Occurrence and Molecular Characterization of Abundant tet(X) Variants Among Diverse Bacterial Species of Chicken Origin in Jiangsu, China

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INTRODUCTION

Tigecycline is a broad-spectrum antibiotic of glycylcyclines and is one of the last-resort antibiotics to treat serious infections caused by multidrug-resistant (MDR) Gram-negative bacteria (Sun et al., 2013). The mechanisms of tigecycline resistance were mainly the overexpression of non-specific active efflux pumps or mutations within the drug-binding sites in the ribosome, which were limited
by less capability of horizontal transfer among bacteria (Pournaras et al., 2016). However, the emergence and dissemination of plasmid-mediated high-level tigecycline resistance genes tet(X3) and tet(X4) are bringing formidable threats to public health (He et al., 2019; Sun J. et al., 2019). A variety of tet(X) variants containing tet(X3.2) (Li et al., 2019), tet(X5) (Wang et al., 2019), tet(X6) (He et al., 2020; Liu et al., 2020; Peng et al., 2020), and tet(X14) (Cheng et al., 2020) have been detected in Enterobacteriales, and Acinetobacter so far. These widespread tet(X) variants will limit treatment options for MDR bacteria infections. 

The livestock industry has been a critical reservoir of resistance genes due to the abuse and misuse of antibiotics in animal agriculture. Many clinically significant resistance genes, such as mcr-1 (Liu et al., 2016), tet(X3), and tet(X4) (He et al., 2019), were first detected in bacteria of animal origin. According to a retrospective screening project, the prevalence of tet(X)-positive isolates of animal source (6.9%) was much higher than that of human source (0.07%) (He et al., 2019). Recent studies also showed that the detection rate of tet(X) genes in isolates from animals (Cui et al., 2020; Li et al., 2020b,c,d) was higher than that from humans (Wang et al., 2019). Hence, it is critical to enrich more information about the animal source associated with tet(X)-bearing pathogens. The prevalence of tet(X) genes in swine farms and slaughterhouses has been systematically investigated (Li et al., 2020b,c,d), but the comprehensive molecular characterization of tet(X)-bearing bacteria of chicken was unexplored. In this study, we focused on the prevalence of tet(X) variants in cultivable bacteria among chicken fecal microbiota and demonstrated that tet(X) genes in diverse bacteria are worthy of continuous surveillance among different sources.

**MATERIALS AND METHODS**

**Sample Collection and Bacterial Isolates**

A total of 45 chicken fecal samples were collected from a chicken farm in Jiangsu Province, China, in May 2020. We incubated 0.5 g feces in 5 ml of Tryptic Soy Broth (TSB) for 6 h to perform bacteria enrichment. The tet(X)-positive isolates were screened by Tryptic Soy Agar (TSA) plates supplemented with tigecycline (4 mg/L) and further identified by PCR using primers previously described (He et al., 2019). The species of all tet(X)-positive isolates were determined by 16S rRNA gene sequencing.

**Antimicrobial Susceptibility Testing**

The minimum inhibitory concentrations (MICs) of all tet(X)-positive isolates were tested by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). *Escherichia coli* ATCC25922 was used for quality control. The resistance breakpoint for tigecycline was interpreted as >0.5 mg/L according to European Committee on Antimicrobial Susceptibility Testing (EUCAST).  

**Conjugation Experiments**

In order to verify the transferability of tet(X) genes, we conducted conjugation experiments using *E. coli* C600 and a clinical carbapenem-resistant *Acinetobacter baumannii* 5AB as recipients. Briefly, the donor and recipient strains were cultivated to the logarithmic growth phase with an optical density at 600 nm (OD_{600}) of 0.4 in LB broth, mixed at a ratio of 1:1, and cultured overnight on TSA agar plates at 37°C for the tet(X)-positive *Acinetobacter*, we also conducted the conjugation assay at 30°C. Then, the transconjugants were selected using TSA plates containing tigecycline (2 mg/L) and rifampin (300 mg/L) or meropenem (2 mg/L). And we further confirmed the recovered transconjugants by PCR for tet(X) and 16S rRNA genes. The frequencies of conjugation transfer were calculated by the number of transconjugants per recipient.

