Widespread Dissemination of Plasmid-Mediated Tigecycline Resistance Gene \textit{tet}(X4) in \textit{Enterobacterales} of Porcine Origin

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ABSTRACT The emergence of the plasmid-mediated high levels of the tigecycline resistance gene has drawn worldwide attention and has posed a major threat to public health. In this study, we investigated the prevalence of the \textit{tet}(X4)-positive \textit{Enterobacterales} isolates collected from a pig slaughterhouse and farms. A total of 101 tigecycline resistance strains were isolated from 353 samples via a medium with tigecycline, of which 33 carried \textit{tet}(X4) (9.35%, 33/353) and 2 carried \textit{tet}(X6) (0.57%, 2/353). These strains belong to seven different species, with \textit{Escherichia coli} being the main host bacteria. Importantly, this report is the first one to demonstrate that \textit{tet}(X4) was observed in \textit{Morganella morganii}. Whole-genome sequencing results revealed that \textit{tet}(X4)-positive bacteria can coexist with other resistance genes, such as \textit{bla}_{NDM-1} and \textit{cfr}. Additionally, we were the first to report that \textit{tet}(X4) and \textit{bla}_{NDM-1} coexist in a \textit{Klebsiella quasipneumoniae} strain. The phylogenetic tree of 533 \textit{tet}(X4)-positive \textit{E. coli} strains was constructed using 509 strains from the NCBI genome assembly database and 24 strains from this study, which arose from 8 sources and belonged to 135 sequence types (STs) worldwide. We used Nanopore sequencing to interpret the selected 21 nonclonal and representative strains and observed that 19 \textit{tet}(X4)-harboring plasmids were classified into 8 replicon types, and 2 \textit{tet}(X6) genes were located on integrating conjugative elements. A total of 68.42% of plasmids carrying \textit{tet}(X4) were transferred successfully with a conjugation frequency of \(10^{-2}\) to \(10^{-7}\). These findings highlight that diverse plasmids drive the widespread dissemination of the tigecycline resistance gene \textit{tet}(X4) in \textit{Enterobacterales} of porcine origin.

IMPORTANCE Tigecycline is considered to be the last resort of defense against diseases caused by broad-spectrum resistant Gram-negative bacteria. In this study, we systematically analyzed the prevalence and genetic environments of the resistance gene \textit{tet}(X4) in a pig slaughterhouse and farms and the evolutionary relationship of 533 \textit{tet}(X4)-positive \textit{Escherichia coli} strains, including 509 \textit{tet}(X4)-positive \textit{E. coli} strains selected from the NCBI between 2002 and 2022. The drug resistance of tigecycline is widely prevalent in pig farms where tetracycline is used as a veterinary drug. This prevalence suggests that pigs are a large reservoir of \textit{tet}(X4) and that \textit{tet}(X4) can spread horizontally through the food chain via mobile genetic elements. Furthermore, tetracycline resistance may drive tigecycline resistance through some mechanisms. Therefore, it is important to monitor tigecycline resistance, develop effective control measures, and focus on tetracycline use in the pig farms.

KEYWORDS \textit{Enterobacterales}, tigecycline resistance, \textit{tet}(X4), evolutionary relationship, genetic environments

In recent years, antibiotic resistance has become a serious concern, posing a major threat to human health and food safety owing to the increased use of human and agricultural antibiotics, which has created selection pressure contributing to resistance...
Tetracycline antimicrobials are effective against a wide spectrum of pathogens, including Gram-positive and Gram-negative bacteria and atypical organisms (2). Tigecycline is considered a last-resort antibiotic for the treatment of severe infections caused by extensively drug-resistant bacteria (3). However, reports of clinical resistance to tigecycline have increased since 2007, and the initial discovery of tigecycline resistance was largely owing to the expression of different efflux pump genes (tetA-E and tetL) and ribosome protection protein-encoding genes (tetM and tetO) (4).

The plasmid-carrying tet(X) genes can encode a flavin-dependent monooxygenase that not only catalyzes the efficient degradation of a broad range of tetracycline analogs but also confers resistance to these antibiotics in vivo. To date, 47 tet(X) variants have been identified (3–13). tet(X) genes are widely present in a variety of hosts, such as Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Acinetobacter baumannii, and Citrobacter freundii, in humans and animals (14–18). Additionally, tet(X) genes occasionally coexist with blaNDM, mcr-1, and cfr, which confer resistance to carbapenem, colistin, and oxazolidinone, respectively (19–21). Prior to the discovery of the plasmid-carrying tet(X) gene variant, a small number of tigecycline resistance bacteria were reported in 2005 and subsequently discovered, of which most were Gram-negative bacteria (22–26). The rapid spread of the plasmid-mediated tet(X) gene variant to tigecycline resistance would complicate the treatment of multidrug-resistant infections and pose a threat to human health. The plasmid-carrying tet(X) and its variants are involved in a new mechanism of tigecycline resistance in humans and animals. In 2019, a study revealed the plasmid-mediated tigecycline resistance genes tet(X3) and tet(X4) in Enterobacteriaceae and Acinetobacter in China, posing a major threat to global health (3, 20). Subsequently, plasmids including the IncX1 plasmid and fusion plasmids, such as IncX1-IncFIA-IncFIB-IncFIC, IncFIA-IncH1B-IncH1A, IncX1-IncN, and IncY-IncX1-IncFIA-IncFIB, and IncX1-IncFIA-IncFIB, have been reported to mediate the transmission of tet(X) and others (27, 28). The appearance of strains, which simultaneously carry super antibiotic resistance genes, and the multiple types of mobile genetic elements, which mediate the spread of resistance genes, can exacerbate the situation of global antibiotic resistance.

