II
W1R	<i>C. braakii</i>	201902	Sewage	Sewage trench	NDM-5	S/T, FFC, STR, DOX, TET, CIP	IncX3	45,122	II
F3R	<i>C. freundii</i>	201902	Goose	House	NDM-5	AZM, S/T, FFC, STR, GEN, DOX, TET, TIG	IncX3	46,296	V
H2C3R	<i>K. pneumoniae</i>	201807	Fodder	Feeding trough	NDM-5	S/T, FFC, STR, GEN, DOX, TET, CIP, FOS	IncX3	46,161	I
M2	<i>P. stuartii</i>	201902	Mouse	Hatchery	NDM-1	S/T, FFC, STR, DOX, TET, TIG, CIP, CST	UT	293,745	- ^e
M4	<i>P. stuartii</i>	201902	Mouse	Hatchery	NDM-1	S/T, FFC, STR, DOX, TET, TIG, CIP, CST	UT	ND ^d	-
F3W	<i>P. stuartii</i>	201902	Goose	House	NDM-1	S/T, FFC, STR, DOX, TET, TIG, CIP, CST	UT	ND	-
M5	<i>P. stuartii</i>	201902	Mouse	Hatchery	NDM-1	S/T, FFC, STR, DOX, TET, TIG, GEN, CIP, CST	UT	ND	-

^a*E. hormaechei*, *Enterobacter hormaechei*; *E. cloacae*, *Enterobacter cloacae*; *E. asburiae*, *Enterobacter asburiae*; *C. braakii*, *Citrobacter braakii*; *C. freundii*, *Citrobacter freundii*; *K. pneumoniae*, *Klebsiella pneumoniae*; *P. stuartii*, *Providencia stuartii*.

^bHouse, goose house; Outdoor or indoor, outdoor or indoor of goose houses.

^cAZT, aztreonam; S/T, trimethoprim-sulfamethoxazole; FFC, florfenicol; STR, streptomycin; GEN, gentamicin; AMK, amikacin; TET, tetracycline; DOX, doxycycline; CIP, ciprofloxacin; FOS, fosfomycin; CST, collistin; TIG, tigecycline.

Other resistance phenotype that were not included meropenem, imipenem, eropenem, ampicillin, ceftiofur, cefotaxime and ceftazidime, to which all 44 isolates were resistant.

^dND, the sizes of the three *bla*_{NDM}-positive plasmids were not determined.

^e-, Not applicable.

FIG 1 Sankey diagram illustrating an indicative view of links among NDM types, *Enterobacteriaceae* species, and sources.

Antimicrobial susceptibility and ARGs distribution among NDM-producing *Enterobacteriaceae*. The 44 NDM-positive isolates we identified in the present study were resistant to meropenem (MICs 4 to 32 mg/liter), imipenem (MICs 8 to 128 mg/liter), and ertapenem (MICs 8 to >64 mg/liter). They were also concurrently resistant to other β -lactams (ampicillin, ceftiofur, cefotaxime, and ceftazidime) as well as florfenicol, trimethoprim-sulfamethoxazole, and tetracycline (Table 1). Moreover, most were resistant to doxycycline (95.4%), streptomycin (72.7%), and ciprofloxacin (70.5%). However, most showed relatively low rates of resistance to gentamicin (31.8%), aztreonam (36.3%), tigecycline (29.5%), fosfomycin (27.3%), colistin (9.1%), and amikacin (4.5%). Of note, all four clonal *bla*_{NDM-1}-carrying *P. stuartii* isolates were concurrently resistant to tigecycline (8 mg/liter) and colistin (>256 mg/liter). In addition, all five *bla*_{NDM-5}-carrying *E. hormaechei* isolates show resistance to tigecycline, with MICs ranging from 2 to 16 mg/liter.

