Plasmid-encoded tet(X) genes that confer high-level tigecycline resistance in Escherichia coli

Jian Sun1,2,11, Chong Chen1,2,11, Chao-Yue Cui1,2, Yan Zhang1,2, Xiao Liu1,2, Ze-Hua Cui1,2, Xiao-Yu Ma1,2, Youjun Feng1,2, Liang-Xing Fang1,2, Xin-Lei Lian1,2, Rong-Min Zhang1,2, You-Zhi Tang1,2,3, Kou-Xing Zhang4, Han-Mian Liu5, Zhi-Hui Zhuang4, Shi-Dan Zhou5, Jing-Nan Lv6, Hong Du7, Bin Huang7, Fang-You Yu8, Barun Mathema9, Barry N. Kreiswirth10, Xiao-Ping Liao1,2,2*, Liang Chen10*, and Ya-Hong Liu1,2,3*

Tigecycline is one of the last-resort antibiotics to treat complicated infections caused by both multidrug-resistant Gram-negative and Gram-positive bacteria. Tigecycline resistance has sporadically occurred in recent years, primarily due to chromosome-encoding mechanisms, such as overexpression of efflux pumps and ribosome protection. Here, we report the emergence of the plasmid-mediated mobile tigecycline resistance mechanism Tet(X4) in Escherichia coli isolates from China, which is capable of degrading all tetracyclines, including tigecycline and the US FDA newly approved eravacycline. The Tet(X4)-harbouring IncQ1 plasmid is highly transferable, and can be successfully mobilized and stabilized in recipient clinical and laboratory strains of Enterobacteriaceae bacteria. It is noteworthy that tet(X4)-positive E. coli strains, including isolates co-harbouring mcr-1, have been widely detected in pigs, chickens, soil and dust samples in China. In vivo murine models demonstrated that the presence of Tet(X4) led to tigecycline treatment failure. Consequently, the emergence of plasmid-mediated Tet(X4) challenges the clinical efficacy of the entire family of tetracycline antibiotics. Importantly, our study raises concern that the plasmid-mediated tigecycline resistance may further spread into various ecological niches and into clinical high-risk pathogens. Collective efforts are in urgent need to preserve the potency of these essential antibiotics.

The emergence and spread of antimicrobial resistance in Enterobacteriaceae bacteria pose a serious threat to human and animal health. Of special concern is the emerging resistance to carbapenems, as these agents are often regarded as the last line of effective therapy for the treatment of infections caused by multidrug-resistant (MDR) Gram-negative bacteria. Antibiotic treatment options for carbapenem-resistant Enterobacteriaceae (CRE) bacteria are becoming limited, and colistin and tigecycline have been regarded as the final armamentarium. However, the recent discovery of a plasmid-mediated colistin resistance gene, mcr-1, threatens the clinical utility of colistin to treat these infections, leaving tigecycline as one of the last options.

Tigecycline resistance has inevitably emerged over the recent years, mostly identified among extensively drug-resistant and carbapenem-resistant isolates. Decreased susceptibility to tigecycline is commonly associated with overexpression of efflux pumps of the resistance-nodulation-cell division protein family, such as AcrAB-ToLC, OqxAB and AdeABC. Meanwhile, mutations in plsC, rpsM, trm, tet(A) and tet(M) have also been found to decrease tigecycline susceptibility. By contrast, tetracycline destructases, such as Tet(X), represent a unique enzymatic tetracycline inactivation mechanism. Tet(X), the flagship tetracycline-inactivating enzyme, has been confirmed for in vitro activity in degrading all tetracyclines, including tigecycline. Since being first described in the obligate anaerobe Bacteroides fragilis, tet(X) and its orthologous genes (namely, tet(X1) and tet(X2)) have now been detected in clinical Enterobacteriaceae and Acinetobacter baumannii isolates, but their distribution, genetic structure and clinical significance remain to be explored. In this study, we describe a plasmid-mediated mobile tigecycline resistance gene, tet(X4), and explore the prevalence of tet(X4)-positive Escherichia coli strains in humans, food-producing animals and the surrounding environment in China (Fig. 1).

Initially, a tigecycline-resistant E. coli strain LHM10-1 (8 mg l−1; Supplementary Table 1) was isolated from the stool sample in an intensive pig farm (Jiangxi, China) in July 2017. The tigecycline resistance could be successfully transferred into the recipient E. coli C600 (8 mg l−1; Supplementary Table 1), suggesting that the gene encoding tigecycline resistance might be plasmid-borne. Whole-genome sequencing of E. coli LHM10-1 showed that it belonged to sequence type (ST) 515, and contained one 4.81-Mb chromosome and six plasmids with sizes of 108,366 bp (pLHM10-1-p1, IncF), 98,254 bp (pLHM10-1-p2, untypeable), 33,301 bp (pLHM10-1-p3, IncX4), 28,942 bp (pLHM10-1-p4, untypeable), 28,519 bp (pLHM10-1-p5, untypeable) and 12,783 bp (pLHM10-1-p6, IncQ1).