Although tigecycline is used in human medicine and is prohibited in veterinary medicine, tetracycline antibiotics are used widely in livestock and poultry breeding in China. In particular, oxytetracycline and aureomycin are used occasionally in pig breeding and may cause cross-drug resistance and the spread of tetracycline resistance genes. However, the prevalence of the plasmid-mediated tigecycline resistance genes in Enterobacterales of pig origin in Sichuan Province remains unknown. In this study, we detected the prevalence of tet(X4) in a pig slaughterhouse and 10 pig farms and analyzed its genetic environmental diversity in Sichuan Province. Furthermore, we investigated the evolutionary relationship of tet(X4)-positive E. coli isolates worldwide. We observed multiple Gram-negative bacteria carrying the tet(X4) gene and demonstrated its genetic environmental diversity.

RESULTS

Prevalence of tet(X4) in a pig slaughterhouse and farms in Sichuan Province. In total, 101 tigecycline resistance strains were selected, of which 33 carried tet(X4) (9.35%, 33/353) and 2 carried tet(X6) (0.57%, 2/353). The 33 tet(X4)-positive strains were observed predominantly in E. coli (72.72%), followed by K. pneumoniae (12.12%), M. morganii (6.06%), K. quasipneumoniae (3.03%), Proteus vulgaris (3.03%), and P. mirabilis (3.03%). The remaining two tet(X6)-positive strains belonged to Proteus terrae subsp. cibarius. To the best of our knowledge, we are the first to report that tet(X4) is observed in M. morganii. The strains isolated from the slaughterhouse belonged to six species, and the strains isolated from the pig farms belonged to four species. Besides, the monoclonal strains were from different samples (Table 1).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing using 17 antimicrobial agents revealed that these positive strains widely exhibited a multidrug resistance (MDR) phenotype. All tigecycline resistance strains were resistant to tetracycline and doxycycline (DOX), and more than 90% of the strains were resistant to...
florfenicol (FFC). A total of 24 E. coli strains carrying tet(X4) were resistant to tetracycline (100%; n = 24), DOX (100%; n = 24), FFC (95.83%; n = 23), trimethoprim-sulfamethoxazole (SXT; 79.17%; n = 19), ceftriaxone sodium (CRO; 45.83%; n = 11), gentamicin (GEN; 41.67%; n = 10), ceftazidine (CAZ; 37.50%; n = 9), aztreonam (ATM; 25%; n = 6), ciprofloxacin (CIP; 20.83%; n = 5), amikacin (AMK; 12.50%; n = 3), norfloxacin (NOR; 12.50%; n = 3), and amoxicillin (AMC; 22.86%; n = 8). All E. coli isolates were susceptible to polymyxin B (PMB), cefoxitin (FOX), meropenem (MEM), and fosfomycin (FOS). The lowest MIC of tigecycline was 16 mg/L; however, strangely, the highest MICs of tigecycline were to polymyxin B (PMB), cefoxitin (FOX), meropenem (MEM), and fosfomycin (FOS). The MICs of tigecycline were 32 mg/L or less for all strains of Proteus mirabilis (P. mirabilis) and Pseudomonas aeruginosa.