WGS analysis revealed that 44 NDM-producing isolates possessed 74 distinct ARGs conferring resistance to nearly all currently available antibiotics (Fig. 2). Multiple ARGs were identified in most of the 44 isolates, and 42 (95.5%) harbored at least one aminoglycoside, one plasmid-mediated quinolone, and one β -lactam resistance gene. In addition to *bla*_{NDM-5}, the most prevalent ARGs whose prevalence was $\geq 50\%$ included *sul2* ($n = 37$), *floR* ($n = 35$), *aph(3')-Ia* ($n = 26$), *mph(A)* ($n = 25$), *aph(6)-Id* ($n = 24$), *qnrS1* ($n = 23$), and *aph(3'')-Ib* ($n = 22$). Notably, some ARGs were unique to a specific species such as the *bla*_{CTX-M-55} variant largely present in 22 *E. coli* isolates (45.4%). In contrast, *bla*_{CMY}, *qnrB*, and *bla*_{DHA} variants were primarily found in *Citrobacter* sp. isolates, and *bla*_{ACT} and *bla*_{CMH} variants were unique to *Enterobacter* isolates.

Population structure analysis of NDM-producing *Enterobacteriaceae*. We conducted a detailed comparison of the 44 NDM-producing *Enterobacteriaceae* isolates. multilocus sequence typing (MLST) analysis revealed 13 sequence types (STs) among 22 NDM-producing *E. coli* isolates, and ST4358 was the most prevalent (6/22, 27.3%), followed by ST48 (3/22, 13.6%) and ST1421, ST515, and ST1011 (2/22, 9.1%). The remaining isolates were each of a single ST type. Of note, compared to ST48, ST4358

FIG 2 Population structure and ARG distribution of NDM-producing *Enterobacteriaceae*. (a) Phylogenetic tree and distribution of NDM-producing *E. coli* Clermont type, MLSTs, lineages, and ARGs. (b and c) Phylogenetic trees and distributions of MLST, lineages, and ARGs for NDM-producing *E. cloacae* (b) and *C. freundii* (c). (d) Phylogenetic tree and ARG distribution of NDM-producing *P. stuartii*. The sources and sampling points of the isolates are differentiated by color of filled circles and squares, respectively. ARGs are denoted by filled squares to indicate presence and empty squares to indicate absence. *, Novel ST type.

possessed two locus variants, and both ST48 and ST4358 *E. coli* isolates were from geese and dust or sewage. These 22 *E. coli* isolates were classified into three phylogenetic groups with the majority belonging to commensal phylogenetic groups A (16/22, 72.7%) and B1 (4/22, 18.2%) that were all from geese except for one dust isolate. However, the remaining two isolates belonged to the potentially virulent phylogenetic

group D and were recovered from mice. Among nine NDM-producing *Enterobacter* isolates, nine STs were identified, including five novel ones. In contrast, only three ST types were identified among the eight NDM-producing *Citrobacter* isolates, and ST388 was predominant (6/8, 75%), including both goose and sewage origins.

We further analyzed population structure by constructing phylogenetic trees based on the core genomes of the isolates. Bayesian analysis revealed eight distinct lineages among the 22 NDM-producing *E. coli* isolates, indicating a high degree of whole-genome sequence heterogeneity among these isolates. However, the major lineage (lineage VIII) included six isolates belonging to ST4358 that were identical (single nucleotide polymorphisms [SNPs] = 0) (Fig. 2a). Nine NDM-producing *Enterobacter* sp. isolates of nine different STs were comprised of four distinct lineages, and the major lineage (lineage IV) was found in four *E. hormaechei* isolates sharing 7,512 SNPs (6.0%) of 124,272 total SNPs (Fig. 2b). All eight *Citrobacter* isolates belonged to two distinct lineages, and the major lineage (lineage II) included six ST388 *C. braakii* isolates possessing extremely high genetic similarity (SNP = 0) (Fig. 2c). Furthermore, the four *P. stuartii* isolates were also highly similar at the whole-genome sequence level (SNPs ≤ 7), suggesting clonal dissemination of *bla*_{NDM-1}-positive *P. stuartii* isolates from mice and geese (Fig. 2d).

