Contaminated in-house environment contributes to the persistence and transmission of NDM-producing bacteria in a Chinese poultry farm

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ABSTRACT

While carbapenem use is prohibited in the poultry production chain and carbapenem-resistant *Enterobacteriaceae* (CRE) are absent from hatchery farms, New Delhi metallo-β-lactamase-producing CRE contamination of commercial broiler chicken farms (grow-out farms) can occur via living hosts such as flies. However, it is not known whether the inanimate factors from in-house environment play a role in the persistence of CRE on commercial farms. Herein, we monitored one typical broiler house in Hebei Province, China, from January 2017 to April 2018. We collected 350 cloacal samples from four broiler batches along with 582 environmental samples (194 in the raising period and 388 in the vacancy period) from sites including the surfaces of drooping boards, feeding troughs, nipple drinkers, corridor floors, sewage trenches, and air. All samples were screened for bla_{NDM} and cultured for NDM-producing isolates. The resistance profiles, genotypes, and genetic context of bla_{NDM} in CRE isolates were further characterized. Results showed that 1-day-old broilers, which were transferred from a hatchery farm and negative for CRE, acquired bla_{NDM} within 24 h of transfer (2 days of age), with a detection rate of up to 18.6%. High bla_{NDM} detection rates (26.8%–31.4%) were obtained among all environmental samples except air after standard cleaning and disinfection during the vacancy period. bla_{NDM} carriage rates (52.9%–72.9%) within the flocks remain stable and high across the next three broiler batches. Overall, 279 NDM-producing bacteria, including 259 *Enterobacteriaceae* (8 species), 14 *Morganellaceae* (3 species), three *Alcaligenes faecalis* and three *Pseudomonas putida* isolates, were recovered from 85 (24.3%) cloacal and 101 (17.4%) environmental samples. Three NDM variants, including NDM-5 ($n = 181$), NDM-1 ($n = 92$), and NDM-9 ($n = 3$), and a novel NDM-like-metallo-β-lactamase (NLM, $n = 3$) were identified among the samples. The predominant NDM-producing CRE species among the samples were *Klebsiella pneumoniae* (CRKP; 32.6%, $n = 91$) and *Escherichia coli* (CREC; 27.2%, $n = 76$). Both clonal and horizontal transmission of bla_{NDM} and an overlap of sequence types (STs) were observed in both CREC and CRKP from chicken and environmental samples. Notably, ST6751 CREC and ST37 CRKP persisted throughout the 16-month surveillance period. IncX3 ($n = 197$, 7 species), IncA/C$_2$ ($n = 41$, 5 species), and IncFII ($n = 8$, *E. coli*) were the three major bla_{NDM}-carrying plasmid types among the isolates. Although routine cleaning and disinfection procedures and "all-in/all-out" management were performed, once introduced to the farm environment, a diverse range of NDM-positive isolates may survive and persist, becoming an important reservoir of NDM-positive CRE for broiler chickens. Therefore, cleaning and disinfection procedures should be improved on poultry farms to avoid cross-contamination of NDM-producing bacteria between different batches of chickens, as well as further downstream in the poultry production chain.

1. Introduction

The rapid and worldwide dissemination of multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative bacteria is of great concern. Carbapenems are critically important for the treatment of infections caused by these pathogens (WHO, 2019); hence, the emergence and global spread of carbapenem-resistant *Enterobacteriaceae* (CRE) is one of the most pressing public health threats relating to antibiotic resistance worldwide (Van Duin and Doi, 2017). First reported in 2008, New Delhi metallo-β-lactamases (NDMs) are one of the main types of carbapenemases and confer resistance to almost all β-lactam antibiotics, including carbapenems. To date, 29 NDM variants (NDM-1 to −29) have been identified, and NDM-producing bacteria have been isolated in 50 countries across six continents (Berrazeg et al., 2014; Logan and Weinstein, 2017). Although *Enterobacteriaceae* species are most commonly associated with NDM gene carriage, bla_{NDM} genes

have sporadically been identified in strains from 10 other Gram-negative bacterial families, including *Aeromonadaceae, Alcaligenaceae, Cardiobacteriaceae, Moraxellaceae, Morganellaceae, Neisseriaceae, Pseudomonadaceae, Shewanellaceae, Vibrionaceae,* and *Xanthomonadaceae* (Wu et al., 2019).

NDM-producing bacteria are predominantly isolated from humans; however, the emergence of bla_{NDM}-positive bacteria in food-producing and companion animals is of increasing concern (Woodford et al., 2014). In particular, high prevalence rates of NDM-producing *Enterobacteriaceae* have been observed on Chinese poultry farms (Wang et al., 2017; Xiang et al., 2018). As a result of increasing demand for poultry meat, poultry production in China has topped 10 billion chickens per year since 2017 (Broilers research, 2017). This huge amount of chickens was mostly reared with the battery cage layer poultry rearing method, characterized by limited space and high density among Chinese poultry farms over the last decade (Chinese poultry
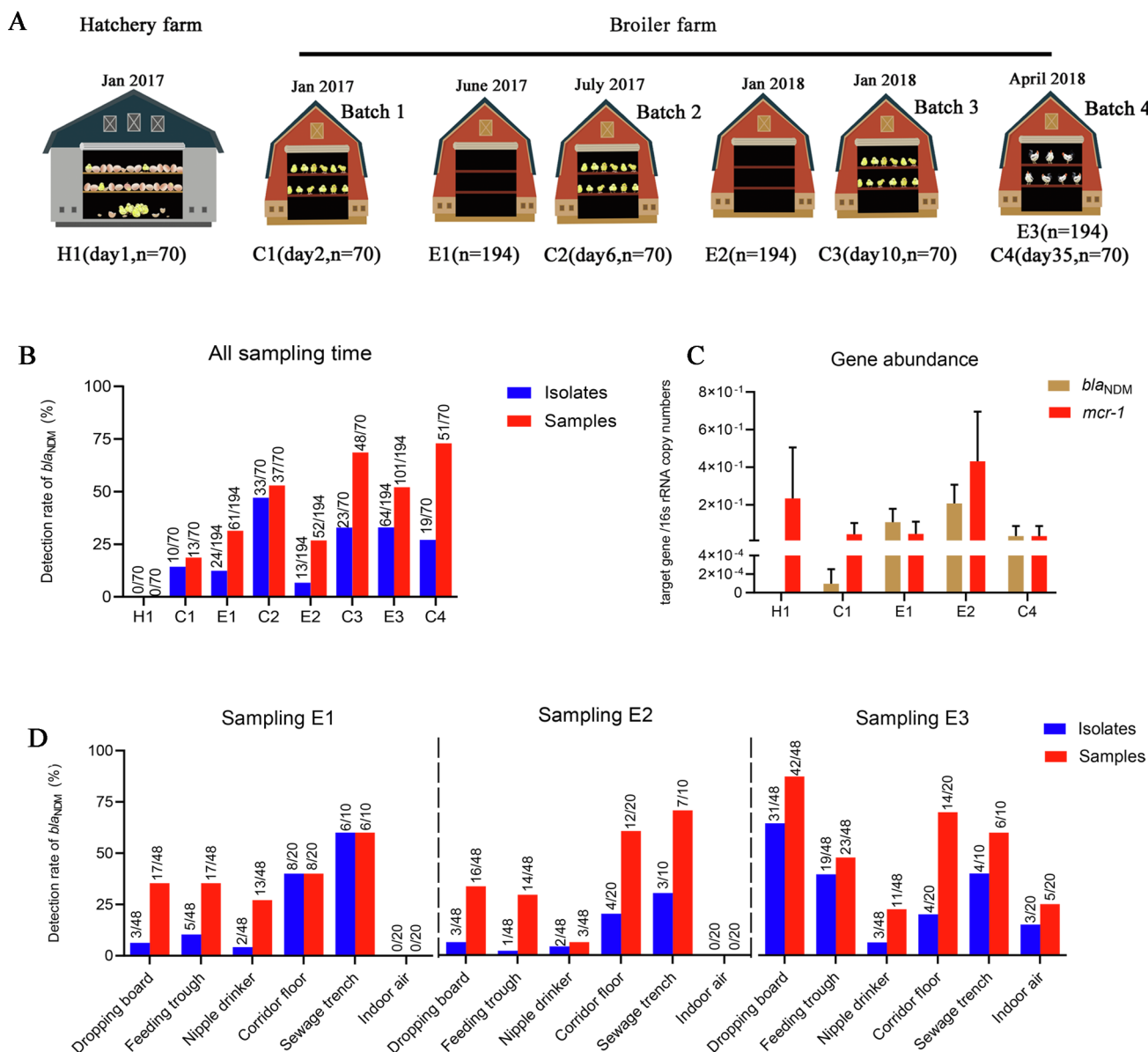