**TABLE 1** Information on 35 tet(X4)/tet(X6)-carrying strains

| Strain | Sourcea | Species | ST | MIC of tigecycline (mg/L) | Resistance phenotypeb | Genome accession |
|--------|---------|---------|----|------------------------|-----------------------|------------------|
| JZ30   | S       | *P. vulgaris* |    | >256                   | TGC, DOX, FFC, PMB, SXT, FOX, GEN, TET, CAZ, AMC, AMK, ATC, CAZ | JALMET000000000 |
| JZ35   | S       | *P. terrae* subsp. *cibaria* | 32 |                        | TGC, DOX, FFC, PMB, SXT, GEN, TET, CAZ, AMC, AMK | JALMEM000000000 |
| JZ49   | S       | *P. mirabilis* |    | >256                   | TGC, DOX, FFC, PMB, SXT, GEN, TET, CAZ, AMC, AMK, ATC | JALMER000000000 |
| JZ47   | S       | *K. pneumoniae* | ST629 | 32                   | TGC, DOX, FFC, SXT, FOX, FOS, GEN, TET, CAZ, AMC, AMK, ATC | JALMEQ000000000 |
| JZ2    | S       | *K. quasipneumoniae* |    | 128                   | TGC, DOX, FFC, MEM, SXT, FOX, FOS, TET, CAZ, AMC, AMK, ATC | JALMEP000000000 |
| JZ18   | S       | *K. pneumoniae* | ST25 | 128                   | TGC, DOX, FFC, SXT, FOX, TET, CAZ, AMC, AMK, ATC | JALMEO000000000 |
| JZ19   | S       | *E. coli* | ST793 | 32                   | TGC, DOX, FFC, SXT, GEN, TET, AMK | JALMEN000000000 |
| JZ50   | S       | *E. coli* | ST195 | 64                   | TGC, DOX, FFC, SXT, TET, CAZ, AMK, ATM | JALMEM000000000 |
| JZ21   | S       | *E. coli* | ST48  | 32                   | TGC, DOX, FFC, SXT, GEN, TET, CAZ, AMK, ATM | JALMEM000000000 |
| JZ27   | S       | *E. coli* | ST10671 | 32   | TGC, DOX, FFC, GEN, TET, TET, AMK | JALMEM000000000 |
| JX26   | F1      | *M. morganii* |    | 256                   | TGC, DOX, FFC, PMB, SXT, FOX, TET | JALMEC000000000 |
| JX10   | F1      | *E. coli* | ST101 | 32                   | TGC, DOX, FFC, SXT, TET | JALMEC000000000 |
| JX3    | F1      | *E. coli* | ST101 | 16                   | TGC, DOX, FFC, CIP, SXT, TET | JALMEC000000000 |
| JX1    | F1      | *E. coli* | ST218 | 16                   | TGC, DOX, FFC, SXT, GEN, TET | JALMEC000000000 |
| JX7    | F1      | *E. coli* | ST1684 | 32    | TGC, DOX, FFC, SXT, TET, ATC | JALMEC000000000 |
| JX14   | F1      | *E. coli* | ST195 | 64                   | TGC, DOX, FFC, SXT, GEN, TET, CAZ, AMK | JALMEM000000000 |
| DM22   | F4      | *E. coli* | ST195 | 64                   | TGC, DOX, FFC, SXT, GEN, TET, CAZ, AMK | JALMEM000000000 |
| DM3    | F4      | *E. coli* | ST195 | 32                   | TGC, DOX, FFC, SXT, TET, CAZ, AMK | JALMEM000000000 |
| DM13   | F4      | *E. coli* | ST8076 | 16     | TGC, DOX, FFC, SXT, TET, AMC | JALMEM000000000 |
| TQ3    | F5      | *E. coli* | ST195 | 64                   | TGC, DOX, FFC, SXT, GEN, TET, CAZ, AMK | JALMEDZ000000000 |
| TQ5    | F5      | *E. coli* | ST10  | 16                   | TGC, DOX, FFC, SXT, TET | JALMEDY000000000 |
| TQ6    | F5      | *E. coli* | ST761 | 64                   | TGC, DOX, FFC, TET, CAZ, AMK | JALMEDX000000000 |
| TQ2    | F5      | *E. coli* | ST10  | 16                   | TGC, DOX, FFC, SXT, TET, AMC | JALMEDW000000000 |
| TQ13   | F5      | *E. coli* | ST877 | 32                   | TGC, DOX, CIP, SXT, GEN, NOR, TET | JALMEDV000000000 |
| TQ41   | F5      | *E. coli* | ST69  | 32                   | TGC, DOX, FFC, GEN, TET, AMK | JALMEDU000000000 |
| TQ25   | F5      | *E. coli* | ST12984 | 16   | TGC, DOX, FFC, CIP, SXT, GEN, TET, CAZ, AMK, ATM | JALMDT000000000 |
| TQ55   | F5      | *E. coli* | ST48  | 16                   | TGC, DOX, FFC, SXT, TET | JALMDM500000000 |
| TQ17   | F5      | *E. coli* | ST165 | 32                   | TGC, DOX, FFC, TET | JALMDR000000000 |
| TQ30   | F5      | *K. pneumoniae* | ST25 | 64                   | TGC, DOX, FFC, CIP, SXT, TET | JALMDQ000000000 |
| TQ11   | F5      | *K. pneumoniae* | ST35 | 128                  | TGC, DOX, FFC, TET | JALMDP000000000 |
| TQ12   | F5      | *P. terrae* subsp. *cibaria* | 128 |                        | TGC, DOX, FFC, SXT, TET, AMC | JALMDQ000000000 |
| TQ28   | F5      | *M. morganii* | 128 |                        | TGC, DOX, FFC, SXT, TET, AMC | JALMDQ000000000 |
| DW10   | F8      | *E. coli* | ST46  | 128                  | TGC, DOX, FFC, TET | JALMEO000000000 |
| DW28   | F8      | *E. coli* | ST617 | 64                   | TGC, DOX, FFC, CIP, SXT, NOR, TET | JALMDL000000000 |