The most prevalent *Enterobacteriaceae* species for *bla*_{NDM-5} carriage was *E. coli*, and this was found in geese, mice, and the environment. We further characterized the genetic relationships between these isolates and another 153 *bla*_{NDM-5}-positive Chinese *E. coli* isolates with known hosts as previously described (10) (see Table S1 in the supplemental material). Core-genome SNP-based phylogenetic analysis revealed that 37 isolates, including 13 identified in the present study, clustered together (SNPs $\leq 8,041$) (Fig. 3). Notably, these 37 isolates belonged primarily to ST48 and ST4358 and originated from diverse sample types, including humans, ducks, geese, and various environments (dust, pond water, sewage, and soil). In addition, ten NDM-positive ST1011 *E. coli* isolates from duck and soil clustered together with two NDM-positive ST1011 *E. coli* isolates from geese (SNPs $\leq 8,419$). One NDM-positive *E. coli* isolate from a human specimen shared 5 to 7,881 SNPs with three NDM-positive *E. coli* isolates from mice and geese (Fig. 3).

NDM-positive plasmid analysis. WGS analysis indicated that each of these 44 NDM-producing isolates possessed one *bla*_{NDM}-carrying contig. The *bla*_{NDM-5} gene was found on IncX3 plasmids in the group of 40 *bla*_{NDM-5}-carrying isolates. In contrast, *bla*_{NDM-1} was found on untypeable plasmids in the remaining four NDM-1-producing *P. stuartii* isolates. We further obtained the complete sequences of the 40 *bla*_{NDM-5}-carrying IncX3 plasmids by PCR mapping using the corresponding reference sequences, and their sizes ranged from 44.3 to 48.7 kb (Table 1). A sequence comparison analysis revealed that all 40 plasmids were similar to *bla*_{NDM-5}-carrying IncX3 plasmid pHNYX644-1 from a pork ST48 *E. coli* isolate from China (accession no. [NZ_MK033578](#)), with 96 to 100% coverage and 99.95 to 99.99% nucleotide identity. These 40 plasmids shared an identical and conserved IncX3 backbone, including the basic core structure *repB-taxD-parA-hns-topB-parB-virB-rfaH-taxCA*. Differences between these plasmids were primarily located within the *bla*_{NDM-5}-containing region between the *parA* and *IS3000* genes. According to the diversity within this region, these 40 plasmids were classified into seven groups and one predominated (type I, 72.5%) (see Fig. S1 in the supplemental material). Furthermore, all 40 *bla*_{NDM-5}-carrying plasmids from seven *Enterobacteriaceae* species could be transferred to the recipient *E. coli* strain C600 by conjugation.

To obtain the complete sequence of *bla*_{NDM-1}-carrying plasmids from our four NDM-1-producing clonal *P. stuartii* isolates, a single M2 strain was randomly selected for further sequence analyses using the Oxford Nanopore Technologies (ONT) platform. Combined with Illumina and ONT data, a complete plasmid, pM2-1 (293.745 kb), containing *bla*_{NDM-1} was obtained. pM2-1 did not possess any known plasmid replicon type, but the *dnaB* gene (882 bp) encoded a DNA replication termination site binding protein. Comparative sequence analysis revealed that pM2-1 shared high synteny and

Tree scale: 0.1

FIG 3 Phylogenetic structures of the 22 NDM-5-positive *E. coli* isolates from this study (highlighted by red points) and another 153 Chinese isolates in NCBI database. A maximum-likelihood tree shows the relationships among the 175 NDM-5-positive *E. coli* isolates. Internal nodes are labeled with circles indicating branch >70% bootstrap support. Isolate hosts are indicated in the outer ring. NDM-positive *E. coli* isolates from this study closely related to those of others from the NCBI database are indicated by black lines.

extensive sequence similarity with plasmid pVSC3 from a chicken *Proteus vulgaris* isolate from China (accession no. [CP034667](#)) (Fig. 4a). The detected variation was primarily concentrated within a large multidrug region (MDR II) (45.094 kb) and inversion region (150.758 kb) that also included the other multidrug region (MDR I) (15.103 kb) and a large insertion region (59.621 kb) (Fig. 4a, b, and c). The large MDR II contained *bla*_{NDM-1} and other 18 ARGs, including a new class 1 integron, as well as two copies of ~3.9-kb mercury resistance operons (*merRTPCDA*), which were heterogeneous with 81.7% nucleotide identity. In addition, the large MDR II possessed four distinct modules that were identified by a comparison with related segments (Fig. 4d).

