Emergence of Plasmid-Mediated Resistance Genes tet(X) and mcr-1 in Escherichia coli Clinical Isolates from Pakistan

Ruichao Li, a,b Mashkoor Mohsin,c Xiaoyu Lu, a Sabahat Abdullah, c Asim Munir, c Zhiqiang Wang a

ABSTRACT The emergence of tet(X) represents a significant threat to human health. In this study, we aimed to investigate the genomic characterizations of tet(X)-positive clinical Escherichia coli isolates and provide genomic insight into the dissemination of antibiotic-resistant bacteria in clinical settings. Four tet(X)-positive E. coli isolates, PKS074, PKS086, PKS095, and PKS097, from 100 human clinical isolates were identified by PCR and were resistant to tigecycline. tet(X) genes were in IncFII plasmids in 4 E. coli isolates. Worryingly, PKS074 also carried an mcr-1-bearing IncHI2 plasmid. Notably, a relatively high cotransfer frequency of tet(X) and mcr-1 in PKS074 was found. PKS086, PKS095, and PKS097 were categorized into sequence type 410 (ST410) and indicated clonal dissemination of tet(X)-positive strains in hospitals, but tet(X)-bearing plasmids in PKS086, PKS095, and PKS097 were nontransferable. We present the first report of clinical E. coli isolates harboring tet(X) in South Asia. Our results support the implication of humans as a potential reservoir for tet(X)-harboring E. coli. We provide insight into the dissemination of tet(X) and mcr-1 in a clinical setting and highlight the current transmission of both critical resistance genes globally.

IMPORTANCE Global transmission of plasmid-mediated tigecycline resistance gene tet(X)-bearing Escherichia coli strains incurs a public health concern. However, the research focusing on the prevalence of tet(X)-positive isolates in clinical specimens is still rare, and to our knowledge, there is no such report from South Asia. Here, we characterized four E. coli clinical isolates harboring tet(X) of human origin in Pakistan and demonstrated clonal dissemination of tet(X)-positive isolates in hospitals. We report the emergence of an mcr-1-bearing IncHI2 plasmid together with a tet(X)-positive IncFII plasmid in one clinical isolate. Cotransfer of tet(X)- and mcr-1-carrying plasmids is worrying and warrants further investigations.

KEYWORDS Escherichia coli, tet(X), mcr-1, plasmids, clinical settings

Tigecycline is used as the last-resort antibiotic to treat infections caused by extensively drug-resistant (XDR) bacteria, particularly carbapenem-resistant Enterobacteriaceae (1, 2). However, the emergence of the plasmid-mediated tigecycline resistance gene tet(X4), the most prevalent tet(X) variant that confers high-level tigecycline resistance in Escherichia coli, represents significant threats to human health (3, 4). Recently, reports on tet(X) in Enterobacteriaceae isolates from humans have increased significantly, mainly from China and Singapore (4, 5). Here, we report the first identification of E. coli clinical isolates harboring tet(X) of human origin in Pakistan and characterize the genetic environment of tet(X). We also describe a relatively high cotransfer frequency of tet(X) and mcr-1 in clinical isolate PKS074, which highlights the current worldwide transmission of both critical resistance genes.

Among 100 human clinical isolates, four tet(X)-positive isolates (including PKS074, PKS086, and PKS097 from hospital A and PKS095 from hospital B) were acquired, and
they were identified as *E. coli* (Table 1). Antimicrobial susceptibility testing revealed that all 4 *E. coli* isolates conferred high-level resistance to tigecycline with the MICs ranging from 32 to 64 mg/liter. Worryingly, mobile colistin resistance gene *mcr-1* was also detected in strain PK5074 (Table 2). MICs for PK5074 revealed that *E. coli* PK5074 exhibited resistance to tigecycline, colistin, kanamycin, doxycycline, ampicillin, enrofloxacin, streptomycin, amoxicillin, florfenicol, terramycin, and tetracycline (Table 1). PK5086, PK5095, and PK5097 exhibited the same resistance spectrum and were resistant to tigecycline, gentamicin, kanamycin, doxycycline, ampicillin, enrofloxacin, cefotiofur, streptomycin, amoxicillin, ceftriaxone, florfenicol, terramycin, and tetracycline (Table 1). Four tet(X)-positive isolates were multidrug-resistant (MDR) bacteria. PCR and Sanger sequencing confirmed the tet(X) present in four strains was tet(X4), which is annotated as tet(X) in the following context.