aS, pig slaughterhouse; F, pig farms.
bTGC, tigecycline; TET, tetracycline.
aph (n = 1), ST46 (n = 1), ST617 (n = 1), and a new ST, ST12984. Three different STs, including ST25 (n = 2), ST629 (n = 1), and ST35 (n = 1), were observed in four K. pneumoniae isolates. All strains carried at least one aminoglycoside resistance gene, such as aac, aph, and ada. Additionally, various kinds of β-lactam resistance genes were observed, such as blaCTX-M-14 (n = 7, 20%), blaCTX-M-55 (n = 1, 2.86%), blaOXA-1 (n = 5, 14.29%), blaCMY-2 (n = 2, 5.71%), blaCMY-38 (n = 2, 5.71%), blaADE-1 (n = 1, 2.86%), and blaOXA-1 (n = 1, 2.86%). Furthermore, the phenicols (flor; 35/35), sulfonamides (sul; 30/35), trimethoprim (dfrA; 28/35), quinolones (gmrS; 26/35), and lincosamide (lin(F); 21/35) resistance genes were also detected. A strain of P. terrae subsp. cibarii carrying both multiple resistance gene cfr and tigecycline resistance gene tet(X6) was observed (see Table S1 in the supplemental material). In addition, the remaining 66 tigecycline resistance strains, including P. mirabilis (n = 52), P. vulgaris (n = 1), K. pneumoniae (n = 7), E. coli (n = 4), and Enterobacter hormaechei (n = 2) without tet(X4) or tet(X6) had some other tetracycline resistance genes like tet (A), tet(B), tet(D), tet(H), tet(J), and tet(M). Furthermore, 3 P. mirabilis strains from a pig slaughterhouse had tigecycline resistance gene cluster tmexCD3-toprJ3.

Phylogenomic analysis. Based on the stand-alone BLAST, a total of 509 tet(X4)-positive E. coli strains were observed in the 27,802 assembled genomes of E. coli from NCBI between 2002 and 2022. These strains were derived from pigs, humans, chickens, ducks, pigeons, cows, and pet dogs around the world, and the pig is the most important source of the tet(X4) gene (see Table S2 in the supplemental material). Based on single nucleotide polymorphisms (SNPs) of core genomes, the phylogenetic tree of 533 tet(X4)-positive E. coli strains, including 24 strains in this study, was constructed. The 533 strains belonged to 135 different ST types, and ST48, ST10, and ST761 were the main ST types. The SNP range of 533 E. coli strains ranged from 0 to 44,532, and there were 304 distinct clones, 66 of which were clonally transmitted between people, animals, and the environment, and they belonged to 45 STs, with the most prevalent, and their lengths ranged between 31,271 bp and 73,084 bp. These plasmids were found in several species as well as various pig farms and slaughterhouses, demonstrating the global dissemination of plasmids of the IncX1 type. Additionally, there were some plasmid types that have been reported in previous studies, such as IncFIA-IncHI1A-IncHI1B (n = 3), IncC (n = 2), IncFIB-IncFIC (n = 1), IncFIA-IncFIB-IncX1 (n = 1), IncFIA-IncHI1A-IncHI1B-Col (n = 1), and IncFIA-IncHI1A-IncHI1B-IncX1 (n = 1). However, the heterozygous plasmid containing IncX3 of IncFIA-IncHI1A-IncHI1B-IncX3 (n = 1) was first reported in this study (Table 2). Importantly, more than 99% similarity existed in pXY14-tet (X4), pXY10-tet(X4), pJZ2-tet(X4), pTQ3-tet(X4), pJZ50-tet(X4), pJZ18-tet(X4), and pJZ19-tet(X4). However, these plasmids were divided from nonclone strains. The tet(X4) gene in M. morganii is located on a heterozygous plasmid, IncFIA-IncHI1A-IncHI1B. Remarkably, tet(X4) and cfr/blaQDM-1 were observed in the same strain, and this is the first study to report that tet(X4) and blaQDM-1 coexisted in a K. quasipneumoniae strain. tet(X4) and blaQDM-1 are located on plasmids IncX1 and IncX3, respectively. Furthermore, the coverage rate and the similarity between ICEPmiChn-JZ35 and ICEPmiChn-TQ12 were 75% and 99.55%, respectively. Compared with the first reported ICE SXT/R391 carrying tet(X6), ICEPmiChn-JZ35 had an 82% coverage rate and 96.93% similarity, whereas ICEPmiChn-TQ12 had a 64% coverage rate and 97.20% similarity.

Based on the results of the bacterial complete genome map, the genetic contexts of tet(X4) were analyzed and categorized into five main groups, as follows: group I had the most normal structure of IS26-abh-tet(X4)-ISCR2-virD2, which consisted of 9 strains.
in this study. There was a different upstream ISCR2 of tet(X4) performing ISCR2-abh-tet(X4)-ISCR2-virD2 in group II \((n = 3)\) compared with that in group I. An analysis of group III \((n = 1)\), namely, ISCR2-abh-tet(X4)-ISCR2, revealed that ISCR2 was located upstream and downstream of tet(X4), whereas group IV \((n = 2)\) had an upstream IS1B, forming the genetic structure of IS1B-abh-tet(X4)-ISCR2-virD2. Compared with group IV, there was no virD2 downstream in group V \((IS1B-abh-tet(X4)-ISCR2, n = 4)\). Besides, interestingly, pJZ21-tet(X4) had seven tet(X4) genes, which was consistent with the second-generation sequencing results depicting that the copy number of tet(X4) was approximately 10 times that of other drug-resistant genes. However, the number of tet(X4) genes was not proportional to the MICs (Fig. 3 and 4). Moreover, 68.42% of plasmids carrying tet(X4) and 100% of ICEs carrying tet(X6) could be transferred successfully with a conjugation frequency \(10^{-4} \) to \(10^{-2}\) and \(10^{-4} \) to \(10^{-5}\), respectively, and pTQ28-tet(X4) in \(M. morganii\) could horizontally transfer in \(E. coli\) strain EC600 with a conjugation frequency \(10^{-6}\) (Table 2).