Fig. 1. Spatiotemporal-transmission of bla_{NDM} during a 16-month period of broiler production, and abundance of bla_{NDM} and *mcr-1* at sampling point. (A) Time line of sampling, including cloacal samples from a hatchery farm (H1) and a commercial broiler farm (C1, C2, C3, C4) with environmental samples collected from the chicken houses at E1 (vacancy period day 108), E2 (vacancy period day 158), and E3 (35-day-old chickens). (B) PCR-based detection rates of bla_{NDM} directly from samples, and isolation rates of bla_{NDM}-carrying bacteria at different sampling points. Numbers above bars indicate the total number of positive samples. (C) Abundance of bla_{NDM} and *mcr-1* at the H1, C1, E1, E2, and C4 sampling points. (D) Distribution of bla_{NDM} and isolation rates of bla_{NDM}-carrying bacteria among various environment samples at sampling points E1, E2, and E3, respectively.

report, 2016). An "all-in-all-out" strategy is often applied in broiler production, whereby 1-day-old chicks are purchased and transferred from hatchery farms to commercial broiler farms where they are placed in battery cages located in poultry houses. The chickens are raised for an average of 42 days before slaughter, after which the poultry houses undergo rigorous cleaning and disinfection during the vacancy period to reduce infection pressure from microorganisms from one flock to the next. However, intensive farming in cages accelerates the transmission of MDR bacteria. Despite the additional disinfection steps and the use of the "all-in-all-out" method, the in-house environment (manure/litter, soil, sediment, air, sewage, residual manure, etc.) of broiler farms may still act as a natural reservoir of MDR bacteria (Kostadinova et al., 2014).

We previously observed that hosts such as flies, wild birds, and dogs play an important role in the transmission of NDM-producing *Enterobacteriaceae* to chicken flocks in broiler houses (Wang et al., 2017). However, few studies focused on the inanimate surfaces of the in-house environment that is linked with the broilers (Dumas et al., 2011; Kostadinova et al., 2014). Whether the in-house environment plays an important role in the persistence and transmission of NDM-producing bacteria remains unknown. Herein, we selected and monitored a typical broiler farm using the cage-farming style to investigate the occurrence of NDM-producing bacteria in different chicken flocks, and to assess the association between these bacteria and the in-house environment. We also examined the molecular epidemiology of NDM-producing isolates in a stand-alone house over a 16-month period.

2. Methods

2.1. Study area and sample collection

To better understand the prevalence of CRE on broiler farms, routine surveillance was carried out at a commercial broiler farm with A-frame cages in Hebei Province, China. The farm contained four separate chicken houses with approximately 16,000 individuals (Ross 308) per flock/house. Broiler farming operations were based on agreements with poultry producers. During the vacancy period between different flocks, each house was depopulated, cleaned, and disinfected using routine cleaning and disinfection procedures (Table S1). For this study, a total of 932 non-duplicate samples were collected and stored in Eswabs (Copan, Brescia, Italy) from a Hatchery farm (chicken cloaca swabs, $n = 70$, abbreviation of H1 in Fig. 1A) and the commercial broiler farm (cloacal swabs from four batches of Chicken, $n = 280$, abbreviation of C1-C4 in Fig. 1A; indoor environment samples, $n = 582$, abbreviation of E1-E3 in Fig. 1A) from January 2017 to April 2018. At the broiler farm, all chickens were reared in the houses from the day of arrival (day 1) until the day of slaughter (day 40–45). Cloacal swabs were randomly collected from 70 chickens from each of the four batches (C1-C4), while the indoor environment samples were collected at three time points: the 108-day vacancy period (E1), the 158-day vacancy period (E2), and the raising period (35 days old) (E3). A total of 194 samples were collected at each of the environmental sampling points (Fig. 1A, Fig. S1, Fig. S2) from various locations: surface samples (dust or residue) from drooping boards ($n = 48$), feeding troughs ($n = 48$), nipple drinkers ($n = 48$), corridor floors ($n = 20$), and sewage trenches ($n = 10$). Additionally, air samples (90–150 cm off the ground, $n = 20$) were collected using a Coriolis μ Biological Air Sampler (Bertin, Montigny-le-Bretonneux, France) containing 15 mL of brain-heart infusion (BHI) broth (Land Bridge, Beijing, China). Air flow (300 L/min) through the air sampler was controlled within 5 min. The sampling of animals and the environment was conducted in accordance with the principles of the Beijing Municipality Review of Welfare and Ethics of Laboratory Animals.

2.2. Direct sample testing and bacterial identification

Samples were enriched in 1.5 mL of BHI broth (Land Bridge, Beijing, China) containing 0.25 mg/L meropenem and 30 mg/L vancomycin at 37 °C overnight. Total DNA was extracted from 1 mL of each enriched culture using the boiling method and used for polymerase chain reaction (PCR)-based screening of bla_{NDM} as previously described (Nordmann et al., 2011). Enriched samples that were positive for bla_{NDM} were streaked directly onto MacConkey agar (Land Bridge, Beijing, China) supplemented with 0.25 mg/L meropenem and 30 mg/L vancomycin and incubated at 37 °C for 18–22 h. Putative colonies of each morphotype were selected for bla_{NDM} gene detection, with the positive isolates then identified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (Bruker, Billerica, MA, USA) and 16S rRNA gene sequence-based analyses.

The total DNA of environmental samples (collected from the drooping boards at E1 and E2) and cloacal samples (obtained at H1, C1, and C4) were extracted using a DNease PowerSoil Kit (QIAGEN, Hilden, Germany) as the manufacturer's protocol. The concentrations and quality of the extracted DNA samples were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Primers NDM-F (5′-CGCCATCCCTGACGATC AAA-3′) and NDM-R (5′-CTGAGCACCGCATTAGCCG-3′) were used to quantify the abundance of bla_{NDM} in the samples by quantitative PCR (qPCR) as previously described (Luo et al., 2014). Considering that the colistin is a last-line antibiotic for treating Gram-negative bacterial infections in clinics and the possibility of transmission of *mcr-1* between animals and humans via the poultry production chain, the colistin resistance gene *mcr-1* was also been tested and quantified by primers *mcr-1*-F (5′-CGGTCAGTCCGTTTGTTC-3′) and *mcr-1*-R (5′-CTTGGTCGGTCT GTAGGG-3′) (Liu et al., 2016). qPCR assays were conducted using a QuantStudio 7 Flex Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) with SYBR Premix Ex Taq II (Thermo Fisher Scientific, USA).

