Tigecycline-resistant \textit{Escherichia coli} ST761 carrying \textit{tet}(X4) in a pig farm, China

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This study aimed to investigate the prevalence and characterization of \textit{tet}(X4) in \textit{Escherichia coli} isolates from a pig farm in Shanghai, China, and to elucidate \textit{tet}(X4) dissemination mechanism in this swine farm. Forty-nine (80.33\%) \textit{E. coli} strains were isolated from 61 samples from a pig farm and were screened for the presence of \textit{tet}(X). Among them, six (12.24\%) strains were positive for \textit{tet}(X4) and exhibited resistance to tigecycline (MIC $\geq$ 16 mg/L). They were further sequenced by Illumina Hiseq. Six \textit{tet}(X4)-positive strains belonged to ST761 with identical resistance genes, resistance profiles, plasmid replicons, and cgMLST type except that additional ColE10 plasmid was present in isolate SH21PTE35. Isolate SH21PTE31, as a representative ST761 \textit{E. coli} strain, was further sequenced using Nanopore MinION. The \textit{tet}(X4) in SH21PTE31 was located on IncFIA18/IncFIB(K)/IncX1 hybrid plasmid pHUSHP31-1, highly similar to other \textit{tet}(X4)-carrying IncFIA18/IncFIB(K)/IncX1 plasmids from ST761 \textit{E. coli} and other \textit{E. coli} lineages in China. These IncFIA18/IncFIB(K)/IncX1 plasmids shared closely related multidrug resistance regions, and could reorganize, acquire or lose resistance modules mediated by mobile elements such as IS\textit{CR2} and IS\textit{26}. Phylogenetic analysis were performed including all \textit{tet}(X4)-positive isolates obtained in this pig farm combined with 43 \textit{tet}(X4)-positive \textit{E. coli} from pigs, cow, pork, wastewater, and patients with the same ST from NCBI. The 50 \textit{tet}(X4)-carrying \textit{E. coli} ST761 isolates from different areas in China shared a close phylogenetic relationship (0-49 SNPs). In conclusion, clonal transmission of \textit{tet}(X4)-positive \textit{E. coli} ST761 has occurred in this swine farm. \textit{E. coli} ST761 has the potential to become a high-risk clone for \textit{tet}(X4) dissemination in China.

\textbf{KEYWORDS}

plasmids, ST761, tigecycline resistance, \textit{tet}(X4), \textit{Escherichia coli}
Introduction

Tigecycline is considered as a last-resort antimicrobial agent to treat serious infections caused by multidrug-resistant bacteria, particularly carbapenem-resistant Enterobacteriaceae (Yaghoubi et al., 2021). However, the recent identification of novel plasmid-borne tigecycline resistance genes tet(X) in Acinetobacter baumannii and tet(X4) in Escherichia coli from animals in China significantly impairs the clinical efficacy of tigecycline (He et al., 2019). Thus far, tet(X) and its variants [tet(X1)–tet(X7)] have been identified in Gram-negative pathogens and encode flavin-dependent monooxygenase that modify tigecycline (Aminov, 2021; Li R. et al., 2021; Umar et al., 2021; Zhang et al., 2021). Among them, the mobile tet(X4) gene has been increasingly identified in E. coli from various sources including food-producing animals, wild birds, food products, humans, and the environment, mainly in China (He et al., 2019; Fang et al., 2020; Li et al., 2020; Li Y. et al., 2021; Dong et al., 2022; Liu et al., 2022). It has sporadically reported in countries outside of China, e.g., Singapore, Pakistan, Vietnam, United Kingdom, and Norway (Ding et al., 2020; Marathe et al., 2021; Mohsin et al., 2021; Dao et al., 2022; Martelli et al., 2022). The tet(X4) has subsequently detected in various Enterobacteriaceae species, such as Proteus, A. baumannii, Aeromonas caviae, Citrobacter freundii, Enterobacter cloacae, E. hormaechei, Klebsiella pneumoniae, and Shewanella xiamenensis (Chen et al., 2019; He et al., 2019; Zeng et al., 2021; Dao et al., 2022; Li et al., 2022; Wu et al., 2022; Zhai et al., 2022).