**Genomic DNA Extraction and Whole-Genome Sequencing**

Genomic DNA of tet(X)-positive isolates were extracted using FastPure Bacteria DNA Isolation Mini Kit (Vazyme™, Nanjing, China) following the manufacturer’s instruction. The quality and purity of genomic DNA were evaluated by Qubit 4 Fluorometer (Thermo Fisher Scientific™, Hennigsdorf, Germany) and Titerk-Berthold Colibri (Berthold, Hennigsdorf, Germany) and Titerk-Berthold Colibri (Berthold™, Bad Wildbad, Germany). The genomic DNA of all tet(X)-positive isolates was subjected to the short-read sequencing (2 × 150 bp) by Illumina Hiseq 2500 platform. According to the assembly result of short-read sequencing, the genomic DNA of isolates with different tet(X) genetic contexts was further sequenced by long-read sequencing platform Oxford Nanopore Technologies MinION with a rapid barcoding library preparation strategy.

**Bioinformatics Analysis**

*De novo* short-read assembly was performed using SPAdes (Bankevich et al., 2012). The complete bacterial genomes were obtained using a hybrid assembly strategy combining long-read Nanopore and short-read Illumina sequencing data (Wick et al., 2017; Li R. et al., 2018). Antibiotic resistance genes, insertion sequence (IS) elements, and plasmid replicon types were identified by CGE services. The draft genomes were annotated by Prokka (Seemann, 2014). Functional annotation of the complete genome sequences was annotated automatically using the RAST® and modified manually. Multilocus sequence typing (MLST) of assembled bacterial genomes was performed using the mlst tool4 and Pubmlst.5 The complete genomes of tet(X4)-bearing *E. coli* were downloaded from nr database in the National Center for Biotechnology Information (NCBI). The phylogenetic tree of strains was constructed using Roary and FastTree based on single-nucleotide polymorphisms (SNPs) of core genomes with default parameters (Price et al., 2009; Page et al., 2015). BRIG and Easyfig tools were used to visualize

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1. http://www.eucast.org/clinical_breakpoints/
2. https://cge.cbs.dtu.dk/services/
3. http://rast.nmpdr.org/
4. https://github.com/tseemann/mlst
5. https://pubmlst.org/
Table 1: Antimicrobial susceptibility testing (MICs, mg/L) of 15 tet(X)-positive strains and their transconjugants.