2.3. Genetic characterization of NDM-producing isolates

Total DNA was extracted from NDM-producing isolates using a Magen Genomic DNA Purification Kit (Magen, Guangzhou, China) as per the manufacturer's instructions. Indexed DNA libraries were constructed using a KAPA HyperPrep Kit Illumina Platforms (Roche, Basel, Switzerland) with standard protocols, and sequenced on an Illumina HiSeq X Ten platform (Annoroad, Beijing, China). All 279 draft genomes were assembled using SPAdes version 3.9.0 (Bankevich et al., 2012). In silico phylotyping of *E. coli* was carried out using the ClermonTyping method (Beghain et al., 2018), while multilocus sequence typing (MLST) results were analyzed using MLST Version2 (https://github.com/tseemann/mlst). Resistance genes, virulence-associated genes, and the plasmid types of bla_{NDM}-carrying contigs were identified using abricate (https://github.com/tseemann/abricate). Some of contigs were very short. Thus, to confirm the plasmid types of a larger number of the bla_{NDM}-carrying contigs and to obtain a more precise genetic context, the MinION platform (Oxford Nanopore Technologies, Oxford, UK) was used. All bla_{NDM}-carrying contigs from the whole-genome sequencing (WGS) and MinION sequencing analyses were annotated using the online automated PATRIC server (https://www.patricbrc.org/app/Annotation). Contigs with similar genetic contexts were grouped together based on basic local alignment search tool (BLAST) analysis against the National Center for Biotechnology Information (NCBI) databases. Comparison of genetic context in the different bla_{NDM}-carrying plasmids was undertaken using BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011). Separate phylogenetic trees for *E. coli* and *K. pneumoniae* based on the core genome sequences of the isolates were structured using Harvest version 1.1.2 (Treangen et al., 2014), with the corresponding characteristics of each isolate visualized using online tool iTOL version 4 (Letunic and Bork., 2007). The
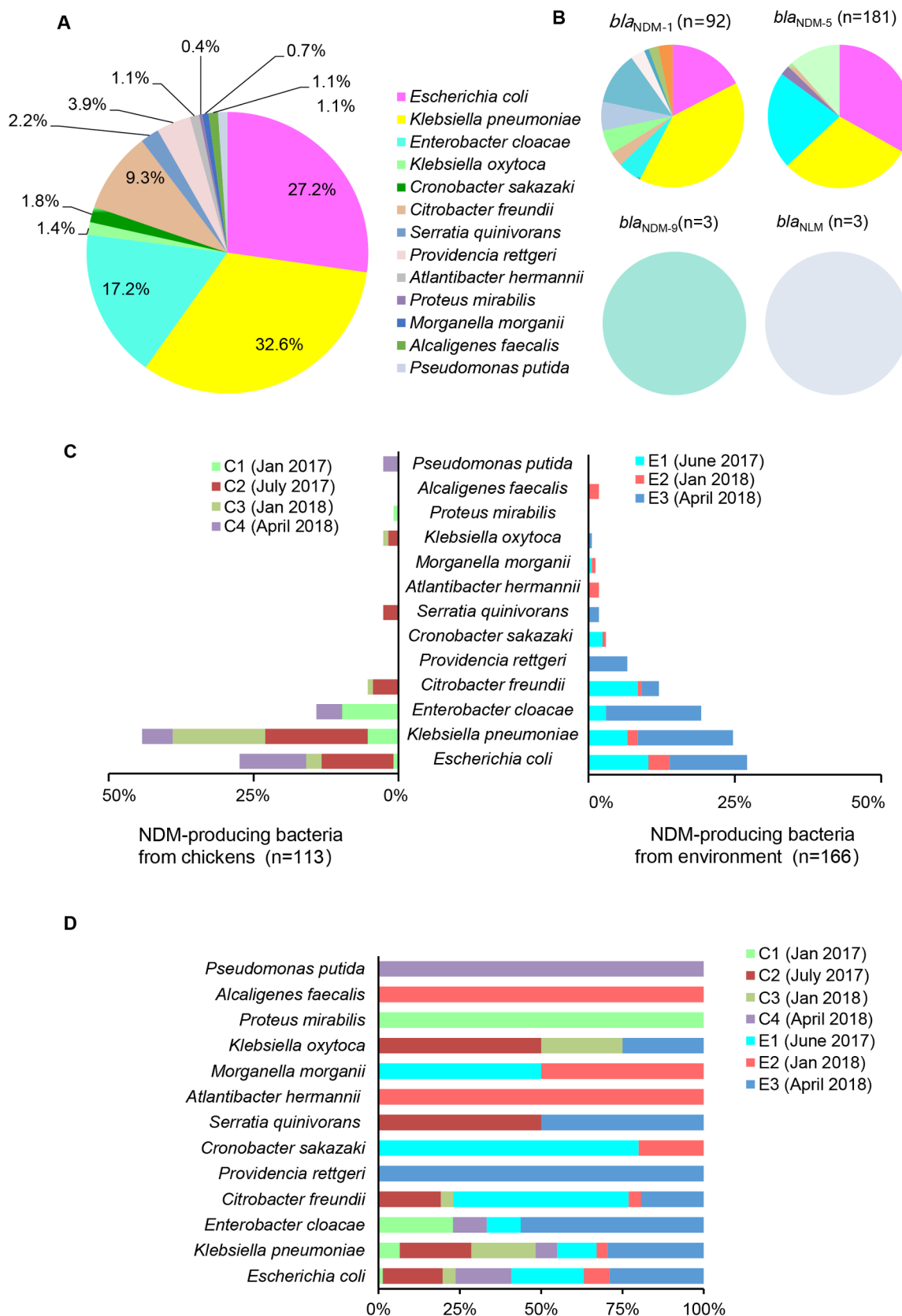
Fig. 2. Diversity and abundance of NDM-producing bacteria isolated from a broiler farm over the 16-month study period. (A) Distribution of the 279 isolates. Each bacterial genus is indicated by a different color, and the sizes of the pie fragments indicate the relative abundance. (B) Distribution of NDM variants among the different bacterial genera. (C) Percentages of NDM-producing isolates from chicken and environmental samples at each sampling point. (D) Distribution of NDM-producing isolates at each sampling point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

population structure of each phylogenetic tree was defined using hier-BAPS version 6.0 (Cheng et al., 2013). All WGS data have been deposited in the NCBI BioProject database: PRJNA601101.

2.4. S1 nuclease-pulsed-field gel electrophoresis (S1-PFGE), Southern blotting, and stability of bla_{NDM}-carrying IncX3 plasmids

The genomic location of bla_{NDM} in the NDM-producing strains were identified by S1-PFGE and Southern blotting using a digoxigenin-labeled bla_{NDM} probe and a Detection Starter Kit II (Roche Diagnostics, Basel, Switzerland). The stability of bla_{NDM}-carrying plasmid IncX3 was confirmed by sub-culturing successive generations as previously reported (De Gelder et al., 2007). Isolates of seven species of *Enterobacteriaceae* (*E. coli, K. pneumoniae, Enterobacter cloacae, Klebsiella oxytoca, Cronobacter sakazakii, Citrobacter. freundii*, and *Serratia quinivorans*) containing bla_{NDM}-carrying plasmids were selected. Briefly, stability experiments were performed with starting to streak seven species of Enterobacter containing bla_{NDM}-carrying plasmids on LB plates with the appropriate antibiotics concentrations and incubated at 37 °C, then three separate colonies of each strains were individually inoculated into 10 mL of LB broth supplemented with 0.5 µg/mL meropenem. Following incubation for 24 h at 37 °C with shaking at 200 rpm, the antibiotics were removed by centrifuging 1 mL of culture and resuspending the pellet in 1 mL of saline. Aliquots (9.77 µL) of the suspensions were transferred into 10 mL volumes of LB broth without antibiotics so that approximately 10 generations were obtained per 24 -h growth cycle (1:2^{10} dilution rate). Subsequently, 9.77 µL of each culture were transferred every 24 h to 10 mL volumes of LB broth and incubated at 37 °C with shaking at 200 rpm. At each time point, culture aliquots were diluted and plated onto LB agar plates. The proportion of plasmid-free cells in the population was determined by replica-plating 50 randomly selected colonies per culture from the LB plates onto LB medium containing meropenem (0.5 µg/mL) and standard LB medium and scoring suspect clones that had lost the bla_{NDM}-carrying plasmid.

2.5. Conjugation assay

The transferability of the bla_{NDM}-carrying plasmid among different *Escherichia coli* strains with the 11 most prevalent ST types was examined using *E. coli* strain J53 as the recipient (Liu et al., 2017). Briefly, the donor and recipient strains were mixed at a ratio of 1:3 on a microporous membrane and incubated for 16 h. Transconjugants were selected on LB agar containing azide (100 µg/mL) and meropenem (1 µg/mL), and confirmed by PCR and sequencing (Nordmann et al., 2011).