To investigate the transferability of tet(X) or mcr-1, conjugation assays were performed. Resistance genes tet(X) and mcr-1 in strain PK5074, with corresponding resistance phenotypes for tigecycline and colistin, were able to successfully transfer from *E. coli* PK5074 into the recipient *E. coli* J53, suggesting that the tet(X) and mcr-1 genes were located in conjugative plasmids or other mobilizable genetic elements in PK5074. The tet(X)-positive genetic structure exhibited good transferability into *E. coli* J53 at a frequency of $(4.34 \pm 0.07) \times 10^{-1}$ cells per recipient. Comparatively, the mcr-1-bearing genetic structure transferred with a frequency of $(6.46 \pm 0.82) \times 10^{-6}$ cells per recipient. Cotransfer of tet(X) and mcr-1 was at a frequency of $(6.18 \pm 0.99) \times 10^{-6}$ cells per recipient. However, tet(X) in strains PK5086, PK5095, and PK5097 was nontransferable.

All the 4 tet(X)-carrying isolates were sequenced using the Illumina HiSeq 2500 platform generating $2 \times 150$-bp paired-end read data, and draft genome sequences were obtained successfully. Whole-genome sequencing (WGS) analysis provided comprehensive information for the tet(X)-carrying bacteria and their phylogenetic relationship. Multilocus sequence typing (MLST) analysis revealed that PK5074 positive for tet(X) and mcr-1 belonged to sequence type 48 (ST48), and tet(X)-bearing strains PK5086 and PK5095 along with PK5097 belonged to ST410. We further determined the clonal relationship of strains PK5086, PK5095, and PK5097 based on their single nucleotide polymorphism (SNP) of the core genome. The numbers of differences in SNPs were only up to three between the three strains. In addition, PK5086, PK5095, and PK5097 contained identical antimicrobial resistance genes, insertion sequences, virulence-associated genes, and plasmid replicons (Fig. 1), indicating that clonal dissemination of tet(X)-positive strains existed in two different hospitals. Multiple antimicrobial resistance genes were identified in four isolates (Fig. 1).

To learn the genetic contexts of tet(X), PK5074 coharboring tet(X) and mcr-1 and the representative PK5086 of the remaining three strains were sequenced with the Oxford Nanopore Technologies MinION long-read platform. PK5074 harbored a chromosome and five plasmids consisting of pPK5074-MCR1, pPK5074-tetX, pPK5074-91kb, pPK5074-69kb, and pPK5074-2kb (Table 2). The tet(X) gene was located on the IncFII plasmid pPK5074-tetX, which is an MDR plasmid coharboring *aph(3’)-la, aph(6)-Id, aadA22, aph(3’)-lb*, sul2, *floR*, and *blaTEM-215* genes (Table 2). pPK5074-MCR1 was a typical mcr-1-bearing IncHI2 plasmid carrying various resistance genes, including *mcr-1, aph(3’)-la, aadA8, Inu(F), sul3, tet(A),

