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Genomic Insights into Carbapenem-Resistant Organisms Producing New Delhi Metallo-β-Lactamase in Live Poultry Markets

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Abstract: The widespread dissemination of the *bla*NDM gene, which encodes New Delhi metallo-β-lactamase, in animal-derived settings poses a threat to public health security. Live poultry markets represent critical nodes in public health surveillance. However, there is currently limited reporting on the spread of the *bla*NDM gene within these markets under One Health approach. This study investigated the prevalence of the *bla*NDM gene in live poultry markets and performed an in-depth analysis of its association networks with other genetic elements across species, by integrating newly sequenced genomes with publicly available database entries. The samples for this study were collected from two live poultry markets in Jiangsu, China. Among the *bla*NDM-positive strains identified, we detected multiple variants, primarily *bla*NDM-5, followed by *bla*NDM-1, *bla*NDM-13, *bla*NDM-27, and *bla*NDM-39. We detected the coexistence of *bla*NDM-5 and *mcr-1* in five *Escherichia coli* strains. Additionally, we found one *E. coli* strain in which *bla*NDM-5 coexisted with *estT* and *tet*(X4), and another *E. coli* strain where *bla*NDM-5 coexisted with *estT*. Network analysis of publicly available genomes revealed that the genetic element preferences of *bla*NDM variants vary significantly across species. The genetic element preferences of *Escherichia coli* carrying *bla*NDM-5 are similar to those of *Klebsiella pneumoniae* harboring *bla*NDM-1. In *Klebsiella aerogenes*, *Enterobacter cloacae*, and *Proteus mirabilis*, strains carrying *bla*NDM-1 have opposite genetic element preferences compared to strains harboring *bla*NDM-5 or *bla*NDM-7. Notably, we report the first evidence of the *bla*NDM-1 gene transfer mediated by IS*Kpn13*, IS*Spu2*, and MITE*Kpn1*. The findings highlight live poultry markets were important transmission hotspot of AMR, which requires continuous surveillance.

Keywords: live poultry; carbapenem resistance; *bla*NDM; genomic analysis; transmission

Introduction

Antibiotics are the primary weapons for humans to combat various infectious diseases and have made significant contributions to human and animal health in the fields of medicine, animal husbandry, and food safety. However, with the widespread use of antibiotics, antimicrobial resistance (AMR) has become a major threat to global public health, and the increasing multidrug resistance (MDR) in clinical pathogens has further exacerbated the problem. Horizontal gene transfer of antibiotic resistance genes (ARGs) across ecological niches amplifies the risk of clinical resistance. Globally, live poultry markets are high-risk interfaces for human-animal contact. These markets aggregate poultry from diverse regions, facilitating ARGs transfer and pathogen dissemination[1]. Live poultry markets have been proven to be reservoirs and dissemination centers for ARGs[2]. The ARGs detected in people, poultry, and the environment within the markets are more diverse than

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those detected in poultry farms[3]. This indicates that the risk of ARGs spreading through food animals is high, and they can easily be further disseminated through pathways such as water and air[4], posing a threat to the entire public health security.

Carbapenems, broad-spectrum antibiotics reserved for human MDR infections (and prohibited in veterinary use), are a last-line defense. Nevertheless, the increasing prevalence of carbapenem-resistant Gram-negative bacteria in recent years has raised significant concerns in the global public health community. The *bla*$_{NDM}$ gene, which encodes New Delhi metallo-β-lactamase (NDM), is a clinically significant determinant of carbapenem resistance. Its product can degrade the majority of β-lactam antibiotics, thereby compromising the effectiveness of these agents against pathogens that harbor this gene. To date, the spread of the *bla*$_{NDM}$ gene across different ecological niches has been extensively documented[5–9]. However, information regarding the prevalence of *bla*$_{NDM}$ in live poultry markets remains limited.

In this study, we isolated and identified multiple *bla*$_{NDM}$-positive strains from various ecological niches within live poultry markets. Through whole-genome sequencing (WGS), we elucidated the genomic characteristics of these *bla*$_{NDM}$-harboring strains. By integrating the sequenced genomes with those available in databases, we conducted a comprehensive analysis of the association networks between various *bla*$_{NDM}$ gene variants and other genetic elements across different species.

Materials and Methods

Sample Collection and Strain Identification

In July 2022, a total of 388 non-duplicate samples were collected from two large-scale live poultry markets in Yangzhou to investigate the epidemiology of *bla*$_{NDM}$-positive strains in both animals and the environment. The poultry traded in these markets originated from Anhui Province and several cities in Jiangsu Province, including Huai'an, Nanjing, Nantong, Taizhou, Yangzhou, and Yancheng. The samples comprised animal feces (chicken, n = 159; duck, n = 29; goose, n = 66; pigeon, n = 21) and other samples (soil, n = 17; water, n = 36; environment, n = 57; plant, n = 3) (Table S1). All samples were transported to the laboratory in cool boxes with ice packs (4 ℃) for bacterial cultivation and DNA extraction. The collected samples were transferred into 2 ml Brain Heart Infusion (BHI) liquid broth and incubated at 37 ℃ for 6 h for pre-bacterial growth. Preculture samples were then spread onto MacConkey plates supplemented with 2 mg/L meropenem and incubated for 18 h at 37 ℃. Different colored colonies were selected from each plate to identify carbapenem-resistant isolates. All confirmed carbapenem-resistant strains were tested for the presence of *bla*$_{NDM}$ genes (Table S2). All *bla*$_{NDM}$-positive bacteria were identified using MALDI-TOF MS Axima™ and 16S rRNA gene sequencing (Table S2).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was tested using the broth dilution method. The susceptibility of carbapenem-resistant isolates was evaluated for a range of antimicrobial drugs commonly used in both human medicine and veterinary practice, including meropenem (MEM), imipenem (IMP), ampicillin (AMP), ceftazidime (CAZ), kanamycin (KAN), gentamicin (GEN), ciprofloxacin (CIP) and colistin (CL). Minimum inhibitory concentrations (MICs) were interpreted in accordance with the guidelines provided by the Clinical and Laboratory Standards Institute (2021) [10] and the breakpoint tables specified in the European Committee on Antimicrobial Susceptibility Testing v.12.0. *E. coli* ATCC 25922 was used as a quality control strain.

Plasmid Conjugation Assay

To explore the transferability of genetic elements carrying the *bla*$_{NDM}$ gene, we conducted a conjugation assay using rifampicin-resistant *E. coli* C600 as the recipient strain. The liquid mating method was utilized for this purpose. Initially, overnight cultures of the original isolates and recipient

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strains were prepared in Luria-Bertani (LB) liquid broth. These cultures were subsequently adjusted to an optical density of 0.6 at 600 nm. A volume of 50 μl of the mixed bacterial cultures was then pipetted and evenly spread onto LB solid media containing 100 μg/mL rifampicin and 2.0 μg/mL meropenem. Following an overnight incubation at 37℃, single bacterial colonies were selected for PCR analysis to confirm the successful transfer of the *bla*$_{NDM}$ gene.

Whole genome Sequencing of bla$_{NDM}$*-Positive Strains*

The genomes of 38 *bla*$_{NDM}$-positive strains were extracted using the FastPure Bacteria DNA Isolation Mini Kit (Vazyme, Nanjing, China). The concentration and purity of the extracted DNA were evaluated using NanoDrop 2000 and gel electrophoresis, with the final concentration determined precisely by the Qubit™ 4.0 fluorometer (Invitrogen, CA, USA). Subsequently, short-read sequencing was performed on the extracted DNA using DNBseq, producing paired-end reads of 2×150 bp. The collected raw reads, with a minimum coverage of 100-fold, were then processed for trimming using SOAPnuke v.2.17[11]. De novo assembly was subsequently carried out using SPAdes v.3.13.1 [12].