A tet(X)-like gene (designated as tet(X4)), with 1,158 bp in length, was found to be located on the IncQ1 plasmid pLHM10-1-p6. The tet(X4) gene encoded a 385 amino acid protein (Fig. 2a and Supplementary Fig. 1a), which showed 94.5% amino acid

1National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, South China Agricultural University, Guangzhou, China. 2College of Veterinary Medicine, South China Agricultural University, Guangzhou, China. 3Guangdong Provincial Key Laboratory of Veterinary Pharmacies Development and Safety Evaluation, South China Agricultural University, Guangzhou, China. 4Intensive Care Unit, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. 5Intensive Care Unit, Huizhou Municipal Central Hospital, Huizhou, China. 6Department of Clinical Laboratory, The Second Affiliated Hospital of Soochow University, Suzhou, China. 7Department of Laboratory Medicine, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. 8Department of Clinical Laboratory, Shanghai Pulmonary Hospital, School of Medicine, Tongji University, Shanghai, China. 9Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY, USA. 10Hackensack-Meridian Health Center for Discovery and Innovation, Nutley, NJ, USA. *These authors contributed equally: Jian Sun, Chong Chen. e-mail: xpliao@scau.edu.cn; Liang.Chen@hackensackmeridian.org; lyh@scau.edu.cn
sequence identity to Tet(X), initially found in the obligate anaerobe B. fragilis (GenBank accession number M37699). Meanwhile, Tet(X) displayed 63.9%, 95.1% and 85.7% identities to the other named Tet(X) orthologues Tet(X1) (AJ311171), Tet(X2) (AJ311171) and Tet(X3) (AB097942), respectively (Supplementary Fig. 1a).

Gene cloning experiment showed that the tet(X4) construct, namely, E. coli JM109 + pBAD24-tet(X4), demonstrated a 64-fold increase in the minimum inhibitory concentration (MIC) of tigecycline when compared with the host E. coli JM109 strain carrying pBAD24 (0.25 mg l⁻¹; Supplementary Table 1). Similarly, MICs of the first-generation and second-generation tetracyclines were increased at least 32-fold. In addition, the MIC of the US FDA newly approved eravacycline, which was structurally similar to tetracyclines by Tet(X4) (Fig. 2b). Furthermore, liquid chromatography–tandem mass spectrometry (LC-MS/MS) showed that the level of tetracycline remained (16.1 ± 1.2%) after incubation with E. coli JM109 + pBAD24-tet(X4) for 16h (verse (80.2 ± 1.2%) in the negative control) (Fig. 2c; P < 0.0001), and eravacycline remained only (7.8 ± 0.5%) after incubation for the same time (verse (79.0 ± 3.8%) in the negative control) (Fig. 2d; P < 0.0001). Our above results demonstrated that Tet(X4) was able to confer resistance to the entire family of tetracyclines.

In E. coli LHM101-1, the tet(X4)-harbouring plasmid pLHM101-1-p6 was 12,783 bp in size with an average G + C content of 55.4% and contained 15 predicted open reading frames (ORFs), of which tet(X4) was adjacent to two copies of ISCR2 (one is truncated; Fig. 3a). Plasmid replication analysis showed that pLHM101-1-p6 belonged to the broad host-range IncQ1 group, possessing the IncQ1 plasmid backbone genes for plasmid replication (repA, repB and repC) and mobilization (mobA, mobB and mobC). A comparison of several IncQ1 plasmids from the public National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) demonstrated that they shared similar replication and mobilization regions, but diverse acquired regions (between repC and mobC) (Fig. 3b). The results suggested that pLHM101-1-p6 originated by acquisition of tet(X4) into an IncQ1 plasmid, probably associated with the translocation of ISCR2.

Interestingly, a mcr-1-carrying IncX4 plasmid pLHM101-1-p3 (33,301 bp) was identified in the same E. coli LHM101-1, which shared >99.0% sequence identity and 100% query coverage to several other mcr-1-harbouring plasmids (for example, KX084392 and CP018773) from animal and human sources in China, the United States and some other countries. In addition, an extended-spectrum β-lactamase gene blaCTX-M-14 was identified on the chromosome of E. coli LHM101-1.

Further conjugation assays showed that tigecycline resistance could be successfully transferred from E. coli LHM101-1 to laboratory strains E. coli C600, Salmonella enterica serovar Typhimurium American Type Culture Collection (ATCC) 14028 and Klebsiella pneumoniae ATCC 700603, at a frequency of (1.2±0.5)×10⁻², (1.2±0.3)×10⁻⁴ and (6.2±1.3)×10⁻² cells per recipient cell, respectively (Fig. 3c). Notably, plLHM101-1-p6 also exhibited good transferability into clinical CRE strains, including NDM-5-producing ST167 E. coli 1314 and KPC-2-producing ST11 K. pneumoniae 1332, at a frequency of (2.2±0.5)×10⁻⁴ and (8.0±0.5)×10⁻⁴, respectively (Fig. 3c). MICs of tetracyclines, including tigecycline and eravacycline, among these transconjugants, were increased at least four-fold (Supplementary Table 1). After 22 d (approximately 220 generations) of serial passage without antibiotic treatment, plLHM101-1-p6 was still stably maintained (75–100% fraction of plasmid-containing lineages) in the parental E. coli LHM101-1 and transconjugants hosts, including the clinical CRE strains (Fig. 3d).

Given the high transferability and stability of tet(X4) in clinical CRE isolates harbouring blaKPC or blaTEM, further acquisition of carbapenem resistance genes by tet(X4)-harbouring strains, or alternatively, the spread of tet(X4)-carrying plasmids into clinical MDR strains, had the potential to create truly pan-drug-resistant strains, resulting in untreatable infections.