**DISCUSSION**

Since the discovery of tet(X4), many studies have investigated the prevalence of tigecycline resistance in various regions of China, indicating different levels of...
prevalence and MDR and the risk of cloning transmission in different regions and sources (29–32). However, in the Sichuan Province, one of the largest pig farming provinces, studies on the prevalence of \( tet(X4) \)-positive bacteria are limited, and an analysis of their genetic environments is not sufficiently comprehensive. Therefore, we isolated \( tet(X4)/tet(X6) \)-positive \textit{Enterobacterales} in pig samples from a pig slaughterhouse and 10 pig farms in Sichuan Province and analyzed the epidemic situation of \( tet(X4) \)-positive strains in this study. We isolated 35 \( tet(X4)/tet(X6) \)-positive strains and observed that Sichuan Province had a high prevalence rate (9.44%) of \( tet(X4) \)-positive bacteria in pig breeding, which was higher than that observed in previous studies. Because the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Core-genome MLST allelic profiles of \textit{E. coli}, including 24 \( tet(X4) \)-positive \textit{E. coli} isolates in this study and 509 \( tet(X4) \)-positive \textit{E. coli} isolates from the NCBI genome assembly database.}
\end{figure}
TABLE 2 The results of 21 tet(X4)/tet(X6)-carrying strains by long-read Nanopore sequencing

| Strain | Location of tet(X) | Length (bp) | MGE* type | Resistance genes on tet(X)-harboring MGE | Conjugation | Accession no. |
|--------|--------------------|-------------|------------|------------------------------------------|-------------|---------------|
| JZ30   | pJZ30-tet(X4)      | 185,363     | IncA/C2    | tet(X4), sul1, aac(3)-I-Vla, aadA1, erm(42), blq(CMY-2), qacE, floR, tet(X6), aph(3')-I-Vla, aadA2b, aadA2, aph(6)-Id, aph(3')-Ib, sul1, sul2, dfrA19, floR, qacE | 10−3        | ON390809     |
| JZ35   | ICEPmiChn-JZ35     | 142,200     | ICE        | tet(X6), aph(3')-I-Vla, aadA2b, aadA2, aph(6)-Id, aph(3')-Ib, sul1, sul2, dfrA19, floR, qacE | 10−5        | ON390821     |
| JZ49   | pJZ49-tet(X4)      | 197,640     | IncA/C2    | tet(X4), aac(3)-Vla, aadA1, erm(42), blq(CMY-2), sul1, floR, qacE | 10−5        | ON390810     |
| JZ2    | pJZ2-tet(X4)       | 31,272      | IncX1      | tet(X4), aadA2, floR, lnuf(F), tet(A) | 10−2        | ON390804     |
| JZ18   | pJZ18-tet(X4)      | 57,105      | IncX1      | tet(X4), aadA2, blq(Dmp-12), floR, lnuf(F), tet(A) | 10−2        | ON390805     |
| JZ19   | pJZ19-tet(X4)      | 57,105      | IncX1      | tet(X4), aadA2, blq(Dmp-12), floR, lnuf(F), tet(A) | 10−2        | ON390806     |
| JZ50   | pJZ50-tet(X4)      | 31,250      | IncX1      | tet(X4), aadA2, floR, lnuf(F), tet(A) | 10−3        | ON390811     |
| JZ21   | pJZ21-tet(X4)      | 73,084      | IncX1      | tet(X4), aph(6)-Id, blq(TEM-1b), sul3, dfrA14, floR, mef(B), qnrS1, qnrS2, tet(A) | 10−4        | ON390808     |
| JZ27   | pJZ27-tet(X4)      | 159,632     | IncFIB, IncFIC | tet(X4), aph(3')-Ia, aadA1, aadA2b, erm(42), sul3, floR, lnuf(F), qnrS1, tet(A) | 10−4        | ON390808     |
| XY36   | pXY36-tet(X4)      | 190,661     | IncFIA, IncHI1A, IncHI1B | tet(X4), aadA2, blq(Dmp-12), qnrS1, floR, lnuf(G) | 10−6        | ON390820     |
| XY10   | pXY10-tet(X4)      | 31,272      | IncX1      | tet(X4), aadA2, floR, lnuf(F), tet(A) | 10−6        | ON390818     |
| XY14   | pXY14-tet(X4)      | 31,272      | IncX1      | tet(X4), aadA2, floR, lnuf(F), tet(A) | 10−6        | ON390819     |
| DM13   | pDM13-tet(X4)      | 241,960     | IncFIA, IncHI1A, IncHI1B, IncX1 | tet(X4), aph(3')-Ia, aadA2, blq(TEM-1b), blq(Dmp-12), dfrA14, floR, lnuf(G), qnrS1, tet(A) | 10−7        | ON390802     |
| TQ3    | pTQ3-tet(X4)       | 31,271      | IncX1      | tet(X4), aadA2, floR, lnuf(F), tet(A) | 10−4        | ON390813     |
| TQ6    | pTQ6-tet(X4)       | 201,404     | IncFIA, IncHI1A, IncHI1B | tet(X4), blq(TEM-1b), floR, lnuf(G), aadA22, qnrS1 | 10−4        | ON390814     |
| TQ2    | pTQ2-tet(X4)       | 198,381     | Col, IncFIA, IncHI1A, IncHI1B | tet(X4), blq(TEM-1b), floR, lnuf(G), aadA22, qnrS1 | 10−4        | ON390815     |
| TQ17   | pTQ17-tet(X4)      | 90,608      | IncFIA, IncFIB, IncX1 | tet(X4), aadA2, blq(Dmp-12), floR, lnuf(F), qnrS1, tet(A) | 10−5        | ON390817     |
| TQ30   | pTQ30-tet(X4)      | 225,359     | IncX3, IncFIA, IncHI1A, IncHI1B | tet(X4), blq(TEM-1b), floR, lnuf(G), aadA22, qnrS1 | 10−3        | ON390817     |
| TQ12   | ICEPmiChn-TQ12     | 147,546     | ICE        | tet(X6), aadA2, aph(3')-Ia, aph(3')-Ib, aph(6)-Id, sul2, floR, tet(A) | 10−4        | ON390822     |
| TQ28   | pTQ28-tet(X4)      | 187,191     | IncFIA, IncHI1A, IncHI1B | tet(X4), blq(TEM-1b), floR, aadA1, qnrS1, lnuf(G) | 10−6        | ON390816     |
| DW28   | pDW28-tet(X4)      | 46,856      | IncX1      | tet(X4), floR, lnuf(F), aadA2, tet(A) | 10−5        | ON390803     |