Bioinformatics Analysis of Assembled Genomes

Mlst v.2.23.0 (https://github.com/tseemann/mlst) was used to determine multi-locus sequence type (MLST) of all assembled genomes. Resfinder[13], ISfinder[14], Plasmidfinder[15], VFDB core dataset[16] and ICEberg[17] were run with 80% coverage and 80% identity in Abricate (https://github.com/tseemann/abricate) to identify ARGs, insertion sequences (ISs), plasmid replicons, integrating conjugative elements (ICEs). ECTyper[18] was used to identify serotypes of all *E. coli* genomes. Prokka v.1.14.6[19] was used to conduct genome annotation. Phylogenetic trees were constructed using Roary v.3.13.0[20] and FastTree v.2.1.11[21] and visualized using Chiplot (https://www.chiplot.online). Heatmap was drawn using Chiplot. Genetic environment of plasmids was visualized using BRIG v.0.95 [22].

Genetic Environment Analysis of bla$_{NDM}$*-Positive Strains*

In order to analyze the differences in the genetic environment among different *bla*$_{NDM}$ gene variants, we used Abricate (https://github.com/tseemann/abricate) to identify 4,072 *bla*$_{NDM}$-positive strains (Table S3) from the Carbapenem-resistant *Escherichia coli* (CREC) dataset of a previous study[23]. We also downloaded 66,609 genomes from *Klebsiella* genus, 10,762 genomes from *Enterobacter* genus and 3,446 genomes from *Proteus* genus from the NCBI database (as of Dec 10, 2023). CheckM2 [24]was used to identify genomes with over 95% completeness and less than 5% contamination. Feature information including collection date, host, country, species and isolation source of these genomes were collected using a homemade python script. Linear genomic comparison and bar plot were visualized using ChiPlot. The network graph depicting the coexistence patterns of different *bla*$_{NDM}$ gene variants with other ARGs, ISs and plasmid replicons was constructed using Gephi [25].

Statistical Analysis

Statistical analysis and plotting were performed using R v.4.3.1 (R Foundation for Statistical Computing, Vienna, Austria). Spearman correlation analysis was used to determine the correlation among *bla*$_{NDM}$ gene, other ARGs, ISs and plasmid replicons.

Results

bla$_{NDM}$*-Positive Strains Profile*

A total of 388 original samples were collected from two live poultry markets in Yangzhou, China. A total of 351 meropenem-resistant strains were isolated from these samples, among which 233

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strains were *bla*NDM-positive (isolated from 144 original samples). The *bla*NDM detection rates were 37.11% (144/388) among samples and 66.38% (233/351) among meropenem-resistant isolates. Among the 233 *bla*NDM-positive strains, there were 218 *Escherichia coli* strains (93.56%), 4 *Enterobacter cloacae* strains (1.72%), 7 *Klebsiella pneumoniae* (3.00%), 2 *Klebsiella aerogenes* (0.86%), 1 *Providencia rettgeri* (0.43%), and 1 *Proteus mirabilis* (0.43%). Conjugation assays were conducted on the 233 *bla*NDM-positive strains, and ultimately 91 *E. coli* C600 transconjugants were obtained, with a conjugation success rate of 39.10%. Among the strains that successfully transferred the *bla*NDM gene through conjugation, all were *E. coli* except for 3 *E. cloacae* strains and 1 *Proteus mirabilis* strain.

A total of 233 *bla*NDM-positive strains were tested for susceptibility to a variety of antibiotics (Table 1). The tested strains exhibited extremely high resistance to meropenem, imipenem, ampicillin, and ceftazidime, with resistance rates approaching 100%. Among the aminoglycoside antibiotics, resistance rates to kanamycin and gentamicin were also high, reaching 87.12% and 82.40%, respectively. Additionally, the tested strains showed a resistance rate of 74.25% to ciprofloxacin and 20.12% to colistin. Only 11.19% (15/134) of strains isolated from chickens were resistant to colistin, while 50% (17/34) of strains from environmental sources were resistant to colistin. Although colistin demonstrated relatively good antimicrobial activity against *bla*NDM-positive strains, the presence of resistance must be taken seriously and monitored more closely.

Table 1. Antimicrobial susceptibility profiles of 233 *bla*NDM-positive strains. MEM: Meropenem; IMP: Imipenem; AMP: Ampicillin; CAZ: Ceftazidime; KAN: Kanamycin; GEN: Gentamicin; CIP: Ciprofloxacin; CL: Colistin.

Strains	Source	Species	Conjugation recipient	MIC							
				MEM	IMP	AMP	CAZ	KAN	GEN	CIP	CL
MTHAC-1-1	**Chicken**	***Escherichia coli***	-	>128	8	>128	>128	>128	>128	≤0.25	≤0.25
MTHAC-1-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	8	1
MTHAC-2-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	2	0.5	0.5
MTHAC-2-2	Chicken	*Escherichia coli*	-	128	>128	>128	>128	>128	4	1	≤0.25
MTHAC-3-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	128	0.5	≤0.25
MTHAC-3-3	Chicken	*Escherichia coli*	-	128	>128	>128	>128	>128	>128	16	≤0.25
MTHAC-4-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	64	8
MTHAC-4-2	Chicken	*Escherichia coli*	-	128	>128	>128	>128	>128	>128	2	≤0.25
MTHAC-5-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	0.5	≤0.25
MTHAC-6-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	≤0.25
MTHAC-6-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	128	1	1
MTHAC-8-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	≤0.25
MTHAC-8-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	32	16	0.5	1
MTHAC-9-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	1	8
MTHAC-9-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	0.5	1
MTHAC-10-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	1
MTHAC-10-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	1	0.5
MTHAC-10-3	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	1	≤0.25
MTHAC-11-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	4	1
MTHAC-11-2	Chicken	*Escherichia coli*	-	64	>128	>128	>128	>128	>128	≤0.25	≤0.25
MTHAC-12-1	Chicken	*Escherichia coli*	-	64	>128	>128	>128	>128	>128	8	≤0.25
MTHAC-12-2	Chicken	*Escherichia coli*	-	128	>128	>128	>128	>128	>128	2	0.5
MTHAC-13-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	16	2
MTHAC-13-2	Chicken	*Escherichia coli*	-	128	>128	>128	>128	>128	>128	1	≤0.25
MTHAC-14-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	0.5
MTHAC-15-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	0.5
MTHAC-15-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	2	0.5
MTHAC-16-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	64	1
MTHAC-16-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	16	0.5
MTHAC-17-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	8	2
MTHAC-17-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	8	0.5
MTHAC-17-3	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	8	1

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MTHAC-18-1	Chicken	*Escherichia coli*	-	64	128	>128	>128	>128	128	8	≤0.25
MTHAC-18-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	16	≤0.25
MTHAC-24-1	Chicken	*Escherichia coli*	-	64	128	>128	>128	>128	64	0.5	≤0.25
MTHAC-25-4	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	128	32	4
MTHAC-27-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	8	2
MTHAC-27-2	Chicken	*Escherichia coli*	-	>128	4	>128	>128	>128	128	8	≤0.25
MTHAC-28-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	64	2
MTHAC-31-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	128	16
MTHAC-31-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	128	16
MTAHC-1-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	128	2	4
MTAHC-1-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	8	128	1
MTAHC-2-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	4	8
MTAHC-3-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	4	1
MTAHC-4-1	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	128	>128	2
MTAHC-4-3	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	64	4
MTAHC-4-4	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	2
MTAHC-5-1	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	32	1
MTAHC-5-2	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	64	2
MTAHC-6-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	128	1
MTAHC-6-2	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	2	64	≤0.25
MTAHC-6-3	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	4
MTAHC-8-1	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	128	4
MTAHC-12-1	Chicken	*Escherichia coli*	-	64	>128	>128	>128	>128	>128	64	2
MTAHC-12-2	Chicken	*Escherichia coli*	-	128	>128	>128	>128	>128	>128	64	0.5
MTAHC-13-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	128	0.5
MTAHC-13-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	4
MTAHC-13-3	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	>128	4
MTAHC-14-3-1	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	128	-
MTAHC-14-3-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	-
MTAHC-15-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	64	-
MTAHC-15-2	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	8	-
MTAHC-16-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	32	-
MTAHC-16-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	-
MTAHC-16-3	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	64	-
MTH-1-3-2	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	≤0.25	≤0.25
MTH-3-1	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	16	≤0.25
MTH-4-1	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	64	64	≤0.25
MTH-4-2	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	64	64	≤0.25
MTH-6-1-1	Environmental	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	32	≤0.25
MTH-6-1-2	Environmental	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	32	≤0.25
MTH-6-2-1	Environmental	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	32	>128
MTH-6-2-2	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	32	2	>128
MTH-6-3-1	Environmental	*Klebsiella aerogenes*	-	>128	>128	>128	>128	>128	>128	0.5	>128
MTH-6-3-2	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	32	≤0.25
MTH-7-2-1	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	16	≤0.25