2.6. Functional cloning of NDM-like metallo-β-lactamase

The gene encoding of NDM-like-metallo-β-lactamase (accession number MN699650) was PCR-amplified using primers NDM-like-F (5′-GGCTAAACAGGAGGAATTAACATGATTACGAAATCGAACATCG CGC-3′) and NDM-like-R (5′-AAACAGCCAAGCTTCGAATTAATCAGCG CAGCTTGTCGGC-3′). The amplicon was then integrated into cloning vector pBAD by homologous recombination, resulting in recombinant plasmid pBAD /NDM-like. The plasmid was then transformed into *E. coli* DH5α via electroporation. The resulting transformant was confirmed by PCR and sequencing analysis, and its sensitivity to meropenem was determined as previously reported (Liu et al., 2017).

3. Results

3.1. High prevalence of bla_{NDM} in chicken houses in both the raising and vacancy periods

Samples collected from 1-day-old broilers at the hatchery prior to their transfer to the commercial broiler farm (H1, January 2017, Fig. 1)

showed that all chickens were negative for bla_{NDM} based on both PCR-based direct sample testing and qPCR analysis. However, after only 24 h at the broiler farm, 13 (18.6%) of the 70 randomly selected 2-day-old chickens (C1, January 2017) were positive for bla_{NDM}, with NDM-producing isolates also recovered from ten chickens. The relative abundance of bla_{NDM} in cloacal samples was 9.60×10^{-5} copies/16S rRNA gene copy. Notably, no flies or birds were observed or captured during the winter period (H1 and C1 sampling points), suggesting that the bla_{NDM} gene contamination of the chicken flock within 24 h may not be associated with other potential hosts previously identified in the farm environment. At the subsequent sampling points, 52.9% (37/70) of samples collected from 6-day-old broilers (C2, July 2017), 68.6% (48/70) of samples from 10-day-old broilers (C3, January 2018), and 72.9% (51/70) of samples from 35-day-old broilers (C4, April 2018) were positive for bla_{NDM}, with detection rates for NDM-producing strains at the corresponding time points of 47.1% (33/70), 32.9% (23/70), and 27.1% (19/70), respectively (Fig. 1B), although few randomly selected 1-day-old chicks ($n = 4$–6) in the day of arrival in batches of C2, C3 and C4 were negative for bla_{NDM} (Data not shown). In addition, the relative abundance of bla_{NDM} in cloacal samples from 35-day-old broilers (C4, April 2018) was calculated as 3.31×10^{-2} copies/16S rRNA gene copy (Fig. 1C). These results suggested a high prevalence of bla_{NDM}-carrying bacteria in different chicken flocks in the raising period during the 16-month study period. We also sampled the in-house environment during one raising period and two vacancy periods. Although cleaning and disinfection protocols were routinely followed at the commercial farm, bla_{NDM} detection rates of 31.4% (61/194) on day 108 of the vacancy period (E1, June 2017), 26.8% (52/194) on day 158 of the vacancy period (E2, Jan 2018), and 52.1% (101/194) during the raising period (E3) were observed in environmental samples. Isolation rates for NDM-producing bacterial strains at the corresponding time points were 12.4% (24/194), 6.7% (13/194), and 33.0% (64/194), respectively (Fig. 1B). Even in the absence of broiler chickens, bla_{NDM}-carrying bacteria were widely distributed throughout the in-house facilities, including drooping boards, feeding troughs, nipple drinkers, corridor floors, and sewage trenches (Fig. 1D). In addition, the bla_{NDM}-carrying bacterial isolation rate of 25% (5/20) was determined for air samples collected during the raising period (35-day-old broilers, E3). Further, bla_{NDM} was highly abundant in environmental samples collected from drooping boards during vacancy periods, with mean relative abundance values of 1.07×10^{-1} copies/16S rRNA gene copy at sampling point E1 (vacancy period day 108) and 2.07×10^{-1} copies/16S rRNA gene copy at the E2 sampling point (vacancy period day 158).

3.2. Diversity of NDM-producing bacteria in the chicken house

Over the 16-month surveillance period, we identified 279 NDM-producing bacterial isolates: 113 from 24.3% ($n = 85$) chicken cloacal samples and 166 from 17.4% ($n = 101$) environmental samples. These included 8 species of *Enterobacteriaceae* (92.3%, $n = 259$) as well as five species of non-*Enterobacteriaceae* including *Morganella morganii* (0.7%, $n = 2$), *Proteus mirabilis* (0.4%, $n = 1$), *Providencia rettgeri* (3.9%, $n = 11$), *Alcaligenes faecalis* (1.1%, $n = 3$), and *Pseudomonas putida* (1.1%, $n = 3$) isolates (Fig. 2). *K. pneumoniae* (32.6%, $n = 91$), *E. coli* (27.2%, $n = 76$), *Enterobacter cloacae* (17.2%, $n = 48$), and *Citrobacter freundii* (9.3%, $n = 26$) were the four most prevalent *Enterobacteriaceae* species from both chickens and the environment across the multiple sampling points. Three bla_{NDM} variant genes were identified: bla_{NDM-5} was identified in 181 (64.9%) isolates belonging to six species of *Enterobacteriaceae*, bla_{NDM-1} in 92 (33.0%) isolates from *M. morganii, P. mirabilis, P. rettgeri, A. faecalis* and 7 species of *Enterobacteriaceae*, and bla_{NDM-9} in 3 (1.1%) *E. cloacae* isolates. bla_{NDM-5} and bla_{NDM-1} were present in isolates from both chickens and the immediate environment across the entire sampling period. A novel NDM-like-metallo-β-lactamase gene was identified in 3 (1.1%) *P. putida* isolates. This novel chromosome-borne gene contained an 804-bp open reading frame

encoding a putative subclass B1 metallo-β-lactamase. The predicted protein sequence showed 85.0% identity to NDM-14, and expression of the novel gene in *E. coli* DH5α resulted in an 8-fold increase in the minimum inhibitory concentration of meropenem (from 0.03 to 0.25 μg/mL), putatively confirming the function of the corresponding NDM-like-metallo-β-lactamase. Hence, the gene was designated as bla_{NLM} by NCBI.

3.3. Clonal and horizontal transmission of NDM-producing E. coli and K. pneumoniae in a stand-alone chicken house

We carried out a detailed genomic epidemiological analysis of the NDM-producing *E. coli* ($n = 76$) and *K. pneumoniae* ($n = 91$) isolates because these two species accounted for 59.9% of all NDM-producing isolates recovered across all sampling periods. MLST analysis identified 14 sequence types (STs) among the *E. coli* isolates, with ST6751 being the most prevalent (38/76), followed by ST6716 (8/76), ST156 (6/76), ST69 (5/76), ST48 (4/76), and ST10 (4/76). Isolates with STs 6751, 10, 125, and 746 were recovered from both chicken and environmental samples. Noticeably, ST6751 isolates were widely distributed among all sample types (chickens, sewage trenches, corridor floors, drooping boards, feeding troughs, nipple drinkers, and air) (Fig. 3), as well as across all sampling points (Fig. S3). The *E. coli* isolates were classified into four groups using Clermont Typing, with the majority of isolates belonging to Groups A ($n = 54$, 71.1%) and B1 ($n = 16$, 21.1%). Both

of these groups are usually associated with commensal strains (Fig. 6). Bayesian analysis of the population structure revealed five distinct lineages among the 76 *E. coli* isolates. The major lineage, lineage I, included 38 (50%) isolates belonging to ST6751 and showing a high degree of whole-genome sequence similarity (pairwise single nucleotide polymorphisms (SNPs) ≤ 3) (Table S3), suggesting that clonal dissemination is the mainstay in the stand-alone chicken house. Similarly, *K. pneumoniae* isolates were also recovered from both chicken flocks and the indoor environment (Fig. 4, Fig. S4). Unlike *E. coli*, the STs of the 91 *K. pneumoniae* isolates were relatively concentrated, with ST37 isolates being the most prevalent ($n = 55$), followed by ST3410 ($n = 28$) and ST726 ($n = 5$). The *K. pneumoniae* isolates belonged to four distinct lineages. Interestingly, 49 ST37 isolates with high genetic similarity (SNPs ≤ 22, Table S4) clustered into lineage IV. The lineage IV isolates were recovered from diverse sources, including chickens, sewage trenches, corridor floors, drooping boards, feeding troughs, and nipple drinkers.