The 59.621-kb insertion region in plasmid pM2-1 was bracketed by two copies of *insO-insN* of IS911 mobile elements in opposite orientations and possessed three unique modules (Fig. 4c). Importantly, module I was composed of the maltose transport system (15.962 kb) containing nine components, including the maltodextrin ABC transporter MdxEFG. Similar maltose operons were also found in the chromosomes from two *Providencia heimbachae* isolates (accession numbers [CP028384](#) and

LS483422). In addition, module II (4.285 kb) contained the arsenic detoxification system composed of five components, including an ATP-driven As(III)/Sb(III) efflux pump (ArsA and ArsB). The similar arsenical resistance (*ars*) operon was also identified on *Providencia* chromosomes and plasmids (Fig. 4c). Furthermore, the *torA* and *torD* genes that encode the proteins of the trimethylamine N-oxide (TMAO) reductase system were also embedded into the 59.621-kb insertion region. In particular, in addition to strain M2, similar pM2-1-like plasmids bearing *bla*_{NDM-1} were also present in the remaining three *bla*_{NDM-1}-carrying *P. stuartii* isolates (see Fig. S2 in the supplemental material), and all of these *bla*_{NDM-1}-positive plasmids could be transferred into the recipient *E. coli* by conjugation.

DISCUSSION

In the present study, we characterized the prevalence and genetic relationships between NDM-producing bacteria from a typical goose farm in a typical commercial goose farm in Guangdong province, southern China. Our results revealed a wide contamination of NDM-producing *Enterobacteriaceae* from eight different species in almost all tested samples, including geese, mice, sewage, soil, fodder, and dust, with the sole exception of drinking water. Of note, wild house mice have been previously implicated as reservoirs for human pathogens and antimicrobial resistance in buildings (11). In the present study, farm mice carried NDM-5-producing *E. coli* strains of three different STs and NDM-1-producing clonal *P. stuartii* strains. Mice contaminated with NDM-producing *Enterobacteriaceae* can move freely throughout the environment, and most likely exacerbate the transmission of *bla*_{NDM}.

More importantly, we detected NDM-producing bacteria with relatively high prevalence from geese (47.1%), mice (50%), and environmental samples (18%). A high prevalence of *bla*_{NDM} (9 to 43.6%) was reported for *E. coli* isolated from chicken and duck farms (5, 6, 10). Together with the current findings, these data strongly indicated that the Chinese poultry farm environment is likely to be an important reservoir for *bla*_{NDM}-carrying bacteria. Importantly, carbapenems are not used in food animal production. Consequently, we surmise that the high prevalence of *bla*_{NDM}-carrying bacteria in the present study may have originated from the use of amoxicillin on the farm, which probably provided a direct selection pressure for *bla*_{NDM} maintenance. Second, the farm reported frequent use of florfenicol, doxycycline, and enrofloxacin, which is consistent with our panel of *bla*_{NDM}-positive bacteria exhibiting a high prevalence of resistance to these antibiotics (>70%). The use of these antibiotics may have coselected for *bla*_{NDM}, since *bla*_{NDM} frequently coexists with other ARGs, including *floR* (phenicols) and *qnr* (quinolones), as determined by WGS analysis. Third, the goose farm was an open space suitable for goose activities, and this made it difficult to perform complete disinfection and sterilization of the farm environment. There was widespread contamination by NDM-producing *Enterobacteriaceae* in farm environments, including sewage, soil, fodder, and dust.