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MTH-7-2-2	Environmental	*Klebsiella aerogenes*	-	>128	>128	>128	>128	>128	>128	32	128
MTH-10-1	Environmental	*Escherichia coli*	C600	>128	>128	>128	>128	>128	128	≤0.25	≤0.25
MTH-11-1	Environmental	*Escherichia coli*	C600	>128	>128	>128	>128	8	4	4	4
MTH-11-2	Environmental	*Escherichia coli*	C600	>128	>128	>128	>128	8	1	8	8
MTH-12-1	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	8	2	1	0.5
MTH-12-2	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	16	2	1	>128
MTH-12-3	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	8	2	1	>128
MTH-13-2	Environmental	*Klebsiella pneumoniae*	-	128	>128	>128	>128	>128	>128	>128	>128
MTH-16-1	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	16	>128
MTH-16-2	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	16	64
MTH-16-3	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	32	>128
MTH-19-2	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	64
MTH-24-2-1	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	>128
MTH-26-1	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	4	2	≤0.25
MTH-29-1-1	Environmental	*Enterobacter cloacae*	C600	>128	>128	>128	>128	4	1	≤0.25	>128
MTH-29-1-2	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	128	1	>128
MTH-30-1-2	Environmental	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	64	≤0.25
MTW-1-1	Water	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	≤0.25
MTW-1-2	Water	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	8	≤0.25
MTW-2-1	Water	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	32	≤0.25
MTW-3-1	Water	*Escherichia coli*	C600	>128	>128	>128	>128	>128	128	128	≤0.25
MTW-3-2	Water	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	>128
MTW-4-1	Water	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	4	>128
MTW-4-2	Water	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	4	>128
MTW-7-1	Water	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	128	>128
MTW-8-1	Water	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	2	>128
MTW-8-2	Water	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	0.5	>128
MTW-8-3	Water	*Escherichia coli*	-	>128	>128	>128	>128	>128	64	4	>128
MTW-9-1	Water	*Escherichia coli*	-	>128	>128	>128	>128	>128	128	0.5	>128
MTW-18-1	Water	*Escherichia coli*	-	>128	>128	>128	>128	>128	128	64	≤0.25
MTW-18-2	Water	*Escherichia coli*	C600	>128	>128	>128	>128	>128	64	64	4
MTW-18-3	Water	*Escherichia coli*	C600	>128	>128	>128	>128	>128	128	≤0.25	≤0.25
MTAHC18-1	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	64	64	2
MTAHC18-2	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	128	64	≤0.25
MTNJC-3-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	64	≤0.25
MTNJC-3-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	128	0.5	≤0.25
MTNTC-2-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	1	≤0.25
MTNTC-5-1	Chicken	*Klebsiella pneumoniae*	-	>128	>128	>128	>128	>128	32	1	≤0.25
MTNTC-5-2	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	1	≤0.25
MTNTC-6-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	128	0.5	≤0.25
MTTZC-2-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	64	64	≤0.25

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MTTZC-2-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	64	≤0.25
MTTZC-3-1	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	≤0.25	≤0.25
MTAHG-11-1	Pigeon	*Providencia rettgeri*	-	32	>128	>128	>128	16	32	8	>128
MTAHY-2-1	Duck	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	32	≤0.25
MTAHY-4-1	Duck	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	8	≤0.25
MTAHY-4-2	Duck	*Escherichia coli*	-	>128	>128	>128	>128	>128	128	≤0.25	≤0.25
MTAHY-12-1	Duck	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	8	8
MTAHY-12-2	Duck	*Escherichia coli*	-	>128	>128	>128	>128	>128	4	≤0.25	≤0.25
MTAHY-13-1	Duck	*Escherichia coli*	-	>128	>128	>128	>128	>128	32	1	>128
MTAHY-13-2	Duck	*Escherichia coli*	-	>128	>128	>128	>128	16	4	≤0.25	≤0.25
MTYZG-24-3	Pigeon	*Klebsiella pneumoniae*	-	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
MTYZG-33-1	Pigeon	*Klebsiella pneumoniae*	-	32	128	>128	16	1	≤0.25	≤0.25	≤0.25
MSYCC-4-2	Chicken	*Escherichia coli*	C600	2	128	>128	>128	>128	128	≤0.25	≤0.25
MSYCC-5-1	Chicken	*Escherichia coli*	C600	64	>128	>128	>128	8	1	≤0.25	≤0.25
MSYCC-6-1	Chicken	*Escherichia coli*	C600	128	>128	>128	>128	16	4	32	≤0.25
MSYCC-6-2	Chicken	*Escherichia coli*	-	32	128	>128	>128	>128	>128	8	4
MSYCC-11-1	Chicken	*Escherichia coli*	-	8	>128	>128	>128	>128	>128	128	≤0.25
MSYCC-11-2	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	2	≤0.25
MSYCC-13-1	Chicken	*Escherichia coli*	C600	64	>128	>128	>128	>128	128	≤0.25	≤0.25
MSYCC-13-2	Chicken	*Escherichia coli*	C600	2	>128	>128	>128	>128	32	≤0.25	≤0.25
MSYCC-19-1	Chicken	*Escherichia coli*	C600	128	>128	>128	>128	>128	>128	1	≤0.25
MSYCC-20-1	Chicken	*Escherichia coli*	-	128	>128	>128	>128	>128	128	≤0.25	≤0.25
MSYCC-20-2	Chicken	*Escherichia coli*	-	128	>128	>128	>128	>128	>128	≤0.25	≤0.25
MSYCC-21-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	0.5	≤0.25
MSYCC-21-2	Chicken	*Escherichia coli*	C600	32	>128	>128	>128	>128	>128	0.5	≤0.25
MSYCC-24-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	2	≤0.25
MSYCC-25-1	Chicken	*Escherichia coli*	C600	32	>128	>128	>128	>128	>128	64	≤0.25
MSYCC-25-2	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	16	0.5
MSYCC-28-2	Chicken	*Escherichia coli*	-	128	>128	>128	>128	>128	>128	2	≤0.25
MSYCC-31-1	Chicken	*Escherichia coli*	-	32	128	>128	>128	>128	128	32	≤0.25
MSYCC-32-1	Chicken	*Escherichia coli*	C600	16	>128	>128	>128	>128	32	≤0.25	1
MSYCC-34-1	Chicken	*Escherichia coli*	-	16	>128	>128	>128	>128	>128	≤0.25	2
MSYCC-34-2	Chicken	*Escherichia coli*	C600	4	32	>128	>128	>128	64	8	≤0.25
MSYCC-35-1	Chicken	*Escherichia coli*	C600	32	>128	>128	>128	>128	>128	0.5	0.5
MSYCC-37-1	Chicken	*Escherichia coli*	C600	64	>128	>128	>128	>128	64	32	≤0.25
MSYCC-40-1	Chicken	*Escherichia coli*	C600	128	>128	>128	>128	>128	>128	32	≤0.25
MSYCC-42-1	Chicken	*Escherichia coli*	C600	16	16	>128	>128	>128	1	≤0.25	≤0.25
MSYCC-43-1	Chicken	*Escherichia coli*	C600	128	128	>128	>128	>128	>128	32	≤0.25
MSYCC-45-1	Chicken	*Escherichia coli*	C600	64	>128	>128	>128	>128	32	32	≤0.25
MSYCC-45-2	Chicken	*Escherichia coli*	C600	64	>128	>128	>128	>128	64	≤0.25	≤0.25
MSYCC-46-1	Chicken	*Escherichia coli*	-	64	>128	>128	>128	>128	>128	32	≤0.25
MSYCC-47-1	Chicken	*Escherichia coli*	C600	64	>128	>128	>128	>128	>128	2	≤0.25
MSYCC-48-1	Chicken	*Escherichia coli*	-	64	>128	>128	>128	>128	2	2	≤0.25
MSYCC-49-1	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	128	16	≤0.25
MSYCC-51-1	Chicken	*Escherichia coli*	-	128	>128	>128	>128	>128	>128	2	≤0.25
MSYCC-51-2	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	>128	>128	≤0.25
MSYCC-51-3	Chicken	*Escherichia coli*	C600	32	>128	>128	>128	>128	>128	64	≤0.25
MSYCC-52-1	Chicken	*Escherichia coli*	C600	64	>128	>128	>128	>128	128	32	0.5
MSYCC-52-2	Chicken	*Escherichia coli*	C600	128	>128	>128	>128	>128	>128	16	1
MSYCC-54-1	Chicken	*Escherichia coli*	C600	32	>128	>128	>128	>128	2	0.5	≤0.25
MSYCC-54-2	Chicken	*Proteus mirabilis*	C600	>128	>128	>128	>128	>128	>128	16	>128
MSYCC-55-1	Chicken	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	64	1
MSYCC-57-1	Chicken	*Escherichia coli*	-	>128	>128	>128	>128	>128	64	≤0.25	8
MSNTC-3-1	Chicken	*Escherichia coli*	C600	64	128	>128	>128	4	2	16	≤0.25
MSNTC-4-1	Chicken	*Escherichia coli*	C600	8	64	>128	>128	>128	128	8	≤0.25
MSNTC-7-1	Chicken	*Escherichia coli*	C600	64	64	>128	>128	4	1	>128	≤0.25
MSNTC-9-1	Chicken	*Escherichia coli*	C600	32	64	>128	>128	>128	128	>128	≤0.25