In this study, the tet(X4) gene was detected in a total of 42 E. coli strains (sharing 100% nucleotide sequence identity of tet(X4) with each other) from 4,189 samples, consisting of 30 (1.3%) from 2,337 pigs, 8 (0.8%) from 1,061 chickens, 2 (0.8%) from 256 soil samples and 2 (0.9%) from 232 dust samples (Supplementary Table 2). These tet(X4)-positive E. coli strains were found in five provinces in southern and eastern China (Fig. 1): Guangdong (n = 12), Fujian (n = 11), Jiangsu (n = 8), Jiangxi (n = 6) and Guangxi (n = 5). However, the tet(X4)-positive E. coli strains were not detected in others.

Although we have not identified tet(X4)-positive E. coli strains from human sources, a series of bacterial genomes carrying tet(X4)-like genes from human (for example, CP023968), animal (for example, CP002562), water (for example, CP023049) and soil (for example, LT906465) sources were identified in the NCBI (Supplementary Fig. 1b). Furthermore, the sequence analysis also indicated that the tet(X4)-like genes had spread outside China and into other countries, including the United States (for example, NZ_JYQQ01000042), the United Kingdom (for example, CP000685) and Thailand (for example, NZ_JRQZ01000088) (Supplementary Fig. 1b).

The susceptibility testing demonstrated that the 42 tet(X4)-positive E. coli strains were all resistant to tigecycline, tetracycline, sulmefamoxazole–trimethoprim and florfenicol, while 15 (35.7%), 12 (28.6%), 10 (23.8%), and 9 (21.4%) isolates showed resistance to cefotaxime, cefotaxime, cefotaxime and cefotaxime, respectively (Fig. 4). In addition, all isolates exhibited an eravacycline MIC of 4–8 mg l⁻¹, which is significantly higher than the MIC of 0.5 mg l⁻¹ for E. coli strains from the routine surveillance. Notably, the 12 cefotaxime-resistant isolates were all found to carry extended-spectrum β-lactamase genes, including blaCTX-M-14 (n = 7), blaCTX-M-48 (n = 4) and blaCTX-M-27 (n = 1). The nine colistin-resistant E. coli strains all carried the colistin resistance gene mcr-1 and the extended-spectrum β-lactamase gene blaCTX-M-14, or blaCTX-M-48, belonging to two STs (ST515 (n = 5) and ST1196 (n = 4)).
Multilocus sequence typing showed that the 42 tet(X4)-positive *E. coli* strains belonged to nine distinct STs, which were, in general, consistent with the pulsortypes (A–K) from the pulsed field gel electrophoresis (PFGE) analysis (Fig. 4). Strains from the same ST or PFGE pulsortype usually shared similar antimicrobial resistance profiles. Two STs (ST8302 and ST8338) were first identified in the current study, with ST8302 being the most predominant ST (n = 16; 38.1%) overall. Notably, the same STs of strains (ST8302, ST515, ST761 and ST10) in this study were identified in different geographical regions (Guangdong (n = 7), Jiangsu (n = 7), Jiangxi (n = 6) and Fujian (n = 7)), followed by dusts (n = 2) and soils (n = 1).

The detection of the IncQ1 pLHM10-1-p6-like plasmid revealed that 57.1% (24 out of 42) of the tet(X4)-positive *E. coli* isolates harboured the same pLHM10-1-p6-like plasmid (Fig. 4). These strains belonged to 4 distinct STs (ST8302 (n = 16), ST515 (n = 6), ST761 (n = 1) and ST871 (n = 1)) and were isolated from 4 different provinces (Guangdong (n = 7), Jiangsu (n = 7), Jiangxi (n = 6) and Fujian (n = 4)). Most of them were collected from pigs (n = 14) and chickens (n = 7), followed by dusts (n = 2) and soils (n = 1).

Notably, IncQ-type plasmids are a group of relatively small (approximately 5–15 kb), non-conjugative but mobilizable plasmids that can be transferred into a broad range of bacterial hosts in the presence of self-transmissible helper plasmids. Our mating experiment results showed that pLHM10-1-p6 could be successfully transferred in the presence of the mcr-1-carrying IncX4 plasmid pZ6-2 (transfer frequency: (5.4 ± 1.8) × 10^-7), the IncI2 plasmid pHJ179 (transfer frequency: (7.9 ± 1.5) × 10^-5) and the IncFIII plasmid pH12-4 (transfer frequency: (4.3 ± 1.0) × 10^-5) (Supplementary Table 3), but the transfer failed in the absence of helper plasmids (pLHM10-1-p6 alone). Co-transfer of the helper plasmids
**Fig. 3 | Characteristics of the IncQ1 tet(X4)-harbouring plasmid pLHM10-1-p6.** a, Structure of the index plasmid pLHM10-1-p6. GC skew and GC content are indicated from the inside out. The positions and transcriptional directions of the predicted ORFs are denoted with arrows. Genes associated with plasmid replication, antimicrobial resistance, heavy metal resistance, mobile element and conjugative transfer are shown. Other genes are also marked.

b, Linear comparison of the representative IncQ1 plasmid sequences. The results of sequence alignment were generated with Easyfig version 2.1 (ref. 43). The arrows represent the position and transcriptional direction of the ORFs. Regions of homology between 73% and 100% are marked by grey shading. Arrow colours are the same as in a.

c, Conjugation transfer efficiencies of the index plasmid pLHM10-1-p6 into *E. coli*, *K. pneumoniae* and *S. Typhimurium* strains. Transfer efficiency is calculated based on colony counts of the transconjugant and recipient cells in triplicate, and all data points are displayed, along with the mean and standard deviation (error bars).