| Strain            | Source     | Conjugation frequency to C600 | ST type | Species            | AMX | ENR | CFF | MEM | CL | KAN | TIG | FFC |
|-------------------|------------|-------------------------------|---------|--------------------|-----|-----|-----|-----|----|-----|-----|-----|-----|
| SC2-6             | Feces      | /                             | 284     | Citrobacter portucaleensis | >256 | 16  | 1   | <0.25 | <0.25 | >128 | 32  | 128 |
| cSC2-6            | Transconjugant | 2.8 x 10^{-9}                  | /       | Escherichia coli | >256 | 16  | 1   | <0.25 | <0.25 | 4    | 16  | 128 |
| LHC3              | Feces      | /                             | 284     | C. portucaleensis | >256 | 16  | 1   | <0.25 | <0.25 | >128 | 64  | 128 |
| cLHC3             | Transconjugant | 1.9 x 10^{-9}                  | /       | E. coli            | >256 | 16  | 1   | <0.25 | <0.25 | 8    | 32  | >128|
| LHC3-1            | Feces      | /                             | 284     | C. portucaleensis | >256 | 32  | 1   | <0.25 | <0.25 | >128 | 32  | >128|
| cLHC3-1           | Transconjugant | 3.9 x 10^{-11}                 | /       | E. coli            | >256 | 4   | 1   | <0.25 | <0.25 | 4    | 8   | >128|
| XMY1F802-7        | Feces      | /                             | 284     | C. portucaleensis | >256 | 16  | 0.5 | <0.25 | <0.25 | 4    | 64  | 128 |
| cXMY1F802-7       | Transconjugant | 3.9 x 10^{-11}                 | /       | E. coli            | >256 | 4   | 0.5 | <0.25 | <0.25 | 4    | 64  | 128 |
| XM10F302-7        | Feces      | /                             | 284     | C. portucaleensis | >256 | 32  | 1   | <0.25 | <0.25 | >128 | 128 | >128|
| cXM10F302-7       | Transconjugant | 1.4 x 10^{-10}                | /       | E. coli            | >256 | 32  | 1   | <0.25 | <0.25 | >128 | 64  | >128|
| LHC5-1            | Feces      | /                             | novel   | Citrobacter werkmanii | >256 | 16  | 18  | <0.25 | <0.25 | 32   | 64  | >128|
| cLHC5-1           | Transconjugant | 1.3 x 10^{-9}                 | /       | E. coli            | >256 | 0.5 | <0.25 | <0.25 | <0.25 | 32   | 64  | >128|
| XM3F402-1         | Feces      | /                             | 93      | E. coli            | >256 | 4   | <0.25 | <0.25 | <0.25 | 8    | 32  | >128|
| cXM3F402-1        | Transconjugant | 4 x 10^{-11}                  | /       | E. coli            | >256 | 0.5 | <0.25 | <0.25 | <0.25 | 4    | 16  | >128|
| XMC1F102-2        | Feces      | /                             | 93      | E. coli            | >256 | 4   | <0.25 | <0.25 | <0.25 | 4    | 32  | >128|
| cXMC1F102-2       | Transconjugant | /                              | /       | E. coli            | >256 | 4   | <0.25 | <0.25 | <0.25 | 4    | 16  | >128|
| XM3F402-7         | Feces      | /                             | 1,286   | E. coli            | >256 | 4   | <0.25 | <0.25 | <0.25 | >128 | 64  | >128|
| cXM3F402-7        | Transconjugant | 4 x 10^{-11}                  | /       | E. coli            | >256 | 4   | <0.25 | <0.25 | <0.25 | >128 | 16  | >128|
| XM7F102           | Feces      | /                             | 155     | E. coli            | >256 | 32  | >64  | <0.25 | <0.25 | 8    | 32  | >128|
| cXM7F102          | Transconjugant | /                              | /       | E. coli            | >256 | 2   | <0.25 | <0.25 | <0.25 | 4    | 16  | >128|
| LHC3-2            | Feces      | /                             | 327     | Enterobacter hormaechei | >256 | 4   | 32  | <0.25 | <0.25 | 8    | 32  | >128|
| cLHC3-2           | Transconjugant | NA                             | /       | E. coli            | >256 | 4   | <0.25 | <0.25 | <0.25 | 4    | 16  | >128|
| LHC2-1            | Feces      | /                             | /       | Providencia alcalifaciens | >256 | 8   | 32  | <0.25 | <0.25 | >128 | >128| >128|
| XM9F202-2         | Feces      | /                             | /       | Acinetobacter variabilis | 2    | 4   | 4   | <0.25 | <0.25 | >128 | 32  | >128|
| XM5X702           | Feces      | /                             | /       | Acinetobacter lwoffii | 128  | 0.5 | 1   | <0.25 | <0.25 | 8    | 32  | >128|
| LHC22-2           | Feces      | /                             | 1,459   | Acinetobacter baumannii | >256 | 32  | 32  | 1    | <0.25 | 16   | 64  | 128|

NA, not available. The transfer frequencies of these samples were too low to be calculated accurately.
MICs, minimum inhibitory concentrations; ST, sequence typing; AMX, amoxicillin; ENR, enrofloxacin; CFF, ceftriaxone; MEM, meropenem; CL, colistin; KAN, kanamycin; TIG, tigecycline; FFC, fleroxacin.
plasmid comparisons (Alikhan et al., 2011) and genetic context comparisons (Sullivan et al., 2011).