Antibiotic resistance genes carried by the *E. coli* (Fig. 3) and *K. pneumoniae* (Fig. 4) isolates were identified from WGS data. In addition to bla_{NDM-1} (16/76) and bla_{NDM-5} (60/76), several other important resistance genes were identified in the *E. coli* isolates, including tetracycline resistance gene *tet*(A) (24/76), aminoglycoside resistance gene *aac*(*3*)-*Via* (69/76), and macrolide resistance gene *mph*(A) (67/76). Most noticeably, 27 isolates also carried the colistin resistance gene *mcr-1*. Resistance genes were also identified in the *K. pneumoniae*
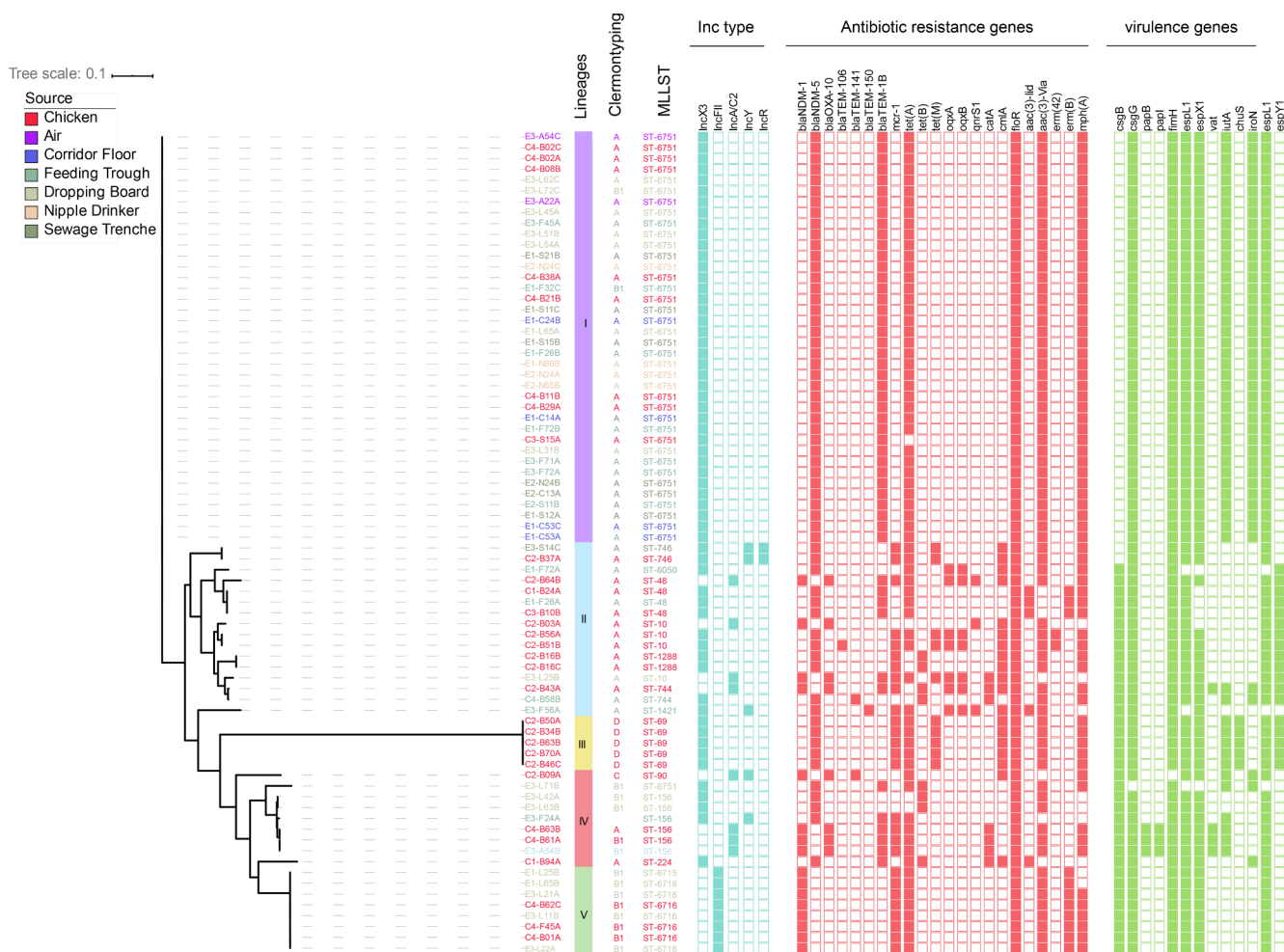

Fig. 3. Phylogenetic tree and distribution of NDM-producing *Escherichia coli* phylogroups, lineages, clermont type, multilocus sequencing types, Inc. type plasmid, antimicrobial-resistance genes, and virulence-associated genes among isolates from chickens and the environment. The sources of the isolates are differentiated by color, resistance genes, and virulence-associated genes are denoted by filled squares for presence and empty squares for absence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
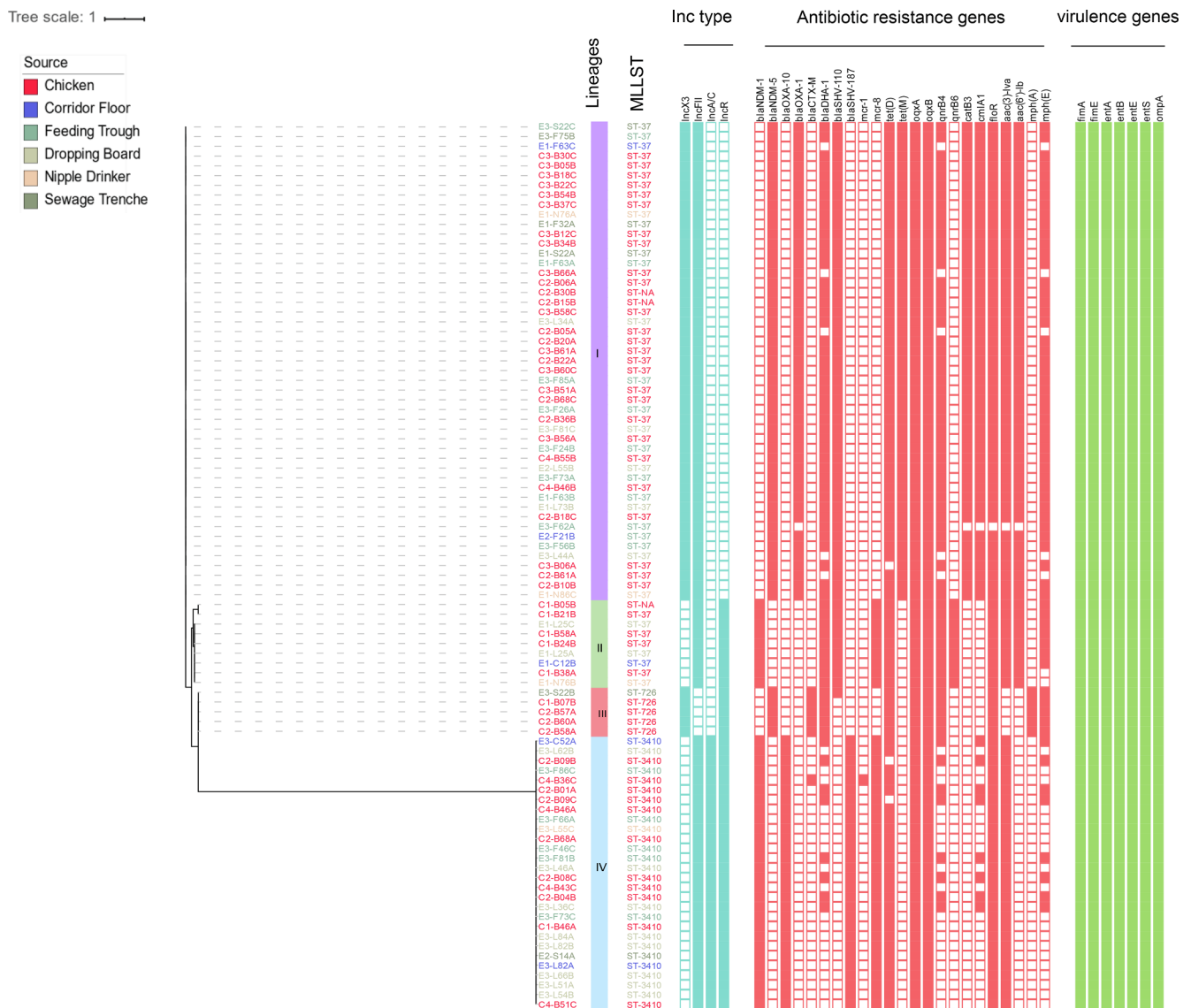
Fig. 4. Phylogenetic tree and distribution of NDM-producing *Klebsiella pneumoniae* phylogroups, lineages, multilocus sequencing types, Inc. Type plasmid, antimicrobial resistance genes and virulence-associated genes among K. *pneumoniae* isolates from chickens and the environment.