| Strain ID | Date of sampling (yr-mo-day) | MICs (mg/liter)* |
|-----------|-------------------------------|------------------|
| PK5074    | 2019-12-17 0.125 0.25 0.5 4   | >128 >128 >512 <0.125 >128 <0.125 4 >128 128 64 |
| PK5086    | 2019-12-23 0.125 0.25 0.5 4   | >128 >128 >128 >128 >128 >128 >128 8 >128 >128 <0.125 <0.125 4 <0.125 4 >128 128 32 |
| PK5095    | 2020-03-01 0.125 0.25 0.5 4   | >128 >128 >128 >128 >128 >128 >128 8 >128 >128 <0.125 <0.125 4 <0.125 4 >128 128 32 |
| PK5097    | 2020-07-01 0.125 0.25 0.5 4   | >128 >128 >128 >128 >128 >128 >128 8 >128 >128 <0.125 0.25 <0.125 4 >128 128 32 |
| ATCC 25922| 0.25 2 0.5 4                  | ≤0.125 ≤0.125 4 4 4 4 ≤0.125 4 ≤0.125 0.25 4 0.5 ≤0.125 |

*GEN, gentamicin; KAN, kanamycin; DOX, doxycycline; AMP, ampicillin; ENR, enrofloxacin; CFF, cefotiofur; STR, streptomycin; AMC, amoxicillin; RIF, rifampicin; CEF, ceftriaxone; FFC, florfenicol; MEM, meropenem; CST, colistin; TER, terramycin; TET, tetracycline; TIG, tigecycline.*
TABLE 2 Genomic information of the chromosomes and plasmids of *E. coli* PK5074 and PK5086 resolved by hybrid assembly strategy

| Strain   | MLST  | Components            | Size (bp) | Accession no. | Replicon type(s) | Resistance genes                                                                 | Virulence-associated genes(s) |
|----------|-------|-----------------------|-----------|---------------|------------------|----------------------------------------------------------------------------------|----------------------------------|
| PK5074   | ST48  | PK5074-chromosome      | 4,746,945 | CP072802      | IncHI2, IncHI2A  | mdf(A), dfrA1, mcr-1, aph(3')-la, aadA8, lnu(F), sul3, tet(A), aph(6)-ld, floR, ARR-2, dfrA14 | gad, ompT, terC, terC |
| pPK5074-MCR1 |     | 267,744               | CP072803  |               |                  | tet(X), aph(3')-la, aph(6)-ld, aadA22, aph(3')-lb, sul2, floR, bla<sub>TEM-215</sub> | traT                            |
| pPK5074-tetX |     | 110,313               | CP072807  |               |                  | qnrS2, tet(A), floR, sul2, aph(6)-ld, aph(3')-lb                                    | ND                             |
| pPK5074-91kb |     | 91,224                | CP072806  | IncFIB(K)     |                  | mdf(A), dfrA1, mcr-1, aph(3')-la, aadA8, lnu(F), sul3, tet(A), aph(6)-ld, floR, ARR-2, dfrA14 | traT |
| pPK5074-69kb |     | 69,302                | CP072804  | IncY          |                  | tet(X), aph(3')-la, aph(6)-ld, aadA22, aph(3')-lb, sul2, floR, bla<sub>TEM-215</sub> | traT |
| pPK5074-2kb  |     | 1,943                 | CP072805  | CoIRNA1       |                  | qnrS1, aph(6)-ld, aph(3')-lb, aadA2, aph(3')-la, bla<sub>TEM-1B</sub>, lnu(F)     | ND |
| pPK5074-91kb |     | 1,943                 | CP072807  |               |                  | mdf(A), dfrA1, mcr-1, aph(3')-la, aadA8, lnu(F), sul3, tet(A), aph(6)-ld, floR, ARR-2, dfrA14 | traT |
| pPK5074-69kb |     | 95,348                | CP080373  | IncY          |                  | tet(X), fosA4, mph(A), dfrA12, floR, bla<sub>TEM-215</sub>                         | traT |
| pPK5074-2kb  |     | 2,088                 | CP080374  | CoIRNA1       |                  | tet(X), fosA4, mph(A), dfrA12, floR, bla<sub>TEM-215</sub>, dfrA17, sul1, sul2, tet(B), bla<sub>TEM-1B</sub>, bla<sub>CTXA-1</sub>, bla<sub>CTX-M-15</sub> | traT |
| PK5086   | ST410 | PK5086-chromosome      | 4,781,220 | CP080370      |                  | mdf(A), dfrA1, mcr-1, aph(3')-la, aadA8, lnu(F), sul3, tet(A), aph(6)-ld, floR, ARR-2, dfrA14 | gad, lpfA, terC, terC |
| pPK5086-tetX |     | 100,261               | CP080371  |               |                  | tet(X), fosA4, mph(A), dfrA12, floR, bla<sub>TEM-215</sub>, dfrA17, sul1, sul2, tet(B), bla<sub>TEM-1B</sub>, bla<sub>CTXA-1</sub>, bla<sub>CTX-M-15</sub> | traT |
| pPK5086-97kb |     | 97,614                | CP080372  | IncFIB (AP001918), IncFIA, IncQ1, IncFII (pRSB107) |                  | tet(X), fosA4, mph(A), dfrA12, floR, bla<sub>TEM-215</sub>, dfrA17, sul1, sul2, tet(B), bla<sub>TEM-1B</sub>, bla<sub>CTXA-1</sub>, bla<sub>CTX-M-15</sub> | traT |
| pPK5086-95kb |     | 95,348                | CP080373  | IncY          |                  | tet(X), fosA4, mph(A), dfrA12, floR, bla<sub>TEM-215</sub>, dfrA17, sul1, sul2, tet(B), bla<sub>TEM-1B</sub>, bla<sub>CTXA-1</sub>, bla<sub>CTX-M-15</sub> | traT |
| pPK5086-2kb  |     | 2,088                 | CP080374  | CoIRNA1       |                  | tet(X), fosA4, mph(A), dfrA12, floR, bla<sub>TEM-215</sub>, dfrA17, sul1, sul2, tet(B), bla<sub>TEM-1B</sub>, bla<sub>CTXA-1</sub>, bla<sub>CTX-M-15</sub> | traT |