d, Plasmid stability experiment results. All experiments were conducted in triplicate. Error bars denote the means (middle lines) and standard deviations.
along with pLHM10-1-p6 into the recipient *E. coli* C600 was not observed under the selection by tigecycline. Our results indicated that a helper plasmid is needed for the mobilization of *tet*(X4)-harbouring pLHM10-1-p6-like plasmids, and importantly, common

| Isolate | Date       | Province | Source | PFGE pattern       | MLST                      | Resistance phenotype |
|---------|------------|----------|--------|--------------------|---------------------------|----------------------|
| G3P11-1 | 16/08/2017 | Guangxi  | Pig    | A                  | ST1196                    | TGC/TC/SXT/FFC/CTX/CS/CIP |
| G3X12-2 | 16/08/2017 | Guangxi  | Pig    | A                  | ST1196                    | TGC/TC/SXT/FFC/CTX/CS/CIP |
| G3X16-2 | 16/08/2017 | Guangxi  | Pig    | A                  | ST1196                    | TGC/TC/SXT/FFC/CTX/CS/CIP |
| G3P8-2  | 16/08/2017 | Guangxi  | Pig    | A                  | ST1196                    | TGC/TC/SXT/FFC/CTX/CS/CIP |
| LHZ2-1  | 16/07/2017 | Jiangxi  | Pig    | B                  | ST761                     | TGC/TC/SXT/FFC/CTX/GEN  |
| XFP18-1 | 15/07/2017 | Fujian   | Pig    | C                  | ST8338                    | TGC/TC/SXT/FFC         |
| XFPY7-1 | 15/07/2017 | Fujian   | Pig    | C                  | ST8338                    | TGC/TC/SXT/FFC         |
| XFPY19-2 | 15/07/2017 | Fujian   | Soil   | C                  | ST8338                    | TGC/TC/SXT/FFC         |
| YSB3-1  | 27/07/2017 | Guangdong| Pig    | D                  | ST10                      | TGC/TC/SXT/FFC/CTX/CS/CIP |
| YSB7-2  | 27/07/2017 | Guangdong| Pig    | D                  | ST10                      | TGC/TC/SXT/FFC/CTX/CS/CIP |
| CDX6-3  | 14/07/2017 | Fujian   | Pig    | E                  | ST515                     | TGC/TC/SXT/FFC/CTX/CS/CIP/GEN |
| LHS1-1  | 16/07/2017 | Jiangxi  | Soil   | E                  | ST515                     | TGC/TC/SXT/FFC/CTX/CS/CIP/GEN |
| DDR1-1  | 14/07/2017 | Jiangxi  | Dust   | E                  | ST515                     | TGC/TC/SXT/FFC/CTX/CS/CIP/GEN |
| DDX16-1 | 14/07/2017 | Fujian   | Pig    | E                  | ST515                     | TGC/TC/SXT/FFC/CTX/CS/CIP/GEN |
| LHM10-1 | 16/07/2017 | Jiangxi  | Pig    | E                  | ST515                     | TGC/TC/SXT/FFC/CTX/CS/CIP/GEN |
| LHP6-1  | 16/07/2017 | Jiangxi  | Pig    | E                  | ST515                     | TGC/TC/SXT/FFC/CTX/CS/CIP/GEN |
| THP14-1 | 14/07/2017 | Fujian   | Pig    | F                  | ST761                     | TGC/TC/SXT/FFC         |
| THX8-2  | 14/07/2017 | Fujian   | Pig    | F                  | ST761                     | TGC/TC/SXT/FFC         |
| STB20-1 | 08/05/2017 | Jiangsu  | Pig    | F                  | ST761                     | TGC/TC/SXT/FFC         |
| THX2-1  | 14/07/2017 | Fujian   | Pig    | F                  | ST761                     | TGC/TC/SXT/FFC         |
| THX3-2  | 14/07/2017 | Fujian   | Pig    | F                  | ST761                     | TGC/TC/SXT/FFC         |
| GQ65-1  | 14/11/2017 | Guangxi  | Chicken| G                  | ST10                      | TGC/TC/SXT/FFC         |
| LHZ4-1  | 10/07/2017 | Jiangxi  | Pig    | H                  | ST871                     | TGC/TC/SXT/FFC/CTX/CS/CIP |
| YSP4-1  | 27/07/2017 | Guangdong| Pig    | I                  | ST101                     | TGC/TC/SXT/FFC/CTX/CS/CIP |
| HCP21-2 | 27/07/2017 | Guangdong| Pig    | J                  | ST542                     | TGC/TC/SXT/FFC/CTX/CS/CIP/GEN |
| YSP8-1  | 27/07/2017 | Guangdong| Pig    | J                  | ST542                     | TGC/TC/SXT/FFC/CTX/CS/CIP/GEN |
| DDPR2-2 | 14/07/2017 | Fujian   | Dust   | K                  | ST8302                    | TGC/TC/SXT/FFC         |
| GDX17-2 | 20/06/2017 | Jiangxi  | Pig    | K                  | ST8302                    | TGC/TC/SXT/FFC         |
| JS100   | 08/07/2017 | Jiangsu  | Chicken| K                  | ST8302                    | TGC/TC/SXT/FFC         |
| JS104-2 | 09/07/2017 | Jiangsu  | Chicken| K                  | ST8302                    | TGC/TC/SXT/FFC         |
| JS105-1 | 09/07/2017 | Jiangsu  | Chicken| K                  | ST8302                    | TGC/TC/SXT/FFC         |
| JS116-2 | 09/07/2017 | Jiangsu  | Chicken| K                  | ST8302                    | TGC/TC/SXT/FFC         |
| JS77-1  | 09/07/2017 | Jiangsu  | Chicken| K                  | ST8302                    | TGC/TC/SXT/FFC         |
| JS78-1  | 09/07/2017 | Jiangsu  | Chicken| K                  | ST8302                    | TGC/TC/SXT/FFC         |
| JS80-1  | 09/07/2017 | Jiangsu  | Chicken| K                  | ST8302                    | TGC/TC/SXT/FFC         |
| XDBN7-1 | 20/07/2017 | Guangdong| Pig    | K                  | ST8302                    | TGC/TC/SXT/FFC         |
| XDP15-1 | 20/07/2017 | Guangdong| Pig    | K                  | ST8302                    | TGC/TC/SXT/FFC         |
| XDP6-1  | 20/07/2017 | Guangdong| Pig    | K                  | ST8302                    | TGC/TC/SXT/FFC         |
| XDP9-1  | 20/07/2017 | Guangdong| Pig    | K                  | ST8302                    | TGC/TC/SXT/FFC         |
| XDXN5-3 | 20/07/2017 | Guangdong| Pig    | K                  | ST8302                    | TGC/TC/SXT/FFC         |
| XDXN5-2 | 20/07/2017 | Guangdong| Pig    | K                  | ST8302                    | TGC/TC/SXT/FFC         |
| XDXN8-1 | 20/07/2017 | Guangdong| Pig    | K                  | ST8302                    | TGC/TC/SXT/FFC/CTX/CS/CIP |
| XDXN15-1| 20/07/2017 | Guangdong| Pig    | K                  | ST8302                    | TGC/TC/SXT/FFC/CTX/CS/CIP |