RESULTS

Characterization of Tigecycline-Resistant Strains

Out of 45 chicken fecal samples, a total of 15 tet(X)-positive strains were isolated from 13 samples (13/45, 28.89%). These tet(X)-positive strains consisted of eight different species including five Citrobacter portucalensis, four E. coli, one Enterobacter hormaechei, one Citrobacter werkmanii, one Acinetobacter variabilis, one Acinetobacter lwofii, one A. baumannii, and one Providencia alcalifaciens. Meanwhile, four different tet(X) variants were detected in these strains, containing tet(X3), tet(X4), and tet(X6) reported previously and a novel tet(X) variant, designated as tet(X15) in another study (Li et al., 2021; Figure 1). Among them, tet(X4) carried by Enterobacteriaceae was the most pervasive. Although three different tet(X) variants, tet(X3), tet(X6), and the novel tet(X15), were found in Acinetobacter, all of them showed low prevalence. Notably, the phenomenon that such a number of tet(X) variants were distributed in bacteria with different species within a farm was not observed in other studies. The extensive prevalence of tet(X) genes in this chicken farm

![FIGURE 2](image-url) Structure analysis of tet(X4)-bearing plasmids. (A) Comparison analysis of the plasmid pLHC5-1-tetX-240k with other similar plasmids including pRW7-1_235k_tetX (GenBank accession_number: MT219825) and pT16R-1 (CP046717). (B) Structure features of tet(X4)-bearing plasmids carried by Enterobacteriaceae in this study. The structural diversity of these plasmids existed within a multidrug-resistant (MDR) region. Resistance genes in plasmid pLHC5-1-tetX-240k were highlighted in red arrows. The reference sequence in panel (B) is pLHC5-1-tetX-240k, and colored circles indicate the sequences in draft genomes, which are mapped to the reference sequence.

![FIGURE 3](image-url) Linear comparison of the tet(X6)-bearing integrative and conjugative element (ICE) ICEPaChnLHC2-1 with other similar ICEs. The multidrug-resistant (MDR) region encoding tet(X6) was inserted into variable region III conserved in ICEs.
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suggested that poultry may be an important reservoir of tet(X), and the tet(X) genes are likely to be transmitted to humans through environmental interactions and chicken consumption (Sun et al., 2020).

Although tet(X4)-harboring E. coli was the most dominant in other research (He et al., 2019; Sun C. et al., 2019; Li et al., 2020a,b; Mohsin et al., 2021), tet(X4)-harboring E. coli of chicken source was rarely reported previously (Mohsin et al., 2021). To
investigate the clonal relationship of tet(X4)-harboring E. coli between chicken and other sources, the genomes of 26 tet(X4)-positive E. coli with different hosts including pig, dog, chicken, cow, and human were downloaded from NCBI database and analyzed. Phylogenetic analysis based on the core genome indicated that the prevalence feature of tet(X4)-harboring E. coli has no clear clonal relationship with their sources (Supplementary Figure 1). It is worth noting that a tet(X4)-harboring E. coli we detected showed a close relationship with a tet(X4)-positive E. coli detected in human gut microbiota (Ding et al., 2020; Supplementary Figure 1). Hence, the serious prevalence of tet(X) of animal source has a potential threat to human health.

### Antimicrobial Susceptibility Testing and Transfer of Different tet(X) Variants

Resistance phenotype analysis found that 15 tet(X)-positive strains showed resistance to multiple antibiotics and were all resistant to ticarcillin and enrofloxacin, but all strains were still susceptible to meropenem. Subsequently, we analyzed the distribution of resistance genes in tet(X)-positive strains according to draft genome sequences constructed by Illumina sequencing data. These strains contained multiple antibiotic resistance genes ranging from 8 to 19 (Supplementary Table 1). Besides, an extended-spectrum beta-lactamase (ESBL) gene blaCTX-M–55 and a carbapenemase gene blaOXA–58 were detected in some tet(X)-positive strains.