ND, not detected.
FIG 1  Distributions of antimicrobial resistance genes, insertion sequences, virulence-associated genes, and plasmid replicons in four tet(X)-bearing isolates. The color in figure legend indicates the percentage of sequence homology.
aph(6)-Id, floR, arr-2, and dfrA14 dispersed among insertion sequences (Table 2). PKS086 contained a chromosome and four plasmids consisting of pPKS086-tetX, pPKS086-97kb, pPKS086-95kb, and pPKS086-2kb (Table 2). The tet(X) gene was in plasmid pPKS086-tetX, which is also an MDR IncFII plasmid carying fosA4, mph(A), dfrA12, floR, and blamr-TEM-215.

BLASTn analysis of pPKS074-tetX and pPKS086-tetX against the NCBI nr database showed that they exhibited 99% identity at 78% coverage with plasmid 3 (LR130554) in E. coli MS14386 from a blood sample, and 97% identity at 64% coverage with plasmid pH1038-142 (KJ484634) in an E. coli isolate from a human (Fig. 2a). Plasmid 3 and pH1038-142 had plasmid backbone structures similar to those of pPKS074-tetX and pPKS086-tetX, but there was no MDR region including tet(X) in plasmid 3. The most obvious difference of pH1038-142, compared with pPKS074-tetX and pPKS086-tetX, was also an MDR region without tet(X) (Fig. 2a). This indicates that the formation of plasmid pPKS074-tetX and pPKS086-tetX may depend on the evolution of MDR regions. Two copies of ISCR2 were adjacent to tet(X) in pPKS074-tetX (Fig. 2a), which may play a role in facilitating the transmission of tet(X) (3, 4). In addition, two repeats of tet(X) were found in pPKS074-tetX, and the repeat structure was ISCR2-hp-abh-tet(X) in 4,608 bp, which was the reported tet(X)-bearing circular intermediate (3, 6). The circular intermediate may play an important role in the formation of tet(X)-bearing tandem repeat structures. pPKS074-MCR1 shared >98% coverage and >99% identity with plasmid pCFA1096 (CP033347) in Salmonella enterica subsp. enterica strain CFA1096 of food origin in China and plasmid p2017_03_03CC (LC511658) in E. coli 2017.03.03CC isolate of human origin (Fig. 2b).