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MSNTC-10-1	Chicken	*Escherichia coli*	-	32	64	>128	>128	>128	>128	≤0.25	≤0.25
MSNTC-10-2	Chicken	*Escherichia coli*	C600	64	128	>128	>128	>128	>128	≤0.25	≤0.25
MSNTC-12-1	Chicken	*Escherichia coli*	C600	64	128	>128	>128	>128	128	64	≤0.25
MSNTC-12-2	Chicken	*Escherichia coli*	-	64	>128	>128	>128	>128	>128	>128	≤0.25
MSNTC-13-1	Chicken	*Escherichia coli*	C600	2	>128	>128	>128	4	2	32	≤0.25
MSNTC-13-2	Chicken	*Escherichia coli*	C600	32	64	>128	>128	4	2	16	≤0.25
MSNTC-14-1	Chicken	*Escherichia coli*	C600	16	64	>128	>128	>128	1	8	≤0.25
MSNTC-14-2	Chicken	*Escherichia coli*	C600	32	64	>128	>128	>128	2	8	≤0.25
MSNTC-17-1	Chicken	*Escherichia coli*	C600	32	64	>128	>128	>128	1	≤0.25	≤0.25
MSNTC-21-1	Chicken	*Escherichia coli*	C600	4	8	>128	>128	>128	>128	8	≤0.25
MSNTC-21-2	Chicken	*Escherichia coli*	C600	4	8	>128	>128	>128	>128	4	≤0.25
MSNTC-29-1	Chicken	*Escherichia coli*	C600	64	128	>128	>128	4	1	16	≤0.25
MSY-2-1	Duck	*Escherichia coli*	-	64	64	>128	>128	8	32	64	≤0.25
MSY-2-2	Duck	*Escherichia coli*	-	64	128	>128	>128	>128	128	0.5	≤0.25
MSY-3-1	Duck	*Escherichia coli*	-	64	64	>128	>128	16	32	32	≤0.25
MSY-3-2	Duck	*Escherichia coli*	C600	8	16	>128	>128	>128	128	≤0.25	≤0.25
MSY-4-1	Duck	*Escherichia coli*	-	32	64	>128	>128	8	32	64	≤0.25
MSY-4-2	Duck	*Escherichia coli*	-	≤0.25	8	>128	>128	>128	16	≤0.25	≤0.25
MSY-6-2	Duck	*Escherichia coli*	-	2	8	>128	>128	>128	64	≤0.25	≤0.25
MSY-7-1	Duck	*Escherichia coli*	-	≤0.25	1	>128	>128	>128	8	≤0.25	≤0.25
MSY-8-1	Duck	*Escherichia coli*	C600	32	128	>128	>128	>128	64	≤0.25	≤0.25
MSY-9-2	Duck	*Escherichia coli*	C600	64	64	>128	>128	128	64	1	>128
MSY-10-1	Duck	*Escherichia coli*	-	32	128	>128	>128	4	1	16	≤0.25
MSY-10-2	Duck	*Escherichia coli*	C600	16	32	>128	>128	>128	64	≤0.25	≤0.25
MSY-11-1	Duck	*Escherichia coli*	C600	32	32	>128	>128	>128	16	4	≤0.25
MSY-13-1	Duck	*Escherichia coli*	C600	128	128	>128	>128	>128	64	8	≤0.25
MSY-13-2	Duck	*Escherichia coli*	C600	2	8	>128	>128	>128	64	≤0.25	≤0.25
MSY-14-1	Duck	*Escherichia coli*	-	32	64	>128	>128	16	32	64	≤0.25
MSY-14-2	Duck	*Escherichia coli*	-	128	128	>128	>128	>128	>128	1	>128
MSY-15-1	Duck	*Escherichia coli*	C600	128	128	>128	>128	>128	128	≤0.25	≤0.25
MSG-2-1	Pigeon	*Escherichia coli*	-	64	64	>128	>128	>128	1	8	≤0.25
MSG-4-1	Pigeon	*Escherichia coli*	-	32	128	>128	>128	>128	>128	64	≤0.25
MSG-5-1	Pigeon	*Escherichia coli*	C600	128	128	>128	>128	>128	64	64	≤0.25
MSE-1-1	Goose	*Escherichia coli*	-	1	8	>128	>128	>128	16	≤0.25	≤0.25
MSE-2-1	Goose	*Escherichia coli*	-	64	128	>128	>128	>128	16	2	≤0.25
MSW-2-1	Water	*Escherichia coli*	C600	16	8	>128	>128	>128	>128	8	2
MSW-4-1	Water	*Klebsiella pneumoniae*	-	32	16	>128	>128	>128	0.5	0.5	≤0.25
MSW-4-2	Water	*Escherichia coli*	-	16	16	>128	>128	8	2	0.5	≤0.25
MSW-5-1	Water	*Escherichia coli*	C600	16	8	>128	>128	8	1	≤0.25	≤0.25
MSW-5-2	Water	*Escherichia coli*	-	8	4	>128	>128	>128	32	16	≤0.25
MSW-6-1	Water	*Escherichia coli*	C600	64	8	>128	>128	>128	128	≤0.25	≤0.25
MSW-7-1	Water	*Escherichia coli*	C600	64	8	>128	>128	>128	32	128	4
MSW-7-2	Water	*Klebsiella pneumoniae*	-	32	16	>128	>128	>128	64	0.5	≤0.25
MSW-9-1	Water	*Escherichia coli*	C600	32	8	>128	>128	>128	16	1	≤0.25
MSW-11-1	Water	*Escherichia coli*	-	32	16	>128	>128	>128	2	≤0.25	≤0.25
MSW-11-2	Water	*Escherichia coli*	C600	8	16	>128	>128	>128	128	0.5	≤0.25
MSW-12-1	Water	*Escherichia coli*	C600	32	16	>128	>128	8	2	8	≤0.25
MSW-12-2	Water	*Enterobacter cloacae*	C600	32	16	>128	>128	4	1	≤0.25	≤0.25
MSW-13-1	Water	*Enterobacter cloacae*	C600	>128	>128	>128	>128	1	0.5	≤0.25	≤0.25
MSW-13-2	Water	*Escherichia coli*	C600	64	16	>128	>128	4	0.5	16	≤0.25
MSW-14-1	Water	*Escherichia coli*	-	32	8	>128	>128	8	≤0.25	8	≤0.25
MSW-15-1	Water	*Escherichia coli*	C600	>128	>128	>128	>128	>128	>128	128	≤0.25
MSH-11-1	Environmental	*Escherichia coli*	C600	>128	>128	>128	>128	>128	128	1	≤0.25
MSH-14-1	Environmental	*Enterobacter cloacae*	-	>128	>128	>128	>128	>128	>128	128	>128