**Fig. 4 | PFGE-Xba dendrogram and details about *tet*(X4)-positive *E. coli* isolates.** The PFGE assay was conducted successfully according to the standard protocol, and then used for the following analysis. The full gel images have also been provided in the Supplementary Information. PFGE patterns with a cut-off at 85% similarity (the dashed line) are considered to be the same PFGE cluster and are indicated as groups A–K, respectively. *E. coli* strains carrying the pLHM10-1-p6-like plasmids are underlined. The strains isolated from environmental (soil and dust) samples were taken from pig farms. CIP, ciprofloxacin; CS, colistin; CTX, cefotaxime; FFC, florfenicol; GEN, gentamicin; MLST, multilocus sequence typing; SXT, sulfamethoxazole–trimethoprim.
4.78-Mb chromosome and two plasmids in the sizes of 265,575 bp (pG3X16-2-p1) and 138,950 bp (pG3X16-2-p2), respectively. Plasmid pG3X16-2-p1 belonged to the IncH12 group, and harboured mcr-1 and blal_\text{CTX-M-63}. The tet(X4) gene was located on an InF plasmid (F: A18-B-), pG3X16-2-p2, co-harbours aadA1, aadA2, aph(3’)-Ib, aph(6)-Id, blas2_\text{TM-18BP}, erm(M), cmlA1, floR, sul3, tet(A), tet(M) and dfra12 (Supplementary Fig. 2), which suggested that the tigecycline resistance may be co-selected by other anti-microbial resistances. Intriguingly, pG3X16-2-p2 harboured four tandem repeated ISCR2-catD-tet(X4)-ISCR2 gene cassettes on the same plasmid, suggesting that the tet(X4)-harbouring genetic elements are highly active and may further transfer to other plasmids or chromosome.

Results of the in vivo experiment showed that the mice infected with the plasmid-cured isogenic strain E. coli STB20-1S (tet(X4) negative) were highly susceptible to tigecycline treatment, with more than 2 log.,(colony-forming unit (cfu)) per gram reduction in E. coli counts (P < 0.0001), whereas tigecycline had no significant effect on animals infected by E. coli STB20-1 after 24 h of treatment (Fig. 2e). The results indicated that the presence of tet(X4) may contribute to tigecycline treatment failure.

In this study, the tet(X4)-positive E. coli strains were identified in pigs, chickens and their surrounding environmental samples from different geographical regions, indicating that Tet(X4)-encoding tigecycline resistance might have already spread in China. We suspected that the usage of ‘older’ generations of tetracyclines had largely contributed to the emergence of new mobile tigecycline resistance. In China, tigecycline is only approved for the treatment of clinical infections in humans, whereas its usage in food-producing animals is not authorized. However, the first-generation and second-generation tetracyclines were widely used in the treatment of infections in food-producing animals and for growth promotion at sub-therapeutic doses in China, probably providing a selective pressure for the emergence of tigecycline resistance. As one of the countries with the largest amount of antibiotics usage in the world, 12,000 tons (7.4%) of tetracyclines were consumed by China yearly (data from 2013), and most of them eventually entered into environment. Similarly in Europe, although the European Union had phased out the use of antibiotics as growth promoters, the latest European Centre for Disease Prevention and Control—European Food Safety Authority—European Medicines Agency joint report (2017) on the integrated analysis of antibiotic consumption and antimicrobial resistance in bacteria revealed that tetracyclines were still the most sold antibiotics in Europe, and a statistically significant positive association between tetracycline consumption and resistance in E. coli strains was observed in food-producing animals. Of concern, the selective pressure imposed by the continuous application of tetracyclines in veterinary practice could serve to maintain and spread the tet(X4)-like genes into pathogenic microorganisms.