*MGE, mobile genetic element.

tet(X4) in Enterobacteriales of Porcine Origin

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tigecycline resistance strains were collected using resistant culture medium in this study, which perhaps provided a higher detection rate, the prevalence of tet(X4) observed from ordinary surveillance might have been underestimated. Therefore, continuous and specific monitoring of tigecycline resistance is important.

Additionally, this study demonstrated that except for E. coli, which was the dominant bacterium and a huge reservoir of the tet(X4) gene, there were six other species of bacteria carrying tet(X4) and exhibiting bacterial host diversity. Slaughterhouses had more types of bacteria than pig farms, and more people worked in a slaughterhouse than on farms, which causes a greater risk of drug-resistant bacteria for the workers in the slaughterhouse. There have been several clinical cases of different species of bacteria carrying tet(X4), such as K. pneumoniae and C. freundii (14, 16, 33). And M. morganii was first observed to carry tigecycline resistance gene tet(X4) in this study. The expansion of the host range of tet(X4) has promoted people to pay more attention to the potential threat of tigecycline resistance to human health. Besides, all tet(X4)/tet(X6)-positive strains exhibited high resistance to tigecycline (16 mg/L to 256 mg/L) and MDR. In a previous study, tet(X4) existed in animals, food, and the environments and spread horizontally among them, which posed a major threat to public health security (32, 34–37).

Furthermore, research has indicated that tet(X4) was observed to coexist with other antibiotic resistance genes (ARGs), such as cfr, blq(Dmp-1), and mcr-1 (19, 38, 39). Furthermore, all tet(X4)-positive strains of this study coexisted with floR, of which most were still with aadA, qnrS, dfrA, qacE, lnuf(F), sul, and tet(A), indicating that these ARGs may cotransfer with tet(X4). This cotransfer possibility poses a major challenge to the clinical use of antibiotics in an era where we rely primarily on antibiotics to treat diseases. The phylogenetic tree of 533 tet(X4)-
positive *E. coli* strains exhibited that these strains arise from 8 sources and belong to 135 STs worldwide. The SNP differences among 533 *tet* (X4)-positive strains indicated that the *tet* (X4)-positive *E. coli* could clonally spread (SNPs, <5) and horizontally transfer among animals, humans, and the environment, which had similarities with previous studies (40). Thus, it is necessary to observe the transmission of tigecycline resistance genes. In addition, except for 35 tigecycline resistance strains carrying *tet* (X4) or *tet* (X6), the remaining 66 strains carrying tetracycline resistance genes *tet* (A), *tet* (J) and *tet* (M) were also resistant to tigecycline and 80.3% of these strains were *Proteus*. It has been found that *K. pneumoniae* and *E. hormaechei* carrying the *tet* (A) variant are resistant to tigecycline due to double frameshift mutation of the *tet* (A) gene (41, 42). And the *tet* (M) variant can make *Streptococcus suis*...
resistant to tigecycline (43). Previous studies have reported that *P. mirabilis* is inherently resistant to tetracycline antibiotics and that *P. mirabilis* strains carrying tigecycline resistance gene cluster *tmexCD3-toprJ3* have been detected from slaughterhouses (44–46).