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MSH-17-1	Environmental	*Escherichia coli*	C600	>128	>128	>128	>128	64	128	64	≤0.25
MSH-19-1	Environmental	*Klebsiella pneumoniae*	-	>128	>128	>128	>128	>128	>128	8	≤0.25
MSH-19-2	Environmental	*Escherichia coli*	-	64	16	>128	>128	>128	8	1	≤0.25
MSH-21-1	Environmental	*Escherichia coli*	C600	8	4	>128	>128	>128	16	2	≤0.25

Genomic Analysis of bla$_{NDM}$*-Positive Strains*

To investigate the genetic characteristics of *bla*$_{NDM}$-positive strains, we selected 38 representative *bla*$_{NDM}$-positive strains for whole-genome sequencing and analysis, including 29 *E. coli* strains, 4 *E. cloacae* strains, 2 *K. pneumoniae* strains, 2 *K. aerogenes* strains, and 1 *P. mirabilis* strain. Based on core genome SNPs, we constructed a phylogenetic tree of 29 *bla*$_{NDM}$-positive *E. coli* strains (Figure 1). The 29 *E. coli* strains from this study presented 18 distinct sequence types, with ST226 (13.79%, 4/29), ST6858 (13.79%, 4/29) and ST1630 (10.34%, 3/29) being the most prominent. A total of 19 serotypes were identified, mainly including O1:H45 (13.79%, 4/29), O8:H4 (13.79%, 4/29), and O16:H48 (10.34%, 3/29). We counted the number of virulence genes of all the *E. coli* strains based on the VFDB core datasets. It is worth noting that one strain of serotype O153:H2 *E. coli* carries 122 virulence genes, and one strain of serotype O8:H16 *E. coli* carries 108 virulence genes (Figure 1).

Among the 29 *bla*$_{NDM}$-positive *E. coli* strains, 24 harbored the *bla*$_{NDM-5}$ gene. The remaining strains included three with *bla*$_{NDM-39}$, one with *bla*$_{NDM-13}$, and one with *bla*$_{NDM-27}$. Co-occurring β-lactamase genes included *bla*$_{OXA-10}$ (19/29, 65.52%), with multiple *bla*$_{TEM}$ and *bla*$_{CTX-M}$ variants also present. Moreover, the *floR* gene was carried by almost all strains (96.55%, 28/29), and the majority of strains also harbored the *qnrS1* gene (75.86%, 22/29). It is noteworthy that the coexistence of the colistin resistance gene *mcr-1* and *bla*$_{NDM-5}$ was found in five strains, and the coexistence of the tigecycline resistance gene *tet*(X4) and *bla*$_{NDM-5}$ was detected in one strain. Furthermore, the resistance gene *estT* encoding macrolide hydrolase was identified in two strains (Figure 1).

ARGs harbored by *E. cloacae*, *K. pneumoniae*, *K. aerogenes*, and *P. mirabilis* differed from that harbored by *E. coli* (Figure S1). Except for *P. mirabilis*, which harbored *bla*$_{NDM-1}$, all other strains carry *bla*$_{NDM-5}$. Additionally, *bla*$_{OXA-10}$ was detected in two *K. aerogenes* strains and one *P. mirabilis* strain. Moreover, *bla*$_{TEM-176}$ and *bla*$_{TEM-1B}$ were identified in two *K. pneumoniae* strains. Except for two *E. cloacae* strains, all other strains harbored the *floR* gene. Furthermore, strains from different genera carried different variants of the *fosA* gene: *E. cloacae* carried *fosA2*, *P. mirabilis* carried *fosA3*, *K. aerogenes* carried *fosA5* and *fosA7*, and *K. pneumoniae* carried *fosA6*.

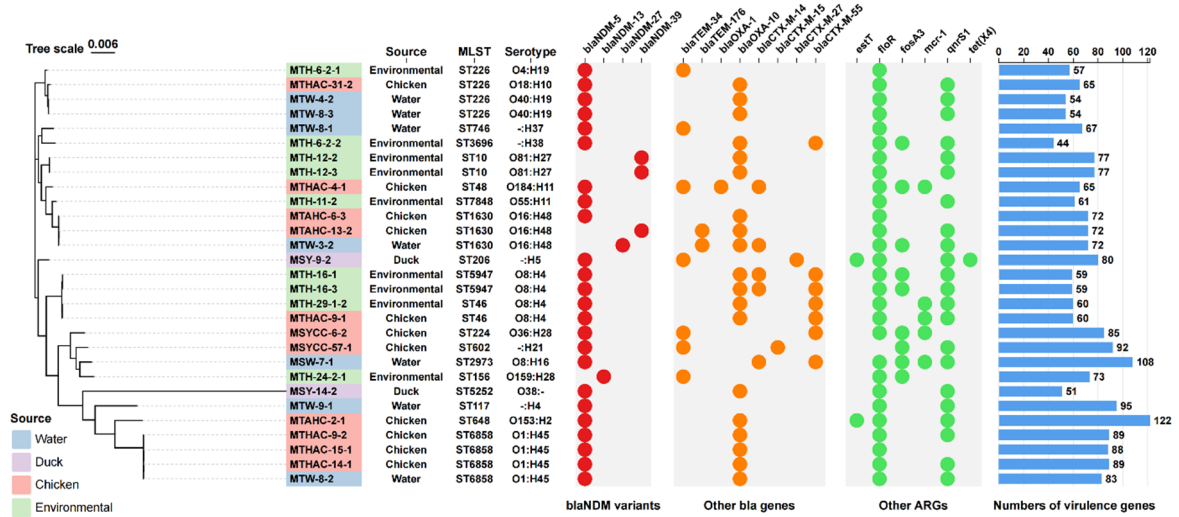

Figure 1. The phylogenetic tree and ARG heatmap of 29 *E. coli* isolates. The phylogenetic tree was generated by FastTree based on core genes alignment using Roary and was visualized using Chiplot. Isolates from different

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sources are highlighted in different colors. The three columns of information marked next to the strain names are isolation source, ST type (identified by MLST), and Serotype (identified by ECTyper). The three sets of heatmaps show the presence of ARGs in the strains. The outermost bar chart shows the number of virulence genes in the isolates based on the VFDB core dataset.

Genetic Environment Analysis of Various bla$_{NDM}$ *Gene Variants*

Multiple plasmid replicon types were detected in all the *bla*$_{NDM}$-positive strains, but we only observed that the *bla*$_{NDM-5}$ gene is directly located on the IncX3-type plasmids in 3 *E. cloacae* and 1 *E. coli* (Figure 2). The transfer of *bla*$_{NDM-5}$ was mediated by the upstream IS*5* or IS*Aba125*. In addition, we observed that in two strains of *E. coli*, the *mcr-1* gene was located on a 60kb Incl2-type plasmid and a 105kb IncHI2A-type plasmid, respectively (Figure S2).