The mobile antimicrobial resistance mechanisms, including Tet(X4), MCR and different families of carbapenemase (for example, NDM), represent a plasmid-mediated antibiotic crisis. However, the reality is that only limited antibiotics on the market or in the pipeline are effective against these highly resistant organisms. Continuous selection pressure from the environment, veterinary usage and clinical practice will probably accelerate the spread of tet(X4) and other resistance genes. The horizontal transfer ability of the resistance genes and plasmids, such as the tet(X4)-harbouring broad host range IncQ1 plasmid, can be a challenge for hospital infection control and public health surveillance. Our study underlines the need for a ‘one-health’ strategy (that is, considering the span of humans, animals and their environment) for antimicrobial resistance, as infectious disease surveillance and containment across different sectors are paramount. To make this last-resort drug available, the prevalence of tet(X4) in both human and veterinary fields should be considered by surveillance programmes, along with judicious administration of tetracyclines in animals and the environment.

Methods

Sample collection and bacterial strains. This study was approved by the institutional review board of hospital A (Huazhou Municipal Central Hospital) and South China Agricultural University (SCAU). The clinical isolates from hospitals B, C and D were retrospectively collected, and patient data were not included in this study; thus, ethical approval was not sought and obtained. The faecal samples were randomly collected from pigs (3–8 months of age) and chickens (7–20 weeks of age), with approximately 50 samples per farm. If possible, the soil, dust, sewage and vegetable samples were also collected, at least three samples per farm.

To examine the spread of mobile tigecycline resistance gene tet(X4), samples and clinical isolates were screened from 4 tertiary-care hospitals and 63 intensive animal farms (pig farms, n = 41; chicken houses, n = 22) in 16 provinces or municipalities in China (Fig. 1). The four hospitals are located in Guangdong (hospitals A and B), Jiangsu (hospital C) and Zhejiang (hospital D) provinces. The pig farms and chicken houses are distributed in four geographical regions of China (Northeast, Southeast, West and Central China) and cover most livestock breeding areas in China. Briefly, a total of 4,189 samples from the animal sector were collected from May 2015 to May 2018 (Supplementary Table 2) and subjected to selection on MAC plates containing tigecycline (2 mg l$^{-1}$). The selected tigecycline-non-susceptible isolates were then subjected to tet(X4) screening. In addition, 299 human specimens from hospital A collected in the same period and a random collection of 720 E. coli clinical isolates from hospitals B, C and D were also screened as described above (Supplementary Table 2).

Porcine E. coli LHM10-1 was selected as the donor strain, while laboratory strains (E. coli C660 (streptomycin resistance), S. Typhimurium ATCC 14028 (tetracycline resistance) and K. pneumoniae ATCC 70060 (ciprofloxacin resistance)) and clinical MDR strains (E. coli 1314 carrying blasaac(6A), ST167) and K. pneumoniae 1332 carrying blasaac(2C), ST11) were chosen as recipient strains for conjugation and plasmid stability tests. Porcine E. coli STB20-1 carrying a single plasmid (tet(X4) positive) was selected for the in vivo infection model.

Whole-genome sequencing. Genomic DNA was extracted from E. coli LHM10-1 using a TIANamp Bacteria DNA Kit (Tiangen), followed by genomic DNA sequencing with a MiSeq platform (Huayin Health). To obtain the complete plasmid sequences, E. coli LHM10-1 was also subjected to single-molecule real-time sequencing with a PacBio RSII system (Nextronic). In addition, another tet(X4)-positive E. coli strain, GSX16-2, was completely sequenced using a combined Illumina HiSeq and Nanopore sequencing approach (Nextronic). Genome assembly was performed with Unicycler version 0.4.1 (ref. 13) using a combination of short and long reads, followed by error correction by Pilon version 1.12 (ref. 14). The functional genes were annotated and classified using the RAST server15. The sequence types and plasmid replicon types were analysed by the CGE server (https://cge.cbs.dtu.dk/services/). Visualization of the genetic structure of tet(X4)-carrying plasmids was generated with DNAPlotter version 1.11 (ref. 14).

Subcloning experiments. To confirm the role of Tet(X4) in tigecycline resistance, the full-length ORF of tet(X4) was amplified from genomic DNA of E. coli LHM10-1 with the addition of an EcoRI restriction site at the 5’ end and a SalI restriction site at the 3’ end (Supplementary Table 4). Both the published recombinant plasmid pBAD24, containing the arabinose pBAD promoter, were digested with restriction endonucleases EcoRI/Sall and then ligated at 16°C overnight following the manufacturer’s instructions (New England Biolabs). Subsequently, the recombinant plasmid pBAD24-tet(X4) was transformed into competent E. coli JM109 by heat shock and then subjected to susceptibility testing against different tetracyclines using a broth microdilution method.