Some studies have found that ISCR2, IS1, IS26, and a variety of conjugative and mobilizable plasmids of different incompatibility groups play an essential role in the acquisition of tet(X) genes from natural reservoirs and further dissemination among different bacterial pathogens (18, 47). In this study, we suggested that ISCR2 was the main insertion sequence to mediate the transmission of tet(X4) among different plasmids. Plasmids, as important mobile genetic elements, play a crucial role in carrying multiple functional genes and transferring them across bacteria through conjugation (48). IncQ1 and IncX1 are reportedly the most widely distributed plasmids carrying the tet(X4) gene. Particularly, small IncQ1 plasmids can be transferred at a high frequency through conjugation.

![Diagram](image-url)
(10^{-2} \text{ to } 10^{-5}) \text{ in the presence of autobiographic helper plasmids (IncX4, IncI2, and IncFII). Additionally, only a few plasmids containing tet(X4) in E. coli isolates were single replicons, whereas most plasmids were multiple replicons, which may be derived through recombination among different plasmids, such as IncX1-IncN, IncX1-IncR, IncX1-IncFIA/B-IncY, IncX1-IncFIA/B-IncH1A/B, and IncFIA/B-IncH1A/B (3, 28, 32, 49). In this study, a structural analysis was carried out in 21 nonclonal strains after Nanopore sequencing. The results demonstrated that the IncX1 plasmid was the most prevalent plasmid. Interestingly, the length of IncX1 plasmid ranged from 31,271 bp to 73,084 bp, and it was discovered from different farms, slaughterhouses, and bacteria, suggesting a wide range of transmission. The similarity between pJZ18-tet(X4) and pYY76-1-2 from a sample of cattle in China was more than 99.9%, suggesting similarity in their origin. The 7 IncX1 types of plasmids had more than 99% similarity, suggesting that the plasmids mediate the horizontal transmission of tet(X4) in different bacteria. Studies have reported that IncX1 plasmids and their hybrid plasmids can cause epidemics in E. coli strains from pig farms in China and that tet(X4)-carrying IncX1 plasmids can spread among bacteria in humans and animals (28, 50). Furthermore, we elaborated on several other types of plasmids and are the first to report that tet(X4) was located on the heterozygous plasmid containing IncX3 in livestock E. coli and the IncA/C plasmid in livestock Proteus. These findings not only expand our understanding of the genetic environments of tet(X4) but also enable bacteria to better adapt and cope with different and complex environments when faced with selection pressure. In addition, since the discovery of tet(X6) in P. tereae subsp. cibarius in 2020, many reports have found that tet(X6) can coexist with the novel tigecycline resistance gene cluster tnfxB3-tnfxCD3-topxJb and that cfr and tet(X6) can be transferred by ICE, which has many similarities with the results of this study (51–53). Finally, 68.42% of plasmids carrying tet(X4) and 100% of ICEs carrying tet(X6) in this study were transferred by conjugation experiments, indicating that plasmid-mediated and ICE-mediated horizontal transfer may occur in different pig farms and slaughterhouses since, of 10 pig farms, 2 had breeding pigs and 8 had fat pigs and parts of fat pigs were sent to the slaughterhouse.

In summary, in this study, we conducted in-depth research on the prevalence and genetic environments of the tet(X4) resistance gene in the pig slaughterhouse and farms in Sichuan Province and analyzed a total of 509 tet(X4)-positive E. coli strains observed in the 27,802 genomes of E. coli assembled from NCBI during 2002 to 2022 worldwide, providing a better understanding of the epidemiology and diversity of mobile genetic elements carrying tet(X4). We observed that the proportion of tet(X4)-positive strains was extremely high, and tet(X4) generally coexisted with other ARGs in different species of bacteria. Importantly, this report is the first one to demonstrate that tet(X4) was observed in M. morganii and that tet(X4) coexisted with blacmaa1 in a strain of K. quasipneumoniae. Furthermore, the IncX1 plasmid was the most prevalent plasmid carrying the tet(X4) gene, and the insertion sequence ISCR2 plays a significant role in transferring tet(X4) and in plasmid fusion. It also indicated that tet(X4) was located on an IncA/C plasmid in livestock Proteus and a heterozygous plasmid containing IncX3 in livestock E. coli. We performed an evolutionary phylogenetic analysis of 533 tet(X4)-positive E. coli strains worldwide and observed that these strains arise from 8 sources and belong to 135 STs. The results of this study expanded the host range and diversity of the plasmid of tet(X4), which may pose a serious threat to public health, and hence, more attention should be paid to monitoring tigecycline resistance and developing effective control measures. Additionally, we also should be concerned that tetracycline resistance may drive tigecycline resistance, and perhaps we need to reduce tetracycline use in pig farms.