(n = 91) isolates that differed slightly from those present in the *E. coli* isolates. Tetracycline resistance genes *tet*(D) and *tet*(M) were detected in 88 and 49 isolates, respectively, while *mcr* variant genes, including *mcr-8* and *mcr-1*, were identified in 37 *K. pneumoniae* isolates. For the virulence-associated genes between the chicken and environmental isolates. The *E. coli* isolates contained genes encoding adherence-associated proteins (*csgG*, *fimH*), autotransporters (*espL1*, *espX1*), iron uptake proteins (*iutA*, *iroN*), and non-LEE encoded type III secretion system effectors (*espL1*) (Fig. 3). The virulence-associated genes identified among the *K. pneumoniae* isolates were consistent across the sampling locations, and included genes encoding adherence proteins (*fimA*, *fimE*), enterobactin (*entA*, *entB*, *entE*, *entS*), and outer membrane proteins (*ompA*) (Fig. 4).

3.4. Locations, plasmid profiles, and genetic context of bla_NDM variants

S1-PFGE, Southern blotting (Fig. S5), and WGS analyses revealed that in all except for 33 isolates (nine *K. pneumoniae*, ten *Providencia rettgeri*, three *E. cloacae*, three *A. faecalis*, three *P. putida*, two *C. freundii*, two *Morganella morganii*, and one *Proteus mirabilis*), bla_{NDM} was plasmid-borne. Three major types of bla_{NDM}-carrying plasmids were

identified among the remaining 246 isolates. Of these, 197 isolates contained IncX3-type plasmids carrying either bla_{NDM-5} (n = 181, six species) or bla_{NDM-1} (n = 16, four species), 41 isolates carried IncA/C$_2$-type plasmids with bla_{NDM-1} (five species), and eight *E. coli* isolates contained bla_{NDM-1} positive IncFII-type plasmids (Fig. 5, Table S2). There was no association between plasmid type and isolate source (chickens or the environment). IncX3-type plasmids were detected in 78.9% (n = 60) of CREC isolates and 59.3% (n = 54) of CRKP isolates recovered throughout the surveillance period. Compared with the common IncX3-type plasmids (46,161 bp) found in most *Enterobacteriaceae* species (Fig. 5A), a much smaller (21,120 bp) IncX3-type bla_{NDM}-carrying plasmid was identified in several *E. cloacae* (n = 34) and *E. coli* (n = 5) isolates, and appeared to have lost all genes associated with the type IV secretion system (Fig. S6).

The 279 bla_{NDM}-carrying contigs obtained via WGS showed eight genomic backbone profiles: type I (n = 11, two species), type II (n = 3, *E. cloacae*), type III (n = 197, seven species), type IV (n = 8, *E. coli*), type V (n = 13, three species), type VI (n = 40, four species), type VII (n = 3, *P. putida*), and type VIII (n = 3, *A. faecalis*) (Fig. 6, Table S2). The chromosome-borne bla_{NDM} genes were detected in type I, II, V, VII, and VIII backgrounds, whilst the genetic context of the type III, IV, and
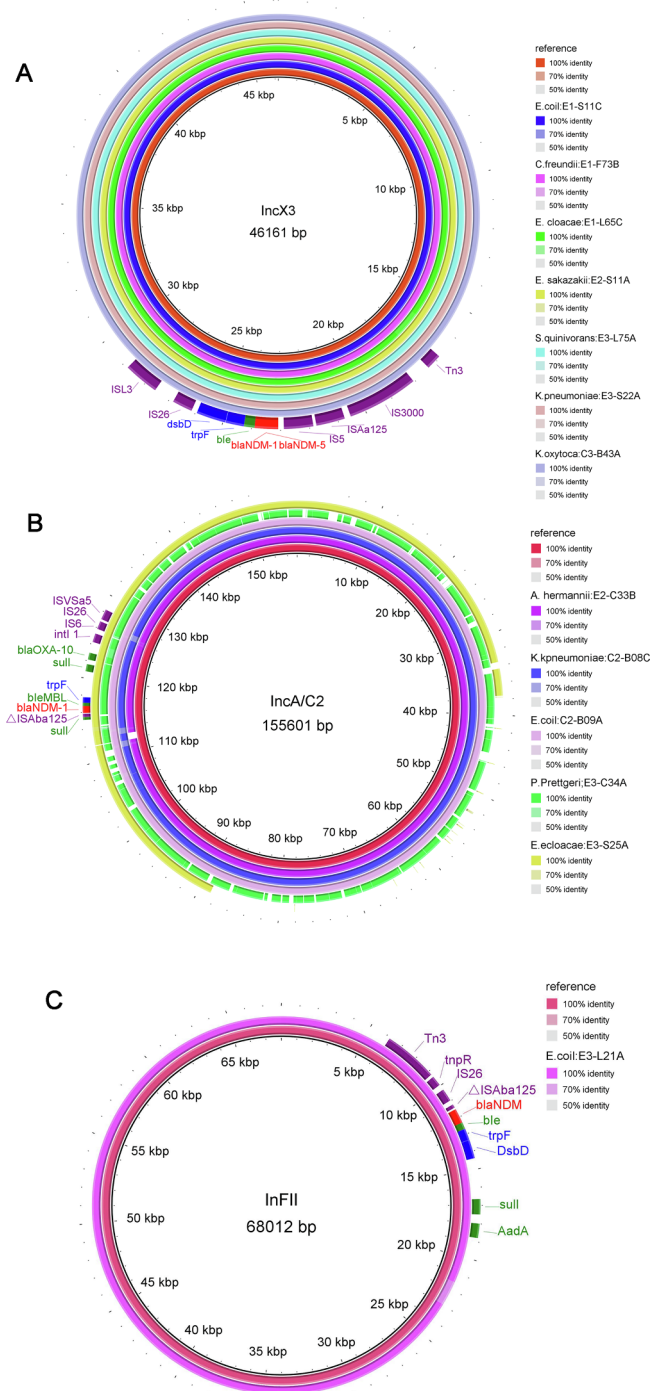
located in *P. putida* and *A. faecalis* isolates. Two mobile IS*91* elements and *Tn3* were shown to flank *bla*$_{NLM}$ in the type VII backbone, with a 4,669-bp circular intermediate carrying *bla*$_{NLM}$ also obtained from these isolates.