The isolate PKS074 belonged to the ST48 E. coli, which was linked to Shiga toxin-producing or extraintestinal pathogenic strains, and three mcr-1-carrying ST48 E. coli isolates were characterized as avian-pathogenic E. coli in Pakistan (7–9). Notably, ST48 strains were found to be a dominant host for the mcr-1-bearing IncX4 plasmid (10) and a host for the carbapenemase gene blamr-TEM occasionally (11, 12). However, the tet(X) gene has also begun to appear in ST48 E. coli isolates, and this should attract our attention. In E. coli PKS074, pPKS074-tetX and pPKS074-MCR1 were MDR plasmids harboring various insertion sequences, such as ISCR2 and IS26 (Fig. 2a and b). It has been reported that ISCR2 and IS26 may facilitate the construction of large fused MDR plasmids (6, 13–15). Therefore, it is possible that the IncFII plasmid pPKS074-tetX and the IncHI2 plasmid pPKS074-MCR1 could form a recombinant plasmid carrying tet(X) and mcr-1 mediated by insertion sequences. This will accelerate the transmission of mcr-1 and tet(X), but the possibility warrants further investigations. In fact, the emergence of the plasmid-mediated tigecycline resistance gene tet(X) in E. coli isolated from poultry, food, and the environment in South Asia was reported in May 2021, and tet(X)-bearing IncFII or IncQ1 plasmid was found to coexist with mcr-1-carrying IncI2 plasmid (16). In combination with this study, we speculate that more tet(X)- and mcr-1-coharboring isolates will appear in the region and constitute a potential public health concern.

In isolate PKS086, the tet(X)-carrying plasmid pPKS086-tetX was highly similar to pPKS074-tetX except for the MDR region, and pPKS086-tetX harbored the transfer elements (Fig. 2a), but they were unable to transfer into J53 by conjugation. Given the high potential of ST410 E. coli to acquire resistance to last-resort antimicrobials (17), the establishment of tet(X)-carrying ST410 E. coli in South Asia should arouse regional and global concerns, as resistance to last-resort antibiotics is already a major public health crisis in the region and worldwide.

To conclude, we report the first identification of E. coli clinical isolates harboring tet(X) and mcr-1 of human origin in Pakistan and report the cotransfer of mcr-1-bearing IncHI2 plasmid with tet(X)-positive plasmid in a clinical isolate. These findings indicate that mobile tigecycline and colistin resistance genes may disseminate in clinical settings in Pakistan and pose a serious global risk in clinical settings. It is recommended to strengthen the monitoring of the coexistence of mcr-1 and tet(X) to avoid the coming of the preantibiotic era.

**Bacterial isolates and identification.** Between 2019 and 2020, a total of 100 human clinical isolates were screened for the presence of mobile tigecycline-resistant E. coli harboring tet(X) in Faisalabad, Pakistan. The human clinical E. coli isolates were collected from two tertiary care hospitals: 70 isolates were collected from hospital A
FIG 2 Sequence comparison of plasmids harboring mcr-1 and tet(X) with structurally similar plasmids available in NCBI database. (a) Circular comparison of the tet(X)-bearing IncFII plasmids, including pPK5074-tetX, pPK5086-tetX, and other IncFII plasmids in the NCBI nr database. The outermost circle indicate reference plasmid pPK5074-tetX with genes annotated. (b) Circular comparison between the mcr-1-bearing IncHI2 plasmid pPK5074-MCR1 and other IncHI2 plasmids in the NCBI nr database.
and 30 isolates from hospital B. All isolates were cultivated on urinary tract infection (UTI) chrome agar supplemented with 2 mg/liter tigecycline and incubated overnight at 37°C for isolation of tigecycline-resistant E. coli strains. PCR was employed to screen the presence of tet(X) in tigecycline-resistant isolates using primers described earlier (3). mcr-1 was further identified in tet(X)-positive isolates (18). 16S rRNA gene sequencing was performed to confirm bacterial species.