In the present study, *bla*_{NDM-5} was the predominant *bla*_{NDM} variant, and *E. coli* was the most prevalent *Enterobacteriaceae* species for *bla*_{NDM-5} carriage in both farm animals and farm environments, which was in consistent with previous reports (5, 10). Our population structure analyses revealed that NDM-producing *E. coli* and *E. cloacae* isolates were highly heterogeneous, whereas most *Citrobacter* sp. and *P. stuartii* isolates possessed extremely high genetic similarities. However, *bla*_{NDM-5}-positive ST48 and its two locus variant ST4358 *E. coli* isolates demonstrated clonal spread between geese and the farm environment. Furthermore, of note, ST48 *E. coli* isolates have been frequently associated with *bla*_{NDM-5} spread on poultry farms and in retail meat in China (9, 10, 12). Our phylogenetic analyses further demonstrated that NDM-5-producing *E. coli* strains (in particular ST48 and ST4358) from the goose farm were closely related to those of other origins, including ducks and farm environments, as well as humans, in China. This indicated that *bla*_{NDM-5} in the goose farm was probably exogenous, such as

FIG 4 Characteristics of the complete nucleotide sequences of *bla*_{NDM-1}-carrying pM2-1 from *P. stuartii* strain M2. (a) Complete nucleotide sequence comparison of pM2-1 identified in this study and pPvSC3 (accession no. CP034667). The broken lines mean that these regions had no similar sequence (Continued on next page)

deriving from human or mouse activity or contaminated feeds, since carbapenems are approved only for clinical use.

IncX3 plasmids have been identified as the primary vectors for the horizontal transfer of *bla*_{NDM-5} on farms (10, 13, 14). In the present study, all 40 transferable *bla*_{NDM-5}-carrying IncX3 plasmids from seven *Enterobacteriaceae* species were remarkably similar, indicating epidemic *bla*_{NDM-5}-bearing IncX3 plasmids circulated in the *Enterobacteriaceae* on this farm. Notably, these epidemic plasmids were highly similar to *bla*_{NDM-5}-bearing pHNYX644-1-like IncX3 plasmids that have successfully disseminated *bla*_{NDM} variants, including *bla*_{NDM-5}, among people, animals, food products, and the environment (12, 13, 15).

In addition, we also identified novel untypeable pM2-1-like plasmids that mediated *bla*_{NDM-1} spread among clonal *P. stuartii* isolates from geese and mice. Plasmid pM2-1 likely originated from plasmid and chromosomal recombination between *Providencia* and *Proteus* species. This resulted in acquisition of diverse gene clusters related to antibiotic (including *bla*_{NDM-1}) and heavy metal (*ars* and *mer* operons) resistance, as well as oligosaccharide metabolism (maltose operon) and small compound utilization (*torA* and *torD*). Of note, the maltodextrin ABC transporter operons were previously implicated in malto-oligosaccharide metabolism in diverse bacteria, including *E. coli* (16) and *Enterococcus faecalis* (17). Furthermore, similar to *E. coli*, the *torA* and *torD* genes most likely contribute to the metabolism of small compounds, including TMAO in *Providencia* species (18). These findings highlight a high degree of plasticity for pM2-1-like plasmids, and acquisition of these specific genes most likely facilitates bacterial adaptation to changing environmental conditions. In addition, it is worth noting that *Providencia* spp. belongs to tribe *Proteeae* in the *Enterobacteriaceae* family and that they are intrinsically resistant to colistin and tigecycline (19). Worrisomely, NDM-producing *P. stuartii* isolates were concurrently resistant to the three remaining antibiotics of last resort (colistin, tigecycline, and carbapenems) and possessed highly plastic pM2-1-like plasmids. Infections caused by these organisms would be a clinically challenging threat.

Conclusions. In the present study, we found a relatively high prevalence of *bla*_{NDM}-carrying *Enterobacteriaceae* (35.0%) from geese, wild mice, and fomites on a typical commercial goose farm in southern China. The population structure and plasmid analyses revealed that both clonal (primarily *Citrobacter* sp., *P. stuartii*, and *E. coli* ST4358 and ST48) and horizontal (IncX3 and pM2-1-like plasmids) spread contributed to the prevalence and transmission of *bla*_{NDM} in *Enterobacteriaceae* of diverse origins in the goose farm. Phylogenetic analysis further revealed *bla*_{NDM}-carrying *E. coli* isolates from the geese farm were genetically similar to those from duck farms and humans. Although the present study was limited to a single commercial goose farm, the results indicate that this farm was an important reservoir for NDM-positive *Enterobacteriaceae*. This widespread contamination with *bla*_{NDM}-positive bacteria is a potential threat to public health. Further studies of other goose farms are needed to determine the generalizability of our findings and to identify practices to limit selection for further dissemination of these bacteria.