To investigate the transmissibility of different tet(X) variants, all strains were performed by conjugation assay. All tet(X4) genes in this study were successfully transferred into the recipient E. coli C600 with low frequencies, resulting in resistance to ticarcillin in transconjugants. The remaining tet(X)-positive strains failed in conjugation assay. The higher horizontal transfer percentage of tet(X4) might explain its high prevalence.

### The Genetic Contexts of tet(X) Variants

In order to investigate the genetic contexts of different tet(X) variants, five strains (one C. werkmanii LHC5-1, one P. alcalifaciens, and three Acinetobacter) were performed with Nanopore long-read sequencing to obtain complete genomes together with short-read data (Supplementary Table 1). Genetic analysis of strain LHC5-1 found that tet(X4) gene was located in a 240-kb IncFIA(HI1)/IncHI1A/IncHI1B(R27)/IncR hybrid plasmid, named pLHC5-1-tetX-240k. Many plasmids with a similar structure to pLHC5-1-tetX-240k were found in the NCBI nr database (Figure 2A), and most of these plasmids were positive for tet(X4) and harbored by E. coli. The emergence of tet(X4)-bearing IncFIA(H11)/IncHI1A/IncHI1B(R27)/IncR plasmid in Citrobacter spp. exacerbated the transmission of tet(X4) among different species of bacteria. Comparative analysis of plasmid pLHC5-1-tetX-240k and other similar hybrid plasmids found that a ca. 190-kb backbone region with replicons IncFIA(H11)/IncHI1A/IncHI1B(R27) in these hybrid plasmids was conserved (Figure 2A). Some small plasmids with replicons IncX1, IncX4, and IncR could integrate into a hybrid plasmid with replicons IncFIA(H11)/IncHI1A/IncHI1B(R27) and form larger and more complex plasmids, such as pRW7-1-235k_tetX (Li et al., 2020b). Subsequently, we investigated the genetic feature of tet(X4) in other strains in this study. The result showed that all tet(X4) genes in this study were carried by plasmids with a similar backbone to pLHC5-1-tetX-240k and located in a conserved ca. 190-kb region harboring IncFIA(H11)/IncHI1A/IncHI1B(R27) plasmid replicons (Figure 2B). In addition, we found that these hybrid plasmids were widely distributed in different species of bacteria. Therefore, the diffusion of tet(X4) was strongly associated with the IncFIA(H11)/IncHI1A/IncHI1B(R27) hybrid plasmids and their evolved complex plasmids. Apart from these tet(X4)-bearing plasmids in Enterobacteriaceae, one tet(X6) gene was detected in a P. alcalifaciens of Enterobacteriaceae for the first time. tet(X6) gene was located in variable region III (VRIII) of a chromosomal integrative and conjugative element (ICE), designated as ICEPalChnLHC2-1. A total of four tet(X6) genes were detected in VRIII within two tandem repeat units (Figure 3). Although tandem repeats of different tet(X) variants were frequently observed, two tet(X6) in one repeat unit have not been reported. The molecular mechanism of tet(X) tandem repeat deserved further investigations. Then, we searched for homologous ICEs with ICEPalChnLHC2-1 in the NCBI database, and three tet(X)-negative ICEs from Proteus genomosa, P. alcalifaciens, and Vibrio fluvialis were downloaded and compared. The four ICEs showed high similarity with each other, which implied that they originated from one ancestor and were popular because of horizontal transfer between different bacterial chromosomes. Meanwhile, we observed an evolution of genetic context in VRIII of the four ICEs, which was a manifestation of the adaptation of bacteria to changes in the external environment.