Stability testing showed that no *bla*$_{NDM}$-carrying IncX3 plasmid (46,161 bp) loss occurred in the isolates of seven *Enterobacteriaceae* species (*E. coli*, *K. pneumoniae*, *E. cloacae*, *K. oxytoca*, *C. sakazakii*, *C. freundii*, and *S. quinivorans*) after 15 days of successive sub-culturing. (Table S5). Conjugation assays confirmed that all *bla*$_{NDM}$-carrying IncX3 plasmids (46,161 bp) from selected NDM-producing *E. coli* isolates with various STs (Table S6) could be transferred into recipient *E. coli* strain J53.

3.5. Co-existence of mcr and bla$_{NDM}$ in isolates from a stand-alone house

In contrast to the absence of *bla*$_{NDM}$ in 1-day-old chickens, we found that the relative abundance of mobile colistin resistance gene *mcr-1* was high in these animals (mean $= 2.34 \times 10^{-1}$ copies/16S rRNA gene copy). This high prevalence of *mcr-1* carriage persisted throughout the 2-day-old chickens, 35-day-old chickens, and in-house environmental samples collected during the vacancy period (4.38×10^{-2}- 4.31×10^{-1} copies/16S rRNA gene copy) (Fig. 1C). Overall, 24.4% of NDM-producing isolates recovered in this study carried either *mcr-1* (*n* $= 29$, 10.4%) or *mcr-8* (*n* $= 37$, 14.0%), while *bla*$_{NDM-1}$, *mcr-1*, and *mcr-8* were shown to co-exist in one chicken-derived *K. pneumoniae* isolate. Isolates carrying both *bla*$_{NDM}$ and *mcr* genes were identified across almost all sampling points, except in 10-day-old broilers (C3, January 2018). Notably, the 65 isolates harboring both *mcr* and *bla*$_{NDM}$ from the broiler farm were identified as *K. pneumoniae* (*n* $= 37$), *E. coli* (*n* $= 27$), or *C. freundii* (*n* $= 1$) (Table S7). Except for seven isolates (six *E. coli* and one *C. freundii*) with very short *mcr*-carrying contigs, *E. coli* isolates mainly carried *mcr-1* on plasmid types IncHI2 (*n* $= 9$), IncI2 (*n* $= 11$), and IncY (*n* $= 1$), while *K. pneumoniae* isolates generally carried *mcr-8* on plasmid types IncHI1 (*n* $= 8$) and IncFII (*n* $= 29$) (Table S7). Regarding the co-existence of plasmid-borne *bla*$_{NDM}$ and *mcr-1*, the *bla*$_{NDM}$-IncX3 plasmids commonly co-existed with *mcr-1*-IncHI2 or IncY plasmids, *bla*$_{NDM}$-IncFII plasmids co-existed with *mcr-1*-IncI2 plasmids, and *bla*$_{NDM}$-IncA/C$_2$ plasmids were found with *mcr-1*-IncI2 or *mcr-8*-IncFII plasmids (Table S7).

4. Discussion

Similar to a previous report in human clinics (Wu et al., 2019), *Enterobacteriaceae* species were the major NDM-producing bacteria at the chicken farm in this study. In addition, we also identified, for the first time, the *bla*$_{NDM-1}$-carrying *A. faecalis* isolates in broiler house and characterized *bla*$_{NLM}$, a novel NDM-like gene in *P. putida*. Different from the overwhelmingly dominance of *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$ in the clinical sector (Shen et al., 2018), *bla*$_{NDM-5}$ was the most prevalent NDM variant (*n* $= 181$, 64.9%) among the tested chicken farm. However, *bla*$_{NDM-5}$ carriage was concentrated among six species of *Enterobacteriaceae*, the less prevalent *bla*$_{NDM-1}$ (*n* $= 92$, 33.0%) gene was carried by a broader range of bacterial hosts, including 7 *Enterobacteriaceae* species, *M. morganii*, *P. mirabilis*, *P. rettgeri* and *A. faecalis*. The high prevalence of *bla*$_{NDM-5}$ in the chicken production chain has been reported previously (Wang et al., 2017; Xiang et al., 2018), suggesting that despite the differences in prevalence of the NDM variants within the animal sector, *bla*$_{NDM-5}$ is becoming the most prevalent variant in both chickens and the farm environment. Notably, all *bla*$_{NDM-5}$ (*n* $= 181$) and 17.4% (n $= 16$) of the *bla*$_{NDM-1}$ genes identified in this study were carried by IncX3 plasmids. IncX3 plasmids have achieved clinical significance because of their contribution to the dissemination of various *bla*$_{NDM}$ variant genes, such as *bla*$_{NDM-1/4/5/7/13/17}$ (Liakopoulos et al., 2018), with IncX3-*bla*$_{NDM-5}$ plasmids increasingly being observed in both human commensal and pathogenic bacterial strains (Li et al., 2019; Li et al., 2018; Shen et al., 2018; Wang et al., 2018). Whether the

Fig. 5. BLAST ring comparison of *bla*$_{NDM}$-carrying plasmid sequences with homologous contigs from NDM-producing isolates recovered in this study. All reference plasmids were confirmed by MinION sequencing. (A) IncX3 plasmid. (B) IncA/C$_2$ plasmid. (C) IncFII plasmid.

VI backbones matched plasmid types IncX3 (*n* $= 197$), IncFII (*n* $= 8$), and IncA/C$_2$ (*n* $= 41$). In addition, the genetic context of *bla*$_{NDM-1}$ gene were complicated in type I, III, IV, V, VI, and VIII, while *bla*$_{NDM-9}$ in type II, *bla*$_{NDM-5}$ in type III, *bla*$_{NLM}$ in type VII. The context of *bla*$_{NDM-1}$ in *P. rettgeri*, *M. morganii*, *P. mirabilis*, *C. freundii*, and *K. pneumoniae* isolates was most similar to backbone types I and V, both of which had $\triangle$IS*Aba125* and IS*91* elements flanking *bla*$_{NDM-1}$, except that the type V backbone was missing *dsbD*. Likewise, IS*91* elements flanked *bla*$_{NDM-9}$ in the type II backbone. Types VII and VIII genetic backbones were
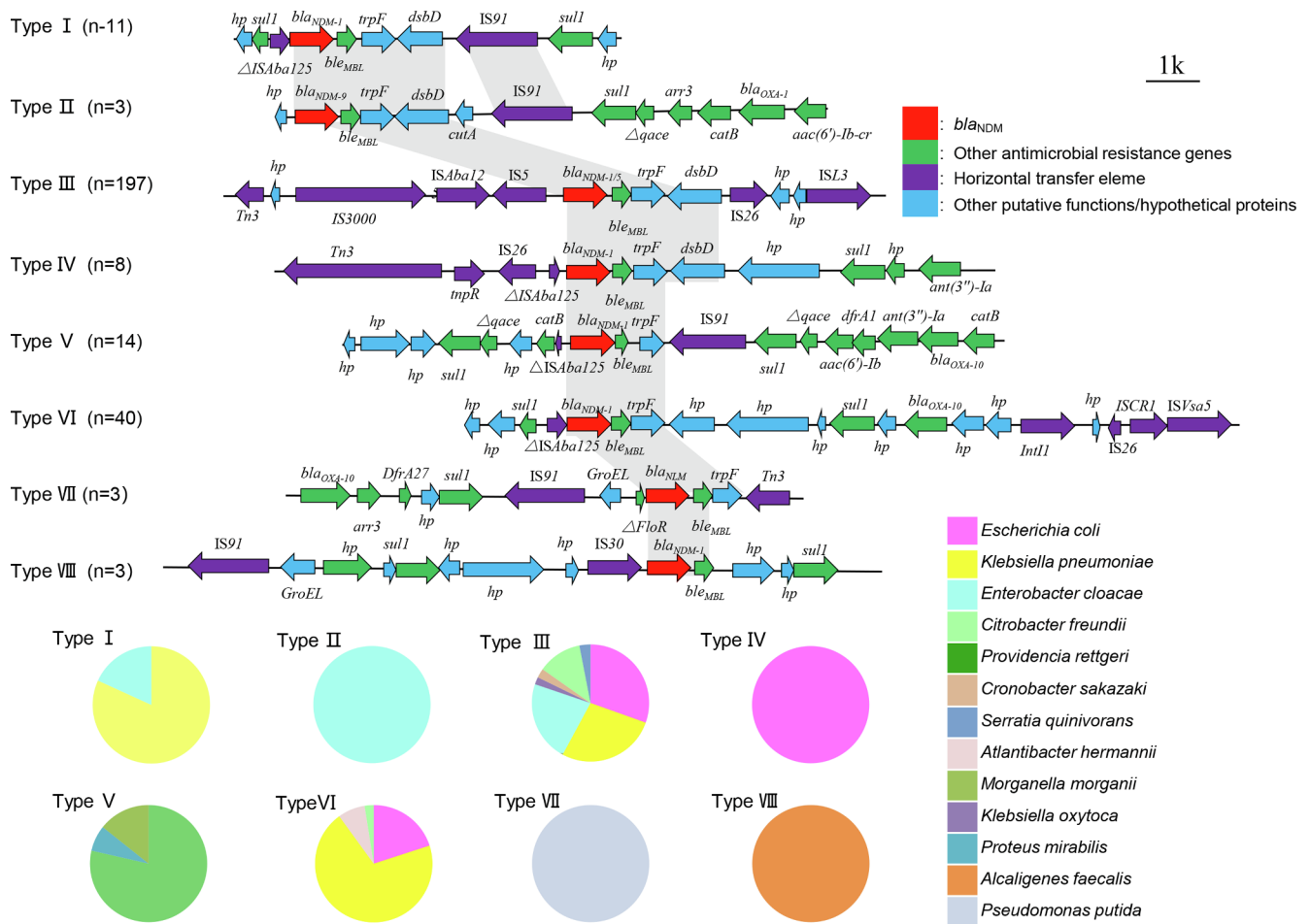
Fig. 6. Genetic environment of bla_{NDM} in all isolates. Schematic representation and comparison of the genetic environment of the bla_{NDM}-flanking region in each genomic backbone type. Arrows indicate the direction of transcription of each of the genes, and different genes are shown in different colors. Regions of ≥99.0% nucleotide sequence identity are shaded in grey. The backbone types associated with the different bacterial genera are shown by pie charts, with different bacterial genera indicated by different colors.