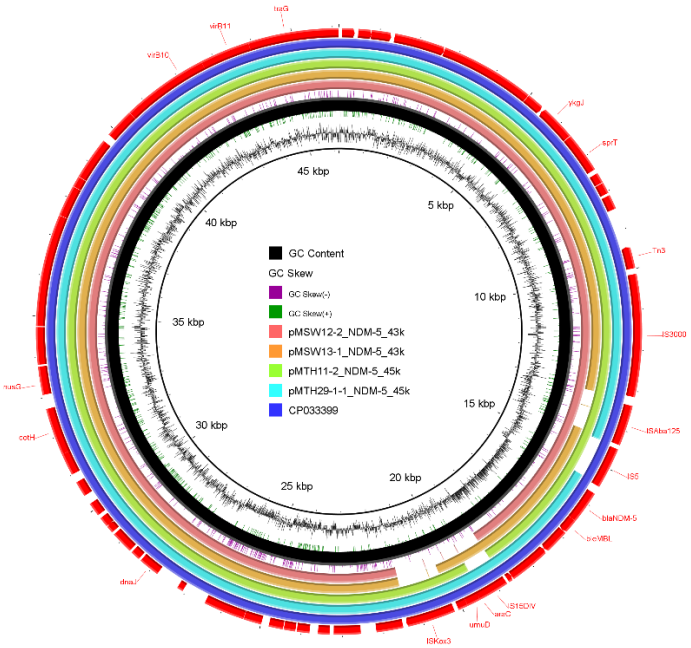

Figure 2. Plasmid profile of the *bla*$_{NDM-5}$-containing IncX3-type plasmid. Plasmid slices (assembled contigs, not complete plasmids) from this study were compared with a plasmid (CP033399.1) derived from *E. coli*. The GC skew and GC content are depicted in an inward-to-outward sequence. The outermost arrows indicate the positions and transcriptional orientations of the open reading frames.

Genetic environment analysis revealed the diversity of *bla*$_{NDM}$ variants-bearing genetic contexts. IS*Aba125*-IS*5*-*bla*$_{NDM-5}$-*ble*$_{MBL}$ was the most common transposable structure found in *E. coli*, *E. cloacae*, and *K. aerogenes* (Figure 3A). In another *K. aerogenes*, we discovered the genetic structure of *ble*$_{MBL}$-*bla*$_{NDM-5}$-IS*5*-IS*1A*-*aph*(3 '')-Ib-*aph*(6)-Id-*aph*(3 '')-Ia. This genetic structure may have been formed by the insertion of IS*1A*-*aph*(3 '')-Ib-*aph*(6)-Id-*aph*(3 '')-Ia mediated by IS*1A*, which replaced the previous IS*Aba125*. Additionally, the IS*Aba125*-*bla*$_{NDM-1}$-*ble*$_{MBL}$ transposon structure was identified in one *P. mirabilis* strain.

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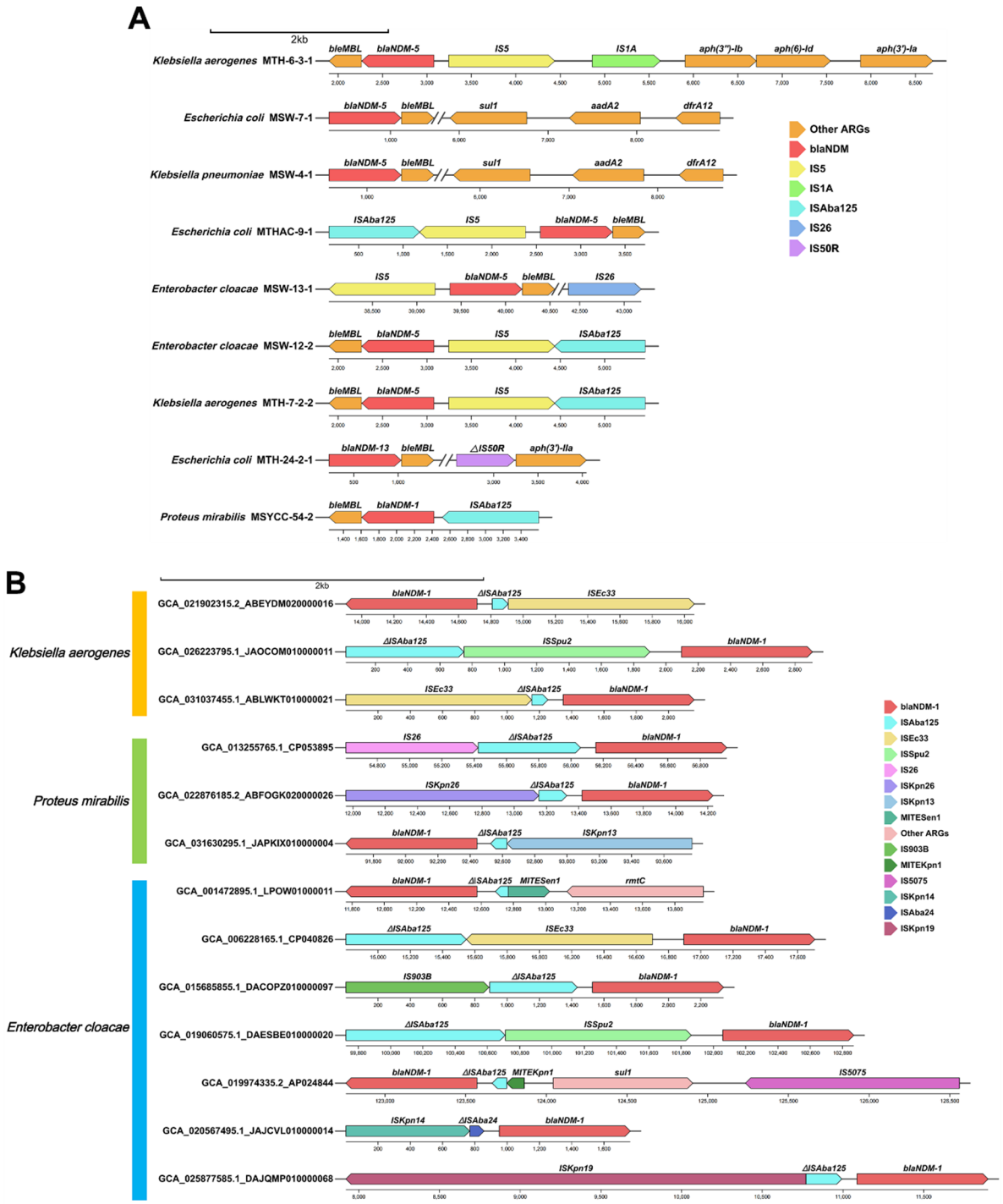

Figure 3. Genetic environments of several *bla*NDM variants in assembled genomes and genomes from database. (A) Different types of genetic environments of *bla*NDM variants in assembled genomes. Different genetic elements are highlighted in different colors. (B) Primary genetic contexts of *bla*NDM-1 across different species. In different species, the transfer of *bla*NDM-1 is mediated by various IS elements.

*Correlation Analysis of bla*NDM *with Other ARGs, ISs and Plasmid Replicons*

To thoroughly investigate the genetic background of the *bla*NDM gene, we collected the CREC samples used in the previous study[23] and downloaded all the genomes of the genera *Klebsiella*, *Enterobacter*, and *Proteus* from the NCBI database. Through sequence alignment, we identified a total of 4,072 *bla*NDM-positive CREC strains (Table S3), 8,465 *bla*NDM-positive *K. pneumoniae* strains (Table S5), 84 *bla*NDM-positive *K. aerogenes* strains (Table S6), 139 *bla*NDM-positive *P. mirabilis* strains (Table S7), and 105 *bla*NDM-positive *E. cloacae* strains (Table S8).