Although only three tet(X)-positive strains belonging to Moraxellaceae were identified, complex genetic contexts of tet(X) variants were found in the three strains. Co-occurrence of two different tet(X) variants in one strain was detected in strains XM9F202-2 and XMC5X702. A plasmid-mediated tet(X3) and a chromosomal novel tet(X) variant, designated as tet(X15), were found in A. variabilis XM9F202-2, which has been investigated in detail in our previous study (Li et al., 2021). In A. lwofii XMC5X702, two different tet(X) variants corresponded to tet(X3) and tet(X6). Genetic analysis found that tet(X3) and tet(X6) were located on a 145-kb plasmid pXMC5X702-tetX-145k with unknown replicon types. Multiple plasmids co-harboring tet(X3) and tet(X6) carried by Acinetobacter were found in the nr database, and they share more than 50% coverage and more than 95% identity to pXMC5X702-tetX-145k (Figure 4A). However, the replicon gene in pXMC5X702-tetX-145k differed from those plasmids co-harboring tet(X3) and tet(X6). The plasmids that harbor the same replicon as pXMC5X702-tetX-145k showed low identity to pXMC5X702-tetX-145k. Hence, the structure of pXMC5X702-tetX-145k was novel, and it enriched the profile of tet(X)-bearing plasmids in Acinetobacter. tet(X6) gene in A. baumannii LHC22-2 was carried by a 162-kb plasmid pLHC22-2-tetX-162k. What is noteworthy is that a carbapenemase gene blaOXA–58 was found in the tet(X6)-bearing
plasmid. Although many plasmids co-harboring tet(X3) and blaOXA−58 have been reported in other species of Acinetobacter (Cui et al., 2020; Ma et al., 2020), tet(X6)- and blaOXA−58-bearing plasmid was rarely reported (Zheng et al., 2020). As a clinically critical opportunistic pathogen, the emergence of carbapenem- and tigecycline-resistant A. baumannii poses a great threat to public health. Phenotype analysis of antimicrobial resistance showed that LHC22-2 was resistant to imipenem but susceptible to meropenem. The expression of blaOXA−58 could be enhanced by an intact upstream ISAba3 and result in resistance to meropenem (Hamidian and Nigro, 2019), but ISAba3 in plasmid pLHC22-2-tetX-162k was truncated. Subsequently, one plasmid pABF9692 co-harboring tet(X6)- and blaOXA−58 from A. baumannii and three plasmids with different sizes from Acinetobacter touneri showing similar backbone with pLHC22-2-tetX-162k were retrieved from the nr database and analyzed (Figure 4B). Notably, the backbone of pABF9692 was different with pLHC22-2-tetX-162k. In contrast, the three plasmids co-harboring tet(X3) and blaOXA−58 showed similar backbone with pLHC22-2-tetX-162k (Figure 4B). Two different tet(X) variants, tet(X3) and tet(X6), were detected in these plasmids, which indicated that such plasmids played a vital role in capturing and propagating the tet(X) genes.

The Core Genetic Structures of tet(X) Variants in This Study

The different tet(X) variants were harbored by various genetic structures and distributed in different bacteria in the chicken farm. However, ISCR2 was always associated with different tet(X) variants except for the novel tet(X15) (Figure 5). This phenomenon was consistent with previous studies (He et al., 2019; Li et al., 2020b,c), implying that ISCR2 was a major driving factor for the dissemination of tet(X) variants. We also found many other IS elements in the surroundings of different tet(X) variants, such as ISAbal in the downstream of tet(X6) in plasmid pLHC22-2-tetX-162k and IS26 in the upstream of tet(X3) in plasmid pXMC5X702-tetX-145k. These IS elements will probably be involved in the transfer of tet(X) variants and hereby have evolved novel genetic context of tet(X) variants. Apart from ISCR2-associated tet(X)-bearing genetic contexts, we identified a novel tet(X15) variant located in an ISAbal-bound composite transposon Tn6866 (Li et al., 2021). The ISAbal in the composite transposon Tn6866 is directly responsible for the movement of tet(X15), which differs from that of tet(X6). Therefore, monitoring the genetic context of tet(X) variants is important for understanding their transmission and evolution destiny.