**Antimicrobial susceptibility testing.** The MICs of gentamicin, kanamycin, doxycycline, ampicillin, enrofloxacin, ceftriaxone, florfenicol, meropenem, colistin, terramycin, tetracycline, and tigecycline for all tet(X)-bearing isolates were determined by the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (19) and were interpreted according to the CLSI standards (M100 and M31-A3) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (http://www.eucast.org/clinical_breakpoints/). Tigecycline and colistin were interpreted in accordance with the EUCAST guidelines (susceptible, ≤2 mg/liter; resistant, >2 mg/liter). E. coli ATCC 25922 served as the quality control strain.

**Conjugation experiments.** To investigate the transferability of tet(X) and mcr-1, conjugation assays were performed using tet(X)-positive strains as donors and E. coli J53 (sodium azide resistant [Azir]) as the recipient. Bacterial strains were streaked onto LB agar plates, followed by inoculation into LB broth overnight. Cultures of donors and the recipient were mixed at 1:1, and then 0.1 ml of mixed culture was applied onto LB agar plates, followed by incubation at 37°C for 16 to 20 h. After incubation, we subsequently collected the mixed culture on LB agar plates and 10-fold serially diluted it in sterile saline. LB agar plates, supplemented with different antimicrobials, including tigecycline (2 mg/liter) and sodium azide (150 mg/liter), colistin (2 mg/liter) and sodium azide (150 mg/liter), and tigecycline in combination with colistin and sodium azide, were used to recover transconjugants [tet(X)-carrying, mcr-1-containing, and tet(X) and mcr-1 cohaboring transconjugants]. The presence of tet(X) or/and mcr-1 genes in transconjugants was confirmed by PCR and antimicrobial susceptibility testing as described above. The frequency of conjugation transfer was calculated by the number of transconjugants per recipient as previously described (20).

**WGS and bioinformatics analysis.** The genomic DNA of all tet(X)-positive isolates was extracted using the FastPure bacteria DNA isolation minikit (Vazyme, China) in accordance with the manufacturer’s recommendations. Whole-genome sequencing was performed via an Illumina HiSeq 2500 platform, and two representative isolates were further sequenced by Oxford Nanopore Technologies (ONT) MinION platform. Short-read Illumina raw sequences were assembled using SPAdes (21). Illumina short-read and Nanopore long-read data were used to perform de novo assembly with Unicycler 0.4.4 (22, 23). The Rapid Annotation using Subsystems Technology annotation website server (https://rast.nmpdr.org/rast.cgi) was then used to annotate the genomes (24). Online tools, including PlasmidFinder 2.1 (25), ResFinder 4.1 (26), VirulenceFinder 2.0 (27), and MLST 2.0 (28), were utilized to assemble and characterize the genomes (https://cge.cbs.dtu.dk/services/). TBtools was used to visualize the distributions of antimicrobial resistance genes, insertion sequences, virulence-associated genes, and plasmid replicons (29). Comparisons of highly homologous complete plasmid sequences available in the NCBI database with plasmids in the study were performed with BRIG (30).

**Data availability.** The nucleotide sequences of the chromosomes and plasmids of E. coli PK5074 and PK5086 have been deposited in the NCBI database with accession numbers CP072802 to CP072807 and CP080370 to CP080374, respectively (Table 2). The draft genomes of E. coli PK5095 and PK5097 were also deposited in NCBI (BioProject identifier [ID] PRJNA751691).

**ACKNOWLEDGMENTS**

This work was supported by the China Postdoctoral Science Foundation (no. 2020M671632), the National Natural Science Foundation of China (no. 31872523 and 31872526), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

We declare no conflict of interest.
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