prevalence of IncX3-bla_{NM-5}-carrying bacteria in farm animals is associated with their increasing colonization of humans in China will require further investigation.

The most striking finding of this study is that although the farm followed the Guidelines for Disinfection and Sterilization in Chicken Farms (Provincial Standard DB13/T 990-2008 of Hebei Province) in combination with a long vacancy period (> 100 days), extensive XDR bacterial contamination was observed in both chickens and environmental samples. The disinfection guidelines were designed to diminish and/or eliminate pathogens (bacteria and viruses) in chicken rearing facilities; however, we observed persistence of bla_{NDM}-carrying bacteria in the in-house environment (mainly in the drooping boards and on corridor floors), which allowed contamination of bla_{NDM}-free 1-day-old chickens soon after their arrival at the broiler farm. Baby chickens have an immature gut and are readily colonized by environmental bacteria (Bordamolina et al., 2018; Wang et al., 2016). The observed persistence of bla_{NDM}-carrying strains in this study is in accordance with previous reports on the stability of antimicrobial-resistant bacteria on animal farms, such as the survival and circulation of ESBL-producing *E. coli* in chicken barns (Projahn et al., 2018) and the continued isolation of vancomycin-resistant *E. faecium* from environmental samples from a chicken farm despite the addition of a disinfection step (Garcia-Migura et al., 2007). Farm animal housing has been previously shown to support the persistence of *E. coli* and *Salmonella* for long periods (Pedersen et al., 2008; Singer et al., 2000). Together with the current findings, these data strongly indicate that the chicken farm environment is a

major reservoir for bla_{NDM}-carrying bacteria.

Although carbapenem use is prohibited in farm animals, there are four possible reasons for the persistence of bla_{NDM}-carrying bacteria at the studied chicken farm. First, drug usage records for the farm revealed that either penicillin or amoxicillin, which are β-lactam antibiotics and similar to carbapenem, was used for treatment of the four previous batches of chicken flocks, which may provide direct pressure for selection and accumulation of bla_{NDM} in bacteria. Second, genetic context analysis revealed the co-existence of bla_{NDM} with other resistance genes, including *sulI* (sulfanilamide resistance), *ant(3″)-Ia* (aminoglycoside resistance), *aac(6′)-Ib-cr* (fluoroquinolone resistance), and *catB* (chloramphenicol resistance). Use of the corresponding antibiotics in farm animals may co-select bla_{NDM}. Third, IncX3 plasmids are the major carriers of bla_{NDM}. These plasmids normally encode a type IV secretion system (*pilX1-11*), which allows the exchange of genetic material within bacteria. IncX3 plasmids are also highly conjugatable and stable, and exert no fitness costs on their bacterial hosts. Because the conjugative rates of animal-derived IncX3 plasmids are higher at 30 °C than at 37 °C, more efficient transfer of these plasmids is observed in the environment than in the animal gut (Liakopoulos et al., 2018). Finally, various mobile genetic elements, including IS*91*, IS*30*, and IS*Aba125* (Fig. 6), flanking bla_{NDM} also increase the likelihood of horizontal transfer in the broiler farm environment.

To meet the enormous consumer demand for poultry meat, the Chinese poultry farming industry employs over 70 million staff (Sohu News, 2018) either directly or indirectly. These workers come into

contact with animals and their waste as they work in and around the enclosed environments with high animal density, operating heavy machinery and performing veterinary procedures, which exposes them to microbes (Neyra et al., 2012). Because chicken farms have the capacity to become reservoirs of CRE and other bla_{NDM}-positive bacteria, the risk of exposure of farm workers to these XDR and potentially pathogenic bacteria cannot be ignored.

We acknowledge that our study has several limitations. First, our investigation used only one chicken farm to examine the persistence of NDM-producing *Enterobacteriaceae*. However, the selected farm utilized A-frame shaped cages and associated facilities that is one of the most popular and representative mode for raising broiler chickens in China (Chinese poultry report, 2016; Sohu News, 2018.). Second, no systematic evaluation of the efficacy of disinfection of the chicken houses during the vacancy period was conducted in this study. Therefore, the association between the persistence of NDM-producing strains and ineffective cleaning and disinfection processes requires further investigation. Third, there are some acknowledged discrepancies between direct sample testing for bla_{NDM} and the isolation of NDM-producing strains using selective media.

5. Conclusions

Our comprehensive analysis of the persistence of NDM-producing bacteria on a typical Chinese commercial broiler farm suggested widespread contamination of the broiler house environment with NDM-producing strains, despite the application of routine cleaning and disinfection procedures and long vacancy period. Our study revealed that, once NDM-producing strains are introduced into a broiler farm, they may persist in the environment for relatively long periods by either clonal or horizontal transmission of bla_{NDM} mainly in *Enterobacteriaceae* from chicken and environmental samples. Therefore, improved or novel cleaning and disinfection procedures should be implemented in conjunction with "all-in/all-out" management to avoid cross-contamination of NDM-producing *Enterobacteriaceae* between batches of chickens, and prevent its further disseminating to humans through either the poultry production chain or environment route.

Data availability

All WGS data obtained in this study have been deposited in the NCBI BioProject database: PRJNA601101.

CRediT authorship contribution statement

Ruidong Zhai: Conceptualization, Methodology, Formal analysis, Writing - original draft, Project administration. **Bo Fu:** Validation, Investigation, Writing - original draft. **Xiaomin Shi:** Investigation, Data curation. **Chengtao Sun:** Formal analysis. **Zhihai Liu:** Formal analysis. **Shaolin Wang:** Software, Data curation. **Zhangqi Shen:** Data curation. **Timothy R. Walsh:** Conceptualization, Data curation, Funding acquisition. **Chang Cai:** Conceptualization, Formal analysis, Data curation. **Yang Wang:** Conceptualization, Resources, Methodology, Project administration, Writing - original draft, Funding acquisition. **Congming Wu:** Conceptualization, Resources, Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105715.

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