Microbiological degradation assays. The activities of Tet(X4) on tetracycline antibiotics degradation were initially assessed by an agar well diffusion method. First, a suspension of the E. coli construct JM109 + pBAD24-tet(X4) was made by suspending the culture from a MH agar plate in 500 µl MH broth containing 0.1% i-arabinose (adjusted optical density at 600 nm of 0.2). The suspension was then inoculated with tetracycline (10 mg l$^{-1}$), oxytetracycline (20 mg l$^{-1}$), chlorotetracycline (20 mg l$^{-1}$), doxycycline (10 mg l$^{-1}$), minocycline (10 mg l$^{-1}$), tigecycline (2.5 mg l$^{-1}$) and eravacycline (2.5 mg l$^{-1}$), and cultured at 37°C for 8 h. Meanwhile, 100 µl of the overnight culture (0.5 McFarland) of Staphylococcus aureus steatothermophilus 7953 (tetracycline susceptible) was spread on a MH agar plate surface. A hole with a 6-mm diameter was punched aseptically with a disposable hole puncher, and its bottom was sealed by a drop of heated liquid MH agar. Following centrifugation and filtration with a 0.22-µm filter, 20 µl of the supernatant from the JM109 + pBAD24-tet(X4) co-culture with different tetracycline antibiotics was added into the prepared agar hole and the plate was incubated at 60°C for 16 h to examine the zone of inhibition. The E. coli JM109 + pBAD24 strain was used as a negative control, and the medium containing only tetracyclines acted as the blank control.
Quantifying the levels of tetracyclines via LC–MS/MS. To further examine whether Tet(X4) was able to inactivate tetracyclines, the levels of tetracycline and eravacycline (tigecycline was not used because of its poor stability in growth media, even with the protection of 0.6 g l\(^{-1}\) asbreakpoint) were quantified in growth media of E. coli JM109-pBAD24-tet(X4) by LC–MS/MS in sextuplicate\(^3\). For tetracycline quantification, E. coli JM109-pBAD24-tet(X4) was grown in 4 ml of M9 minimal media (1x M9 minimal salts, 2 mM MgSO\(_4\) and 100 μM CaCl\(_2\)) containing 0.1% l-arabinose supplemented with 9 μg/ml glucose, 100 μg/ml thiamine, 100 μg/ml leucine and 8 μg/ml tetracycline. The strain was incubated at 37°C with shaking at 200 r.p.m. and protected from light for 16 h. Subsequently, cells were pelleted and the supernatant was passed through a 0.22-μm filter. The filtered supernatant was then diluted tenfold and subjected to LC–MS/MS quantification. Similarly, the level of eravacycline degradation was also determined by incubation with eravacycline (2 μg/ml) for 16 h. The E. coli JM109-pBAD24-tet(X4) was used as a negative control. The linear ranges of standard curves for tetracycline and eravacycline quantification were 25–800 ppb and 10–200 ppb, respectively, with r > 0.995.

Mating experiments and plasmid stability testing. The transferability of tet(X4) was determined by conjugation using E. coli C660, S. Typhimurium ATCC 14028, K. pneumoniae ATCC 700603, E. coli 1314 and K. pneumoniae 1332 as recipient strains. Briefly, the donor and recipient strains were grown at log-phase in LB broth, and were then mixed at the donor/recipient ratio of 1/3 and applied to a 0.22-μm filter, followed by culture at 37°C for 16h. The putative transconjugants were selected as white colonies on MacConkey agar plates. In brief, the host E. coli LHM101-1 strain and its transconjugants were subjected to plasmid stability testing as previously described\(^3\). Briefly, three independent lineages of them were cultured overnight at 37°C in 4 ml antibiotic-free LB broth. Serial passage of 4μl of the overnight culture to 4 ml LB broth was performed daily, yielding approximately 10 generations of growth per passage. For every 20 generations, samples were diluted and plated on LB agar plates.

In addition, the mobilizing efficiency of different helper plasmids on the tet(X4)-harbouring strains was determined by agar dilution and interpreted according to the Clinical & Laboratory Standards Institute guidelines. MICs of tigecycline, eravacycline and colistin were determined by broth microdilution. In particular, the MICs of tigecycline for E. coli strains was interpreted according to the FDA criteria (susceptible, ≤2 μg/ml; intermediate, 4 μg/ml; resistant, ≥8 μg/ml), whereas eravacycline was interpreted with no breakpoint. Colistin was interpreted in accordance with the European Committee on Antimicrobial Susceptibility Testing breakpoints (susceptible, ≤2 μg/ml; resistant, >2 μg/ml).

E. coli ATCC 25922 served as a quality control strain for susceptibility testing.

Molecular typing. All tet(X4)-positive E. coli strains were classified by XbaI-digested (Takara) PFGE according to the PulseNet protocol (http://www.pulsenetinternational.org/protocols/) using a CHEF Mapper System (Bio-Rad). PFGE patterns were compared using BioNumerics version 6.6 (Applied Maths Srl, Belgium) under appropriate optimization (1.5%) and tolerance (1.5%) settings, with a cut-off at 85% similarity to delineate PFGE clusters. Multilocus sequence typing was performed by the primers and protocol specified on the E. coli multilocus sequence typing database website (http://enterobase.warwick.ac.uk/).

Murine thigh infection model. Female Institute of Cancer Research (ICR) mice (6–10 weeks of age; Guangdong Medical Lab Animal Center, Guangzhou, China) were used in this experiment. Mice were maintained in accordance with the National Standards for Laboratory Animals in China (GB 14925–2010). All animal procedures were approved by the Animal Research Committees of SCAU. No specific statistical consideration was taken in determining the sample size for the animal experiments. Investigators were blinded as for group allocation when determining c.f.u. Meanwhile, we were able to cure the tet(X4)-carrying plasmid from wild-type E. coli STB20-1 (tet(X4) positive) using sodium dodecyl sulfate as previously described\(^3\), to create STB20-1S (tet(X4) negative). Tet(X4) was directly caused by treatment would be most likely due to the presence of the plasmid only. Prior to infection, mice were rendered neutropenic by injecting two doses of cyclophosphamide intraperitoneally on 4d (150 mg per kg) and 1d (100 mg per kg). To study the effect of the tet(X4)-curing plasmid on the efficacy of tigecycline, thigh infections were produced by injecting 0.1 ml suspensions of E. coli STB20-1 or its tet(X4)-cured STB20-1S strain at 10\(^6\) c.f.u. per ml. At 2h post-infection, the mice were subcutaneously administered a single dose of tigecycline at 50 mg per kg per 24h, which was approximately equivalent to the dosing regimen in humans given as 50 mg per 12h for 6d\(^\#\#\#\#\)\(^4\). Mice were humanely euthanized at 24 h after treatment and thigh muscles were aseptically homogenized in 3 ml saline. After serial dilutions, suspensions were quantitatively cultured on antibiotic-free LB agar plates for c.f.u. enumeration.