Although tigecycline is not applied in livestock, the tet(X4) gene and tigecycline resistance are frequently described in E. coli from food-producing animals (mainly pigs) in China (He et al., 2019; Fang et al., 2020; Li Y. et al., 2021; Liu et al., 2022). The heavy use of tetracyclines in animal production might facilitate the emergence and spread of tet(X) in livestock (He et al., 2019). In addition, conjugative/mobilizable plasmids and mobile elements play an essential role in the dissemination of tet(X4) in Enterobacteriaceae (Aminov, 2021). In this study, we aimed to investigate the prevalence and characterization of tet(X4) in E. coli isolates from one pig farm in Shanghai, China, to provide insights into the spread of tet(X4) in this swine farm.

Materials and methods

Sample collection and tet(X) detection

On 15 July 2021, 61 non-duplicate samples from pig feces (n = 41) and pig feed (n = 20) were collected from a pig farm in Shanghai, China. Samples were incubated in LB broth for 18~24 h and then cultured on the MacConkey agar with and without 2 mg/L tigecycline. One E. coli isolate per plate was selected and identified by 16S rRNA gene sequencing (Kim et al., 2010). The presence of tet(X) were detected by PCR and sequencing (Wang et al., 2019).

Antimicrobial susceptibility testing

The MICs of tigecycline were determined in all E. coli strains using the broth microdilution method and interpreted according to EUCAST clinical breakpoint (MIC ≥ 1 mg/L). The tet(X4)-positive isolates were further tested susceptibility to other 13 antimicrobial agents including ampicillin, cefotaxime, meropenem, gentamicin, amikacin, streptomycin, tetracycline, chloramphenicol, florfenicol, nalidixic acid, ciprofloxacin, colistin, and sulfamethoxazole/trimethoprim by using the broth microdilution method. The results were interpreted according to Clinical Laboratory Standards Institute (CLSI) M100, 30th edition. Florfenicol (> 16 mg/L) and streptomycin (> 16 mg/L) were interpreted according to the epidemiological cut-off values for E. coli set by EUCAST (see Text Footnote 1). The E. coli strain ATCC 25922 was used for quality control.

Conjugation experiments

Conjugation experiments were conducted according to a previously described protocol (Chen et al., 2007) using E. coli C600 (streptomycin-resistant) as the recipient strain. Transconjugants were selected on MacConkey agar plates supplemented with 2 mg/L tigecycline and 3,000 mg/L streptomycin.

Whole genome sequencing and analysis

The tet(X4)-positive E. coli strains were sequenced on the Illumina Hiseq platform, and the quality-trimmed raw sequence data were assembled into contigs using SPAdes v.3.8.2 with -careful and -cov cut-off auto options. One representative E. coli isolate SH21PTE31 was sequenced using Nanopore MinION, assembling with Unicycler version 0.4.9. The genome sequences of them were analyzed multilocus sequence typing (MLST), resistance genes, and plasmid replicons by using the Center for Genomic Epidemiology (CGE) pipeline. The tet(X4)-carrying plasmid pYUSHP31-1 in strain SH21PTE31 was analyzed by ISfinder, BLAST and the Gene Construction Kit 4.5 (Textco BioSoftware, Inc., Raleigh, NC, United States).

References

1 www.eucast.org
2 http://www.genomicepidemiology.org/
3 https://www-is.biotoul.fr/
4 https://blast.ncbi.nlm.nih.gov/Blast.cgi
pYUSHP31-1 was compared with other similar plasmids using \textsc{BLASTn} and \textsc{BRIG}.

**Phylogenetic analysis of \textit{tet}(X4)-Positive \textit{ST761 Escherichia coli} strains**

The genome sequences of 43 \textit{tet}(X4)-positive \textit{ST761 E. coli} strains in the NCBI database were downloaded (data collected on July 7th, 2022) (Supplementary Table 1). The phylogenetic tree of all the \textit{tet}(X4)-carrying \textit{ST761 E. coli} strains obtained from this pig farm and NCBI was constructed using \textsc{Par}sn\textsc{p}\textsuperscript{5} and visualized by \textsc{iTOL}\textsuperscript{6}. Core genome MLST (cgMLST) profiles based on 2,513 alleles were analyzed using cgMLSTFinder\textsuperscript{7}.