MATERIALS AND METHODS

Sample collection. During July 2018 to February 2019, we collected fecal and environmental samples from a typical commercial goose farm in Guangdong province, southern China, where the goose breeding industry is particularly developed. The tested farm has been in operation for more than 10 years, and it contained two separate buildings that housed about 10,000 geese. The distance between

FIG 4 Legend (Continued)

segments between pM2-1 and pPvSC3. (b) Comparison of the 15.1-kb MDR I on pM2-1 with the corresponding region on pPvSC3 and p99101 (CP023385) and with the chromosome from *P. heimbachae* strains 99101 and NCTC12003 (CP028384 and LS483422) and on chromosomes from *Providencia* sp. WCHPHu000369 (CP031123) and *P. rustigianii* strain NCTC8113 (LR134396) and an unnamed plasmid from *P. heimbachae* strain 99101 (CP028385) and pC131 from *P. rettgeri* strain 30905 (KX774387). (d) Comparison of MDR I on pM2-1 with the corresponding region on pPm60, pHNSD133T1 and pZN3 (MG516911, MG196293, and CP047346) and on the chromosome from *P. rustigianii* strain BML2496 (AP022371).

these two buildings was ~300 m. Each building contained three adjacent sheds and, correspondingly, one pool inhabited by goose was divided into three regions located in front of each shed. Different sheds and pool regions were assigned to the various growth phases of the geese.

A total of 117 nonduplicate samples were randomly collected from geese ($n = 51$), inanimate environments ($n = 50$ [sewage, $n = 12$; soil, $n = 15$; dust, $n = 10$; fodder, $n = 8$; and drinking water, $n = 5$]), and mice ($n = 16$). The goose samples were randomly collected from cloacal swabs from different geese growth phases (days 10 to 80). In each farm building, one to three sewage, soil, and dust samples were collected by convenience sampling. Dust samples were wiped with sterile cotton swabs from surface dust or residue from windows/drooping boards (each from two different regions about 10×10 -cm areas) in goose houses. Sewage samples (~5 ml) were taken from sewage trenches/pools using sterile 1.5-ml microcentrifuge tubes. Soil samples were taken from goose houses or the surrounding area at depths of 5 cm using sterile cotton swabs. Fodder samples (~5 mg) were taken from feeding troughs using sterile bags. Mouse samples were taken from fresh feces in the pharmacy and abandoned hatchery. All samples except for sewage and drinking water samples were rapidly placed or rinsed in sterile Luria-Bertani broth (2 ml). The antimicrobial usage records for this goose farm indicated that the antibiotics ampicillin, florfenicol, doxycycline, and enrofloxacin were in common and extensive use on the farm.

Isolation of bacteria, detection of *bla*_{NDM}, and antimicrobial susceptibility testing. Samples or diluted samples were enriched in Luria-Bertani broth at 37°C for 12 h, and isolates were inoculated onto MacConkey agar containing 1 mg/liter meropenem at 37°C overnight. One or two colonies with different morphologies from each sample were selected and screened for the presence of five major carbapenemase resistance genes, including *bla*_{NDM}, *bla*_{KPC}, *bla*_{IMP}, *bla*_{OXA-48}, and *bla*_{VIM} using PCR as previously described (20). The *bla*_{NDM}-positive isolates were then identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry and 16S rRNA gene sequence-based analyses.