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Distinct distributions of *bla*$_{NDM}$ variants were observed across species (Figure 4A). Upon analysis of the assembled genomes from this study and downloaded genomes, it was observed that 76.88% (3,153/4,101) of *bla*$_{NDM}$-positive CREC strains harbored the *bla*$_{NDM-5}$ gene, 15.51% (636/4,101) possessed the *bla*$_{NDM-1}$ gene, 3.71% (152/4,101) carried the *bla*$_{NDM-7}$ gene, and 1.95% (80/4,101) contained the *bla*$_{NDM-4}$ gene (Table S9). Notably, two CREC strains were found to concurrently harbor *bla*$_{NDM-1}$, *bla*$_{NDM-4}$, *bla*$_{NDM-5}$, and *bla*$_{NDM-24}$. In *K. pneumoniae* strains, the distribution was as follows: 66.78% (5,653/8,477) carried the *bla*$_{NDM-1}$ gene, 27.23% (2,305/8,477) possessed the *bla*$_{NDM-5}$ gene, 3.61% (306/8,477) harbored the *bla*$_{NDM-7}$ gene, and 1.44% (122/8,477) contained the *bla*$_{NDM-4}$ gene (Table S10). For *K. aerogenes* strains, the proportions are 44.18% (38/86) for the *bla*$_{NDM-1}$ gene, 31.40% (27/86) for the *bla*$_{NDM-5}$ gene, and 23.26% (20/86) for the *bla*$_{NDM-7}$ gene (Table S11). In *bla*$_{NDM}$-positive *P. mirabilis* strains, 70.71% (99/140) carried the *bla*$_{NDM-1}$ gene, 22.86% (32/140) possessed the *bla*$_{NDM-7}$ gene, and 6.43% (9/140) harbored the *bla*$_{NDM-5}$ gene (Table S12). As for *bla*$_{NDM}$-positive *E. cloacae* strains, 75.23% (82/109) carried the *bla*$_{NDM-1}$ gene, while 20.18% (22/109) possessed the *bla*$_{NDM-5}$ gene (Table S13).

Network graph analysis revealed that different *bla*$_{NDM}$ gene variants in different species exhibit distinct preferences for genetic elements (Figure 4B). When the absolute value of R is greater than 0.3 and *p* is less than 0.05, we consider that there is a correlation between different genetic elements. In CREC strains, we found that *bla*$_{NDM-5}$ was strongly correlated with *bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, and *bla*$_{OXA-1}$, while *bla*$_{NDM-1}$ was strongly correlated with *bla*$_{SHV-12}$ ($R > 0.3$, $p < 0.05$). In addition, *bla*$_{NDM-5}$ was strongly correlated with ARGs such as *sul1*, *aadA2*, *mph(A)*, and insertion sequence IS*6100*, whereas *bla*$_{NDM-1}$ was strongly correlated with *rmtC* and *aph*(3')-VI, and *bla*$_{NDM-7}$ was strongly correlated with IS*Cfr27* ($R > 0.3$, $p < 0.05$). However, unlike CREC strains, in *K. pneumoniae* strains, *bla*$_{NDM-5}$ only showed positive associations with ARGs such as *rmtB*, *erm*(B), *oqxA*, *oqxB*, and *mph*(A), as well as the plasmid replicon IncX3, while *bla*$_{NDM-1}$ was strongly correlated with *bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, and *bla*$_{OXA-9}$ ($R > 0.3$, $p < 0.05$). Additionally, *bla*$_{NDM-1}$ was also strongly correlated with ARGs such as *oqxB*, *oqxA*, and *sul1* ($R > 0.3$, $p < 0.05$). In *K. aerogenes* strains, a distinct correlation pattern was observed. Genetic elements such as *bla*$_{SHV-12}$, IS*Sen4*, IS*Cfr4*, and IS*Kpn26* were found to be strongly positively correlated with *bla*$_{NDM-1}$ ($R > 0.3$, $p < 0.05$), while *floR*, IS*Aba125*, and IS*5* exhibited negative correlations with *bla*$_{NDM-1}$ ($R < -0.3$, $p < 0.05$). Notably, plasmid replicons including IncN2, IncHI1B, and IncFIB were identified as being strongly positively correlated with *bla*$_{NDM-1}$ ($R > 0.3$, $p < 0.05$), whereas IncX3 showed a negative correlation with *bla*$_{NDM-1}$ ($R < -0.3$, $p < 0.05$). However, IncX3 and IS*5* were positively correlated with *bla*$_{NDM-5}$ ($R > 0.3$, $p < 0.05$). Similar correlation patterns were also observed in *P. mirabilis* strains and *E. cloacae* strains. In both *P. mirabilis* strains and *E. cloacae* strains, *bla*$_{NDM-1}$ was strongly negatively correlated with IS*Aba125*, IncX3, and IS*5* ($R < -0.3$, $p < 0.05$). In *P. mirabilis* strains, *bla*$_{NDM-7}$ was positively correlated with IS*5* and IS*Aba125* ($R > 0.3$, $p < 0.05$), meanwhile in *E. cloacae strains*, *bla*$_{NDM-5}$ was positively correlated with IncX3, IS*5*, and IS*Aba125* ($R > 0.3$, $p < 0.05$). Additionally, in *P. mirabilis* strains, *bla*$_{NDM-1}$ was positively correlated with *bla*$_{OXA-10}$, *sul1*, *arr-3*, *aph*(3')-Ia, and Col3M ($R > 0.3$, $p < 0.05$), and negatively correlated with IncC and *qnrS1* ($R < -0.3$, $p < 0.05$). In contrast, *bla*$_{NDM-7}$ was positively correlated with *qnrS1*, IncC, and *floR* ($R > 0.3$, $p < 0.05$), and negatively correlated with Col3M, *sul1*, *arr-3*, and *aph*(3')-Ia ($R < -0.3$, $p < 0.05$). In *E. cloacae* strains, *bla*$_{NDM-1}$ was positively correlated with *bla*$_{CMH-3}$ ($R > 0.3$, $p < 0.05$), and negatively correlated with IS*Kox3* and *floR* ($R < -0.3$, $p < 0.05$), while *bla*$_{NDM-5}$ was positively correlated with IS*Kox3* and *floR* ($R > 0.3$, $p < 0.05$).

Unexpectedly, *bla*$_{NDM-1}$ exhibited negative associations with IS*Aba125* and IS*5* in *K. aerogenes*, *P. mirabilis*, and *E. cloacae*—despite these IS elements being canonical mediators of *bla*$_{NDM-1}$ transfer. We further investigated the genomic characteristics of *K. aerogenes* strains, *P. mirabilis* strains and *E. cloacae* strains and found that IS*Aba125* was often interrupted by various insertion sequences other than IS*5* (Figure 3B). In *K. aerogenes* strains, IS*Aba125* was interrupted by IS*Ec33* and IS*Spu2*. In *P. mirabilis* strains, IS*Aba125* was interrupted by IS*26*, IS*Kpn26*, and IS*Kpn13*. In *E. cloacae* strains, IS*Aba125* was interrupted by IS*Ec33*, IS*903B*, IS*Spu2*, MITE*Kpn1*, IS*Kpn14*, and IS*Kpn19*. This may suggest that different species capture the heterologous IS*Aba125*-*bla*$_{NDM-1}$ transposon via different types of insertion sequences and integrate it into their own genomes to better adapt the *bla*$_{NDM-1}$ gene to different genetic environments.