**Nucleotide sequence accession number**

The whole genome sequences of \textit{tet}(X4)-positive \textit{E. coli} isolates have been deposited in the GenBank under accession number PRJNA836295.

**Results and discussion**

**Characterization of \textit{tet}(X4)-positive \textit{Escherichia coli} isolates**

A total of 49 \textit{E. coli} strains were obtained from 61 samples. Among them, six strains (12.24\%) from different fecal samples were positive for \textit{tet}(X4), including five strains isolated under selection with tigecycline and one strain isolate without selection. The \textit{tet}(X4)-positive isolates exhibited resistance to tigecycline (MIC $\geq$ 16 mg/L), and the remaining isolates showed susceptibility to tigecycline with MICs of 0.125 to 0.5 mg/L. These \textit{tet}(X4)-positive isolates were also resistant to ampicillin, tetracycline, chloramphenicol, florfenicol, and sulfamethoxazole/trimethoprim, but susceptible to cefotaxime, meropenem, gentamicin, amikacin, streptomycin, colistin, nalidixic acid, and ciprofloxacin (Supplementary Table 2). However, all tigecycline-resistant isolates failed to transfer \textit{tet}(X4) to \textit{E. coli} C600 via conjugation.

The draft genome sequences of six \textit{tet}(X4)-positive \textit{E. coli} strains were obtained by Illumina (Supplementary Table 3). All six \textit{tet}(X4)-positive \textit{E. coli} strains belonged to \textit{ST761} with identical resistance genes \{\textit{bla}TEM\textsubscript{−1}, \textit{tet}(A), \textit{tet}(M), \textit{floR}, \textit{qnrS}1, \textit{su}3, \textit{dfr}A5 and \textit{mef}(B)\} and plasmid replicons [\textit{Inc}FIA, \textit{Inc}FIB(K), \textit{Inc}X1, \textit{Inc}R], except that additional \textit{Col}E10 plasmid was present in isolate SH21PTE35 (Figure 1).

**\textit{tet}(X4)-Carrying plasmid pYUSHP31-1**

The complete sequences of isolate SH21PTE31, as a representative \textit{ST761 E. coli} strain, was obtained. A total of 43,674 reads were obtained, and the sequencing data volume was approximately 1,000 Mbp. The minimal, maximum and average read lengths were 8,260 bp, 150,801 bp and 22,897.3 bp, respectively. The read length N50 of the total sequencing data were 28,637 bp. The isolate SH21PTE31 consisted of one chromosome (4,706,168 bp) and four plasmids (Supplementary Table 3). Among them, \textit{tet}(X4) and another eight resistance genes were co-located on the largest plasmid, designated as pYUSHP31-1. This plasmid had a size of 104,163 bp, and belonged to the hybrid \textit{Inc}FIA18/\textit{Inc}FIB(K)/\textit{Inc}X1 plasmid. It was highly similar to our previously reported plasmid pYUSHP6-tetX (GenBank accession no. MW423609) from \textit{ST761 E. coli} isolate SH19PTE6 collected from the same pig farm in 2019 (Wang et al., 2021), and also showed high identity (99.7\%) to multiple \textit{tet}(X4)-carrying \textit{Inc}FIA18/\textit{Inc}FIB(K)/\textit{Inc}X1 plasmids from \textit{ST761 E. coli} strains in China, such as pNT1W22-tetX (pig, CP075470), pRF108-2\_97k\_tetX (pig, MT219820), pSTB20-1T (pig, CP050174), pS4-tetX (cow, CP041286), pYPE12-101k-tetX4 (pork, CP041443), and pYPE3-92k-tetX4 (pork, CP041453) (Figure 2).

Similar \textit{Inc}FIA18/\textit{Inc}FIB(K)/\textit{Inc}X1 plasmids harboring \textit{tet}(X4) were also present among other \textit{E. coli} lineages obtained from a pig farm in Jiangsu province, China (Li et al., 2021), e.g., pNT1N31-tetX4 (ST716, CP075481), pNT1F25-tetX4 (ST1421, CP075471), pNT1F31-tetX4 (ST206, CP045188), pNT1N25-tetX4 (ST641, CP075485), and pNT1F34-tetX (ST10115, CP075486) (Figure 2), highlighting the importance role of horizontal transfer of plasmids in the \textit{tet}(X4) dissemination between different bacteria.