**MATERIALS AND METHODS**

**Sample collection and bacterial isolates.** In total, 300 fresh fecal samples were collected from 10 pig farms (30 for each farm), and 53 cecal samples were collected from a large-scale pig slaughterhouse in Sichuan Province, China, in 2021. One gram of fresh samples was cultured in 5 mL brain heart infusion (BHI) broth containing tigecycline (4 mg/L) and incubated at 37°C for 10 h in a shaking incubator at 180 rpm, to obtain tigecycline resistance cultures. Cultures were lined with the inoculating ring onto eosin methylene blue (EMB) agar plates containing tigecycline (4 mg/L) to obtain the tigecycline resistance Gram-negative bacteria using the three area marking method. Monoclines of different forms were selected for each plate, and the single colonies were stored in BHI broth with 25% glycerinum at −80°C.
tet(X) resistance genes were determined by PCR and Sanger sequencing (3), and the tet(X)-positive isolates were further subjected to 16S rDNA sequencing for species identification.

**Antimicrobial susceptibility testing (AST).** Susceptibility to 17 antimicrobial agents, including gentamicin (GEN), florfenicol (FFC), polymyxin B (PMB), ciprofloxacin (CIP), trimethoprim-sulfamethoxazole (SXT), meropenem (MEM), ceftodoxin (FOX), fosfomycin (FOS), aztreonam (ATM), doxycycline (DOX), norfloxacin (NOR), tetracycline, tigecycline, cefazidime (CAZ), amoxicillin (AMC), ceftriaxone sodium (CRO), and amikacin (AMK), was determined using the Kirby-Bauer disk diffusion method. E. coli ATCC 25922 was used as the quality-control strain. All results were interpreted per the guidelines of the Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing. The MICs of tigecycline for the strains carrying tet(X) were determined using the broth dilution method (MIC breakpoints of tigecycline from the European Committee on Antimicrobial Susceptibility Testing are as follows: susceptible (S), ≤1 mg/L; intermediate (I), 1 mg/L < I ≤ 4 mg/L; and resistant (R), >4 mg/L).

**Genome sequencing and bioinformatic analysis.** Total DNA of 101 tigecycline resistance strains was extracted using a TiAnamp bacterial DNA kit (Tiangen, China) and was quantified by a NanoDrop 2000 instrument. Genomic DNA was sequenced using the Illumina NovaSeq 6000 platform with paired-end sequencing of 150 bp and assembled using SPAdes version 3.15.3 (54). We downloaded 27,802 E. coli genomes from the RefSeq database of NCBI and used the local blast program to build a library of these genome sequences. We used the tet(X4) gene as the query sequence to screen tet(X4)-positive E. coli. Antimicrobial resistance genes and multilocus sequence type (MLST) were determined using online tools (http://www.genomicepidemiology.org/). Prokka was used to annotate these genomes (55). The phylogenetic trees of positive strains of tigecycline resistance genes were constructed using Roary and FastTree based on single nucleotide polymorphisms (SNPs) of core genomes (56, 57). The resultant phylogeny was visualized and modified using iTol (https://itol.embl.de). A threshold of 5 SNPs among isolates is considered clonally related and likely to have an epidemiological link (58). MLST allelic profiles of E. coli were conducted using GrapeTree (59).

Nonclonal and representative strains (bacteria with large differences in drug resistance phenotype, drug resistance gene, and MIs) were selected for long-read Nanopore sequencing per the results of AST, phylogenetic analysis, and illumina sequencing. The rapid barcoding kit RBB004 was used to construct DNA libraries, which were further sequenced in a MinION sequencer with a Flow-MIN106 flow cell. The genome sequences were completed with a hybrid de novo assembly strategy combining illumina short-read and Nanopore MinION long-read data using Unicycler version 0.4.8 software (60). Plasmid replicons were determined using online tools (https://cge.cbs.dtu.dk/services/). Insertion sequences were discovered using ITagzer (https://www.is.biotoul.fr/index.php). The integrative and conjugative elements (ICEs) were detected by BLASTn analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The complete genome sequences were annotated automatically using RAST (http://rast.nmpdr.org/) and were modified manually. BRIG and Easyfig were used to display plasmid comparison maps (61, 62).

**Conjugation experiments.** A conjugation assay was conducted using the filter mating method, using rifampicin-resistant E. coli EG600 as the recipient, to examine the transferability of tet(X)-positive strains. The donor and recipient were mixed at a ratio of 1:4 in BHI, were cultured until the logarithmic growth period, and were further applied to sterilized 0.22-µm filters in Luria-Bertani agar plates, which were incubated at 37°C overnight. Transconjugants were selected on EMB agar plates containing 4 mg/L tigecycline and 400 mg/L rifampicin and further confirmed with PCR. Transfer frequencies were calculated as the ratio of the number of transconjugants to the total number of recipients.

**Data availability.** The sequences obtained in this paper have been deposited in the GenBank database under BioProject number PRJNA27787. Genome accession and accession numbers can be seen in Table 1 and 2.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.01 MB.

**SUPPLEMENTAL FILE 2**, XLSX file, 0.04 MB.

**SUPPLEMENTAL FILE 3**, XLSX file, 0.8 MB.

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