DISCUSSION

The emergence of high-level tigecycline resistance genes tet(X3) and tet(X4) has caused great concern throughout the world. A large number of tet(X) variants, from tet(X3) to tet(X44), were identified from different bacteria in humans and animals within 2 years (Wang et al., 2019; Cheng et al., 2020; Gasparini et al., 2020; Peng et al., 2020; Zheng et al., 2020; Umar et al., 2021). The current outbreak and widespread situation of tet(X) is rapidly diminishing the effectiveness of tetracycline antibiotics, including tigecycline and the US Food and Drug Administration (FDA) newly approved eravacycline and omadacycline. Tetracyclines have been used in livestock farms for many years in China. However, few studies investigated the epidemiological and genetic features of tet(X) in livestock farms, with limited research focusing on the tet(X)-bearing Enterobacteriales or Acinetobacter (Cui et al., 2020; Li et al., 2020c). Meanwhile, the prevalence of tet(X) in bacteria of chicken origin has not been investigated fully to date. In this work, we systematically explored the distribution and genetic characteristics of different tet(X) variants and their host bacteria in a chicken farm. We found that the prevalence of mobilizable tet(X4) was the highest and more worrisome than that of other variants. Apart from E. coli, Citrobacter spp. was also an emerging host for tet(X4). The high prevalence of tet(X4) might be associated with their host plasmids. Although only three tet(X)-positive strains belonging to Acinetobacter spp. were identified, three different tet(X) variants were identified in them. The epidemic pattern of tet(X) in Acinetobacter differed from that in Enterobacteriales, and the relationship between them warrants further investigations.

Genetic analysis found that plasmids are an important vector for the dissemination of tet(X). However, some chromosomal mobile elements, such as ICEs and transposons, also contribute to the transfer of tet(X). According to transfer experiments, all tet(X4)-positive plasmids in Enterobacteriales could transfer to E. coli C600, and the other tet(X)-bearing genetic structures in Enterobacteriales and Acinetobacter failed to transfer in conjugation assay. The phenomenon explained the high prevalence of tet(X4) in the chicken farm and demonstrated that the prevalence of tet(X) genes was positively related to the horizontal transferability of their vectors within specific bacterial hosts. Notably, the transmission of tet(X4) was associated with various plasmids reported in our previous study (Li et al., 2020b). In this study, we first noticed that serious prevalence of tet(X4) in different bacteria mediated by IncFIA(HI1)/IncHI1A/IncHI1B(R27) plasmids occurred in the chicken farm. Currently, the worldwide dissemination of critical resistance genes was possible with the help of some common types of plasmids, such as blaNDM−5-positive IncX3 plasmid (Li X. et al., 2018; Liu et al., 2019; Zhao et al., 2021) and mcr-1-positive IncI2 plasmid (Elbediwi et al., 2019; Gelbicova et al., 2019; Lu et al., 2020). Hence, the emergence of tet(X4)-positive common plasmids with high mobility might cause an increasing prevalence of tet(X4). In addition, we found that all tet(X)-positive plasmids in Acinetobacter in the chicken farm had no ability of horizontal transfer, which is consistent with the previous reports (Cui et al., 2020; Ma et al., 2020; Wang et al., 2020). Genetic structure analysis found that those tet(X) genes in plasmids harbored by Acinetobacter were adjacent to ISCR2, indicating that ISCR2-mediated mobilization of tet(X) also deserved concerns among bacteria of different genus.

In conclusion, we comprehensively investigated the prevalence of tet(X) in a chicken farm first and identified multiple tet(X) variants from diversified bacteria. The prevalence of tet(X4) in the chicken farm was mainly determined by
their host plasmids. The *Acinetobacter* spp. is an important reservoir for other *tet(X)* variants. Apart from ISCR2, ISAba1 might also be an important element for the mobilization of *tet(X)*. Therefore, we propose that effective measures should be formulated to decelerate the dissemination of *tet(X)* in animal- and human-associated environments.

**DATA AVAILABILITY STATEMENT**

The sequences obtained in this article have been deposited in the GenBank database under BioProject number: PRJNA750704.

**AUTHOR CONTRIBUTIONS**

RL and ZW conceived and designed the experiments, and manuscript reviewing and editing. YL and KP conducted the experiments, analyze the data, and wrote the draft. YY, XS, and WZ conducted long-read sequencing and bioinformatics analysis. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

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