As shown in Figure 3, these \textit{Inc}FIA18/\textit{Inc}FIB(K)/\textit{Inc}X1 plasmids shared closely related multidrug resistance regions (MRRs). The MRRs in all were bounded by one copy of \textit{Is26} and \textit{Isj}, respectively. The \textsc{YUSHP}31-1 MRR (53,134 bp) contained nine resistance genes and consisted of five regions bounded by \textsc{Is26}, respectively. The pYUSHP31-1 MRR (53,134 bp) contained nine resistance genes and consisted of five regions bounded by \textsc{Is26}. This fragment was also present in other \textit{Inc}FIA18/\textit{Inc}FIB(K)/\textit{Inc}X1 plasmids, but differed by 46-bp shorter (limited to pNT1N25-tetX4) or 126-bp longer \textit{Tn}2 except pYUSHP6-tetX (identical to pYUSHP31-1, obtained

\footnotesize{\textsuperscript{5} https://harvest.readthedocs.io/en/latest/content/parsnp.html \textsuperscript{6} https://itol.embl.de/ \textsuperscript{7} https://cge.food.dtu.dk/services/cgMLSTFinder/}
FIGURE 1

The maximum likelihood tree of tet(X4)-positive E. coli ST761 isolates in this study compared with tet(X4)-positive E. coli ST761 isolates from NCBI based on cgSNP analysis. Antibiotic resistance genes and plasmid replicons with > 95% sequence homology and > 60% coverage are shown. The isolates obtained in this study and in the same pig farm were indicated in blue.

The third region corresponded to the core tet(X4) structure [ΔIC2R2-orf1-abh-tet(X4)-IC132R2] and downstream floR-ΔIC2R2 module, as observed in other IncFIA18/IncFIB(K)/IncX1 plasmids with one to four copies of tet(X4) structure (Figures 3A–J). Compared with that of pYUSHP31-1, partial tet(X4) structure [ΔIC2R2-orf1-abh-tet(X4)-IC2R2] with varied copies was identified in plasmids pNT1F10-tetX4, pRF148-2_101k_tetX, pSTB20-1T, pRF148-2_97k_tetX, and pNT1N25-tetX4 (Figures 3K–N); one copy of IC132 was inserted into orf1 within the tet(X4) structure with 9-bp direct repeats in plasmids pNT1F10-tetX4, pRF148-2_97k_tetX, and pNT1N25-tetX4 (Figures 3K–N); one copy of IS1 was inserted into orf1 within the tet(X4) structure with 9-bp direct repeats in plasmids pNT1F10-tetX4 and pNT1N25-tetX4, and the latter plasmid carried the tet(X4) fragment in the opposite orientation and additional two copies of IS26 upstream of floR-ΔIC2R2 module (Figure 3I). As previously described (Liu et al., 2022), IC2R2 is associated with tet(X4) transmission by forming a rolling-cycle transposable unit, thus generating tandem copies of tet(X4)-harboring structures in different IncFIA18/IncFIB(K)/IncX1 plasmids.

The fourth segment (~18.4 kb) included one copy of IS26, an incomplete Tn1721 carrying tetracycline resistance gene tet(A) and an intact Tn2 (tnpA-tnpR-blaTEM–1β), followed by 5,391-bp module [ΔI1H–IS440 tnpA–tet(M)–ΔIS26] and qnrS1 structure (IS26-qnrS1–ΔISKpn19). This region was also found in other IncFIA18/IncFIB(K)/IncX1 plasmids with the same ΔISKpn19/IS26 boundary except pYPE3-92k-tetX (Figure 3F). IS26-mediated homologous recombination could explain the loss or acquisition of this region.

The last segment comprising a 3,507-bp structure (IS26-ΔIS1294-gshB-IS1) was identical to segments in other plasmids except p54-tetX (Figure 3D). Insertion of an extra copy of IS26 downstream of gshB, followed by homologous recombination between it and the first IS26 of MRR, may explain the opposite location of an approximately 50.2-kb fragment within MRR in p54-tetX compared to pYUSHP31-1. Similar recombination between two IS26 elements located in inverse orientations may also occur in pYPE12-101k-tetX4, leading to the presence of ~47.8 kb fragment with the opposite orientation within MRR (Figure 3C).
These tet(X4)-carrying IncFIA18/IncFIB(K)/IncX1 plasmids may evolve from the same ancestor, and form variable but related MRRs by insertions, deletions, or rearrangements of different resistance modules mediated by mobile elements such as IS26 and ISCR2.