Antimicrobial susceptibilities of *bla*_{NDM}-positive isolates and their corresponding transconjugants were determined by the agar dilution method except for colistin and tigecycline that used the broth dilution method. Briefly, *bla*_{NDM}-positive isolates were incubated in Mueller-Hinton broth (Huankai, Guangzhou, China) with serial dilutions of colistin (0.125, 0.25, 0.05, 1, 2, 4, 8, 16, 32, 64, 128, and 256 mg/liter) and tigecycline (0.03, 0.06, 0.125, 0.25, 0.05, 1, 2, 4, 8, 16, 32, and 64 mg/liter). The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI; 2015 guideline M100-S25) (21) and veterinary CLSI (VET01-A4/VET01-S2) guidelines (22). For colistin and tigecycline, the resistance breakpoints were determined according to EUCAST recommendations interpreted with >2 and >0.25 mg/liter, respectively. The following antimicrobials were tested: meropenem, imipenem, ertapenem, ampicillin, ceftiofur, cefotaxime, ceftazidime, aztreonam, streptomycin, gentamicin, amikacin, ciprofloxacin, tetracycline, doxycycline, florfenicol, trimethoprim-sulfamethoxazole, fosfomycin, colistin, and tigecycline. *E. coli* ATCC 25922 was used as the quality control strain.

Genetic characterization of NDM-producing isolates. Genomic DNA samples from all NDM-producing isolates were subjected to 250-bp paired-end whole-genome sequencing using an Illumina MiSeq system (Illumina, San Diego, CA), and the paired-end Illumina reads were assembled by SPAdes v3.6.2 (23). MLST, the presence of ARGs, and plasmid replicon types were analyzed using the CGE server (<https://cge.cbs.dtu.dk/services/>) and ABRicate (<https://github.com/tseemann/abricate>). In addition, *in silico* phylotyping of *E. coli* was carried out using the ClermonTyping method (24). Phylogenetic trees for all NDM-positive isolates were structured based on the core genome sequences of the isolates using Harvest v1.1.2 (25), with the corresponding characteristics of each isolate visualized using online tool iTOL v4 (26). The population structure of each phylogenetic tree was defined using hierBAPS v6.0 (27).

Conjugation assay and *bla*_{NDM}-positive plasmid analysis. The transferability of *bla*_{NDM} genes was investigated by conjugation experiments by the liquid mating-out assay using streptomycin-resistant *E. coli* C600 as the recipient as previously described (28). Transconjugants were selected on MacConkey agar plates supplemented with meropenem (1 mg/liter) and streptomycin (1000 mg/liter). The presence of *bla*_{NDM} was determined by PCR and confirmed as *E. coli* C600 derivatives by ERIC (Enterobacterial Repetitive Intergenic Consensus)-PCR (29). All transconjugants were tested for antimicrobial susceptibility as described above.

To obtain the complete sequence of *bla*_{NDM}-positive plasmids, PCR mapping was performed to fill gaps in *bla*_{NDM}-carrying contigs using the corresponding reference sequences. If the complete sequences of *bla*_{NDM}-carrying plasmids were not available, the host strains and/or corresponding transconjugants were further sequenced using an ONT MinION Flowcell R9.4 (30). *De novo* hybrid assembly using both Illumina and ONT reads was performed using Unicycler v0.4.4 (31), Canu v1.5 (32), and Pilon v1.23 (33). Sequence comparisons and map generation of *bla*_{NDM}-carrying plasmids were performed using BLAST (<http://blast.ncbi.nlm.nih.gov>), Easyfig (34), the BLAST Ring Image Generator (35), and Mauve (36).

Data availability. All WGS data have been deposited in the NCBI BioProject database: PRJNA649083. The complete sequence of plasmid pM2-1 has been deposited in GenBank under accession number MT813046.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

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L.-X.F. and D.-J.C. designed this study. L.-X.F., D.-J.C., J.-L.M., Y.-W.J., and H.Z. collected samples from goose farm. D.-J.C., J.-L.M., Y.-W.J., W.-Y.G., Q.J., H.Z., J.-F.Z., and D.W. performed experiments. L.-X.F., D.-J.C., and R.-Y.S. analyzed and interpreted data. L.-X.F. and J.-L.M. wrote the manuscript. L.-X.F., R.-Y.S., R.-M.Z., and J.S. edited the manuscript. Y.-H.L. and X.-P.L. coordinated the whole project.

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