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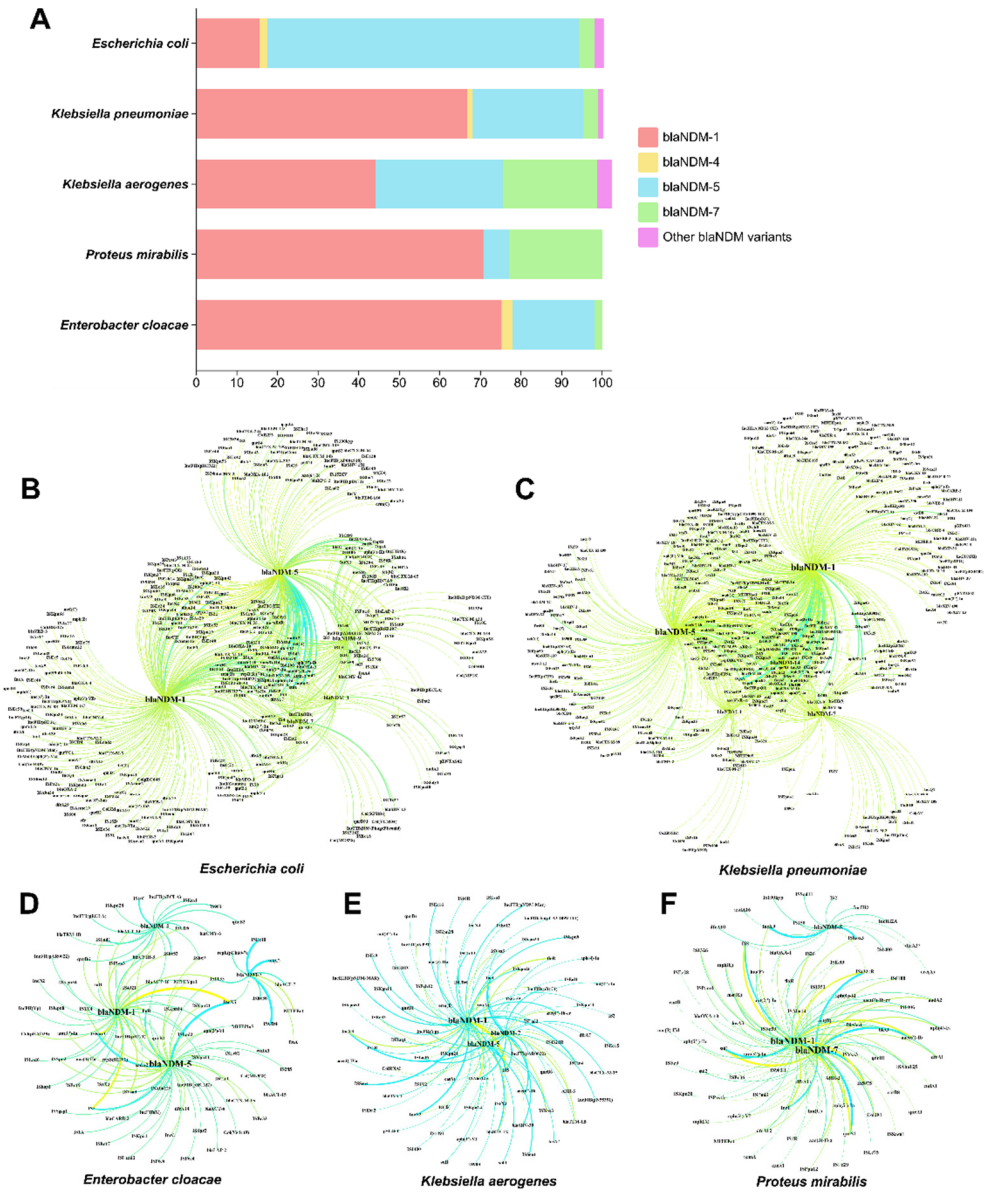

Figure 4. The proportion of *bla*NDM variants across different species and the network graph depicting the coexistence patterns of different *bla*NDM gene variants with other ARGs, ISs and plasmid replicons harbored in different bacteria. (A) The bar chart shows the percentage of *bla*NDM variants in *bla*NDM-positive strains of different species. (B-E) The network graph illustrates the correlations between *bla*NDM variants and other genetic elements in *bla*NDM -positive strains of different species. The nodes represent ARGs, ISs and plasmid replicons identified in all *bla*NDM-positive strains of from different species. The connections between nodes signify their interrelatedness. Blue hues and increased line thickness denote stronger positive correlations. The intensity of the yellow color on the lines indicates the strength of negative correlations, with darker shades of yellow corresponding to stronger negative correlations. Additionally, the thickness of the lines is directly proportional to the correlation strength, where a thicker line signifies a more pronounced relationship between the variables. All associated genes depicted in the figure exhibited p values less than 0.05.

Discussion

Carbapenem-resistant *Enterobacteriaceae* of animal origin represent a critical group of antimicrobial-resistant pathogens. The increasing number of carbapenem-resistant isolates identified poses a severe threat to global public health security. The *bla*NDM gene, which encodes NDM, is an important ARG associated with human clinical medicine. It was first identified in a clinical isolate of *K. pneumoniae* from a hospitalized patient[26]. Although it is only prevalent in Gammaproteobacteria

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[27], it has had a significant impact on human clinical medicine [28–32], markedly reducing the efficacy of clinical treatments.

Live poultry markets serve as reservoirs and dissemination centers for ARGs [2]. The convergence of live poultry from various regions significantly amplifies the risk of ARG spread. Given the close contact between humans, animals, and the environment in live poultry markets, establishing a "One Health" AMR monitoring system in these settings is crucial for preventing the transmission of multidrug-resistant pathogens and for devising effective containment strategies [33]. In this study, we investigated *bla*$_{NDM}$ -positive strains in two live poultry markets in Jiangsu Province, China. We found that over 90% of *bla*$_{NDM}$-positive strains were *E. coli*, indicating the widespread presence of CREC strains in poultry. This may be because the *bla*$_{NDM}$-bearing plasmids have a high fitness cost in other *Enterobacteriaceae* bacteria, but there is still a risk of further dissemination. Additionally, conjugation assays revealed that nearly 40% of *bla*$_{NDM}$-positive strains harbored transferable *bla*$_{NDM}$ genes, suggesting that the *bla*$_{NDM}$ gene can be widely disseminated in live poultry markets.

We obtained assembled genomes of 38 *bla*$_{NDM}$-positive strains through whole-genome sequencing. In five *E. coli* isolates, we detected the coexistence of *bla*$_{NDM-5}$ and *mcr-1*. This once again demonstrates that, despite China's ban on the use of colistin in animal husbandry, animal sources still harbor stable populations of *E. coli* that are resistant to both carbapenems and colistin [34]. Notably, in one strain of O8:H16 serotype *E. coli* isolate coharboring *bla*$_{NDM-5}$ and *mcr-1*, we identified 108 virulence genes, indicating the potential for the spread of highly pathogenic multidrug-resistant bacteria in live poultry markets. Additionally, we identified 122 virulence genes in an O153:H2, ST648-type *bla*$_{NDM-5}$-positive *E. coli* strain. ST648-type *E. coli* is considered a high-risk, globally epidemic clone that can cause human infections [35]. This finding serves as a warning for the sanitation efforts in live poultry markets.

Genetic environment analysis of assembled genomes from this study revealed that the *bla*$_{NDM}$ gene was commonly transferred via IS*Aba125* or IS*5*. However, surprisingly, through network analysis of downloaded *bla*$_{NDM}$-positive strains from the database, we found that in *K. aerogenes* strains, *P. mirabilis* strains, and *E. cloacae* strains, the *bla*$_{NDM-1}$ gene was negatively correlated with IS*Aba125* and IS*5*, which is contrary to the common situation. Upon further investigation, we discovered that in *bla*$_{NDM-1}$-positive *K. aerogenes* strains, *P. mirabilis* strains, and *E. cloacae* strains that lack IS*Aba125* (actually harboring truncated sequences), different insertion sequences interrupt the IS*Aba125*. Among these insertion sequences, the transfer of the *bla*$_{NDM-1}$ gene mediated by IS*Ec33* [36,37], IS*6100* [38], IS*903B* [39], IS*Kpn14* [40], IS*Kpn19* [41], IS*Kpn26* [39], and MITE*Sen1* [39] has been reported. However, to our knowledge, this study is the first to report the transfer of the *bla*$_{NDM-1}$ gene mediated by IS*Kpn13*, IS*Spu2*, and MITE*Kpn1*. In addition, regarding the fact that IS*Aba125* is frequently truncated by various types of insertion sequences across different species, we hypothesize that this phenomenon may result from the adaptation of certain insertion sequences to the genomes of these species. This adaptation allows insertion sequences for the capture of the IS*Aba125*-*bla*$_{NDM-1}$ transposon and its integration into the genetic environment of the respective strains.

Overall, our findings indicate that the prevalence of carbapenem-resistant strains in live poultry markets is a cause for concern. The potential spread of highly virulent, multidrug-resistant pathogens underscores the importance of comprehensive surveillance efforts. Moreover, the molecular mechanisms by which strains of different species capture the *bla*$_{NDM-1}$ gene warrant further investigation. Herein, we call for enhanced sanitation management in live poultry markets, the implementation of appropriate measures to curb the dissemination of *bla*$_{NDM}$-positive strains, and safeguarding food safety in animal husbandry through a One Health approach [42].

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Data Availability Statement: WGS data generated from this study are openly available at the China National GeneBank Database (CNGBdb) with accession number of CNP0007032.

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