Statistical analysis. Descriptive analyses on percentage (together with standard deviation) and prevalence were performed using functions provided in Excel 2007 (Microsoft Software). All statistical analyses (unpaired t-test, two-tailed; two-way analysis of variance (ANOVA)) were performed with GraphPad Prism 5 (GraphPad Software).

Data availability. The whole-genome sequencing data of E. coli LHM101-1 and G3X16-2 strains have been submitted to the NCBI under the BioSample accession number SAMN11087649 and SAMN11180601, respectively. Extra data supporting the findings of this study are available from the corresponding authors on reasonable request.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection | No software was used for data collection. |
|-----------------|----------------------------------------|

Data analysis

- Unicycler version 0.4.1 (ref. 27)
- Pilon version 1.12 (ref. 28)
- RAST server (ref. 29)
- DNAPlotter version 1.11 (ref. 30)
- Phyre2 server (ref. 41)
- AutoDock version 4.2.6 (ref. 42)
- EasyFig version 2.1 (ref. 43)
- ESPript version 3.0 (Supplementary ref. 1)
- MEGA version 5.2 (Supplementary ref. 2)
- BioNumerics version 6.6 (Applied Maths Software)
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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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The nucleotide sequence data of E. coli LHM10-1 and G3X16-2 strains have been submitted to GenBank under the BioSample accession number SAMN11087649 and SAMN11180601, respectively. The sequence types (STs) and plasmid replicon types of them were analyzed by the CGE server (https://cge.cbs.dtu.dk/services/). The tet(X4)-positive E. coli strains were classified by PFGE according to the PulseNet protocol (http://www.pulsenetinternational.org/protocols/). Multilocus sequence typing (MLST) was performed by the primers and protocol specified in E. coli MLST database website (http://enterobase.warwick.ac.uk).

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Life sciences

All studies must disclose on these points even when the disclosure is negative.

Sample size No specific statistical consideration was taken in determining the sample size. The fecal samples were randomly collected from pigs and chickens, with approximately 50 samples per farm. If possible, the soil, dust, sewage, and vegetable samples were also collected at least three per farm. What’s more, 1019 isolates from four tertiary care hospitals in three provinces, which had been detected with tet(X4) from animal sector in this study, were collected for clinical epidemiological study. For the murine thigh infection model, six mice per group were used.

Data exclusions No data were excluded.

Replication Quantifying the levels of tetracycline and eravacycline via LC-MS/MS and the efficacy of Tet(X4) conferring resistance to tigecycline in murine thigh infection model were performed in sextuplicate. Plasmid transfer frequency and stability testings were conducted in triplicate for characteristics of the tet(X4)-harboring plasmid pLHM10-1-p6. All replications were successfully conducted.

Randomization All the samples were randomly collected. All the organisms and participants involved in this study were allocated into groups randomly.

Blinding Investigators were blinded as for group allocation during data collection and analysis.

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Materials & experimental systems

n/a Involved in the study

- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants
- Clinical data

Methods

n/a Involved in the study

- ChiP-seq
- Flow cytometry
- MRI-based neuroimaging

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals The fecal samples were randomly collected from pigs (3 to 8 months old) and chickens (7 to 20 weeks old) in 16 provinces or municipalities in China. Female ICR mice (6-10 weeks old; Guangdong Medical Lab Animal Center, Guangzhou, China) were also used for in vivo experiments in this study.

Wild animals The study did not involve wild animals.
Field-collected samples

The study did not involve samples collected from the field. However, some environmental samples (sewages, soils adjacent to sewage outfalls, dust in vents of breeding house, and vegetables irrigated with sewages in pig farms or chicken houses) were collected for investigation.

Ethics oversight

The animal use procedures were approved by the Animal Research Committees of South China Agricultural University (SCAU). All animal studies were conducted in accordance with SCAU Institutional Animal Welfare and Ethics guidelines.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Human research participants

Policy information about [studies involving human research participants](#)

| Population characteristics | The human research participants were mainly healthy physical examination people and inpatients in Intensive Care Unit (ICU). |
|----------------------------|------------------------------------------------------------------------------------------------------------------------|
| Recruitment                | All participants were recruited in an open manner and informed consent was obtained, respectively. The urine, nasal swabs, and rectal swabs were randomly collected from them. No self-selection bias was present. In addition, all participants held the right to withdraw from the study at any stage. |
| Ethics oversight           | This study was approved by the institutional review board (IRB) of hospital A (Huizhou Municipal Central Hospital) for clinical sample collection. The clinical isolates from hospitals B, C, and D were retrospectively collected, and patient data were not included in this study, therefore ethical approval wasn’t sought and obtained. |

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