**Phylogenomic analysis of tet(X4)-Positive ST761 Escherichia coli strains**

*Escherichia coli* ST761 has been increasingly reported in different sources associated with tet(X4) in China, particularly from pigs (Supplementary Table 1). To further compare the genetic differences between tet(X4)-positive *E. coli* isolates of the same ST, we performed a phylogenomic analysis based on cgSNP. The results revealed a relatively close genetic relationship (0-49 SNPs) among 50 tet(X4)-positive ST761 *E. coli* isolates (Figure 1). Among them, cgST 137253 (n = 44) was the most prevalent type, and it contained two isolates from patients, two from wastewater, three from pork, and 37 from pigs including six strains obtained in this study and SH19PTE6 from the same pig farm (Figure 1). It indicates that clonal transmission has occurred in this swine farm. The plasmid replicons [IncFIA, IncFIB(K), IncX1] possibly associated with tet(X4) were present in all isolates, and the core resistance genes [blaTEM−1, tet(A), tet(M), floR, qnrS1, sul3, afdA5 and mef(B)] within tet(X4)-carrying IncFIA/IncFIB(K)/IncX1 plasmid pYUSHP31-1 were shared by 45 strains (Figure 1).

Although horizontal transfer mediated by plasmids (e.g., IncQ, and IncX1) and insertion sequences (e.g., ISCR2, IS26, and IS1) is the main mechanism for tet(X4) transmission.
FIGURE 3
Genetic organization of the multidrug resistance region of plasmid pYUSHP31-1 and structural comparison with other IncFIA18/IncFIB(K)/IncX1 plasmids. I to IV indicate five regions bounded by IS26 or ISCR2 in pYUSHP31-1. The extents and directions of orientation of resistance genes (thick red arrow) and other genes are indicated by arrows. Regions with >99% identity are shaded in gray. 1 indicates a truncated gene or mobile element. Insertion sequences (ISs) are shown as boxes labeled with the IS name. Labeled vertical arrows with IS boxes denote the insertion position of IS elements. Direct repeats are indicated by arrows and sequences. Tall bars represent the 38-bp IR of transposons (Tn). Arrows labeled with “HR” and dotted lines indicate where homologous recombination could explain differences between structures.

(Aminov, 2021; Liu et al., 2022; Yu et al., 2022), clonal spread of tet(X4)-carrying strains, such as E. coli ST877, ST10, and ST48 clones is also responsible for tet(X4) dissemination between animals and humans (Cui et al., 2022). The E. hormaechei co-harboring tet(X4) and blaNDM could also clonally spread from the slaughterhouse to the retail market (Li et al., 2022). E. coli ST761 isolates carrying tet(X4) has been detected in pigs, cow, pork, wastewater, and patients in different areas from China sharing a close phylogenetic relationship, suggesting that the ST761 lineage has the potential to be
a successful clone to transfer tet(X4) and other resistance genes as well as in China.

**Conclusion**

Our findings suggest that tet(X4)-positive ST761 E. coli was the main reason for spread and persistence of tet(X4) in this pig farm. Importantly, E. coli ST761 has the potential to become a high-risk clone for tet(X4) dissemination in China. On the other hand, the tet(X4)-carrying IncFIA18/IncFIB(K)/IncX1 hybrid plasmids within ST761 E. coli lineage could reorganize, acquire or lose resistance modules mediated by mobile elements such as ISCR2 and IS26. The horizontal transfer of similar IncFIA18/IncFIB(K)/IncX1 plasmids further facilitates the tet(X4) dissemination in distinct lineages.

**Data availability statement**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

**Author contributions**

XJ and JW conceived the study. M.-JL, HW, Z.-YW, and YJ carried out the experiments. JW, Z.-YW, and YJ analyzed the data. JW wrote the manuscript. Z.-MP and XJ revised the manuscript. All authors read and approved the final manuscript.

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**Supplementary material**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.967313/full#supplementary-material
