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Short Communication

Emergence of plasmid-mediated tigecycline resistance *tet*(X4) gene in *Escherichia coli* isolated from poultry, food and the environment in South Asia

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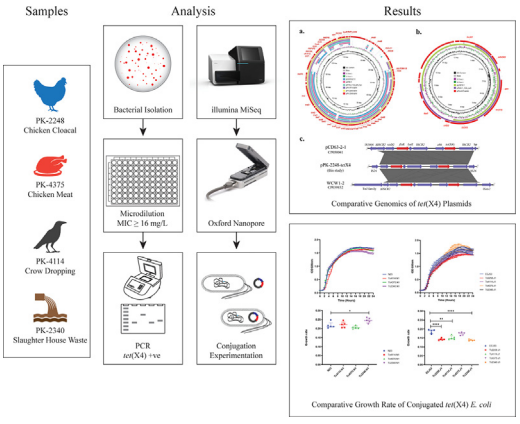
HIGHLIGHTS

- First detection of *tet*(X4)-mediated tigecycline resistance from different non-clinical sources from South Asia
- Co-existence of *tet*(X4) and *mcr-1* is alarming.
- *tet*(X4) gene is transferrable to other host bacteria via conjugation.

GRAPHICAL ABSTRACT



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ABSTRACT

The recent emergence of mobile-tigecycline resistance *tet*(X) genes in human and animals in China seriously threats the clinical utility of tigecycline. Here we focused on the isolation and molecular characterization of plasmid-mediated tigecycline resistance *tet*(X4)-positive *E. coli* from different sources in Pakistan using MinION and Illumina sequencing. The *tet*(X4) gene was detected in four *E. coli* isolates from poultry, chicken meat, wild bird and the slaughterhouse wastewater in Pakistan. Co-existence of colistin resistance *mcr-1* gene was also detected in three isolates. The four isolates belonged to different sequence types and the *tet*(X4) gene was located on plasmids ranging from 12,331 bp to 113,610 bp belonging to IncFII and IncQ replicon types with two genetic contexts IS*CR2*-*tet*(X4)-*abh*-IS*CR2*-*lysR*-*floR*-*virD2* and ΔIS*CR2*-*abh*-*tet*(X4)-IS*CR2*-*virD2*-*floR*, respectively.
In all the four *E. coli* strains, *tet*(X4) was transferable by conjugation to *E. coli* J53 host strain. In addition, three of four strains transferred *tet*(X4) to a wild-type carbapenem resistant *E. coli* strain. To our knowledge, this is the first report of the emergence of plasmid-mediated *tet*(X4) gene from Pakistan. The convergence of tigecycline and colistin resistance in South Asia is a serious threat to human health.

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1. Introduction

Tigecycline is a last resort antibiotic to treat infections caused by extensively resistant (e.g. carbapenem- and colistin-resistant) Gram-negative bacteria (Fang et al., 2020); therefore, the recent emergence of mobile tigecycline resistance highlights the threat of an impending return to the pre-antibiotic *era*. Until recently, mechanisms of tigecycline resistance were restricted to chromosomal mutations which cannot be transferred between bacteria. However, in 2019, He et al. reported two transferable plasmid-mediated tigecycline resistance genes, *tet*(X3) and *tet*(X4) in Gram-negative bacterial isolates (*Escherichia coli* and *Acinetobacter baumannii*) from human and animal sources in China (He et al., 2019). The Tet(X) proteins are flavin monooxygenases that catalyze the degradation of tetracyclines including tigecycline, eravacycline and omadacycline. Recently increased reports of tigecycline resistance genes *tet*(X3/X4) and other variants have emerged, mainly from Mainland China and Singapore (Zhang et al., 2020; Ding et al., 2020). For the first time, we report evidence of plasmid-mediated tigecycline resistance gene *tet*(X4) in *E. coli* isolates from different sources in South Asia.

2. Materials and methods

In an on-going surveillance of mobile colistin resistance in *E. coli*, from human, over 1100 samples were collected from human, animals, food and environmental sources from Faisalabad, Pakistan. We detected four *E. coli* isolates displaying high-level tigecycline resistance (MIC ≥16 mg/L). PCR was conducted to confirm the presence of a tigecycline resistance gene, in this case *tet*(X4), using primers described earlier (He et al., 2019). Antimicrobial resistance phenotypes of isolates were determined by disc diffusion. All *E. coli* isolates carrying *tet*(X4) were subjected to WGS using both MinION (Oxford Nanopore Technologies, UK) and MiSeq (Illumina, USA) platforms followed by *in-silico* analysis for antibiotic resistance genes, virulence genes, multi-locus sequence type (MLST) and genetic context of *tet*(X4) (Supplementary material). Conjugations were performed using sodium azide resistant *E. coli* J53, and wild-type *E. coli* strains from human microbiota (C351, phylogroup A), human clinical (N23 phylogroup D) and animal origin (CX17, phylogroup B2) as recipient strains. Growth curves were constructed for control and conjugated strains using protocols described in Supplementary material.

The complete sequences of the chromosome and plasmids of strains have been submitted to GenBank under project code PRJNA683023.

3. Results and discussion

3.1. Characteristics of tet(X4)-positive E. coli

The four *E. coli* strains were collected in 2018–19 and originated from broilers, chicken meat, slaughterhouse wastewater and a wild-bird. Worryingly, co-occurrence with mobile colistin resistance *mcr-1* gene was also detected in three strains (PK-2248, PK-2340 and PK-4375) (Table 1). All the strains were resistant to three or more antibiotics (multi-drug resistant) and carried ≥6 virulence associated genes (Table 2). MLST analysis categorize the strains into the following ST groups: ST6726 (PK-2248), ST694 (PK-2340), ST4388 (PK-4114) and ST224 (PK-4375), implying the *tet*(X4)-bearing *E. coli* strains are found in diverse phylogenetic clades (Table 1). The genome size of chromosomes and plasmids of all the strains are given in Table S1.

3.2. Genetic characterization of tet(X4) and mcr-1 bearing plasmids

The *tet*(X4)-positive plasmids pPK-2248-*tet*X4, pPK-2340-*tet*X4, pPK-4114-*tet*X4 and pPK-4375-*tet*X4, ranged in size from 12 kbp to 113 kbp (Table 1). The *tet*(X4)-bearing plasmids of PK-2248, PK-2340 and PK-4114 belonged to the IncFII group and showed high-level homology with IncFII plasmids (Fig. 1a). In addition to *tet*(X4), these IncFII *tet*(X4)-positive plasmids also harbored other important resistance genes, such as *mph*(A) (macrolide resistance), *dfrA12* (diaminopyrimidine resistance) and *floR* (phenicol resistance) (Fig. 1a). The immediate genetic context of *tet*(X4) on three plasmids had the same 9 kb region (Fig. 1c). This 9 kb *tet*(X4)-bearing genetic region was also found previously from the chromosome of *Aeromonas caviae* WCW1–2 (CP039832) and the plasmid of *E. coli* CD63-2-1 (CP050041) with an extra truncated IS*CR2* region upstream of *tet*(X4) on both sequences (Chen et al., 2019; Lv et al., 2020). A putative composite transposon with two identical IS*26* elements flanking the 9 kb region were observed in three IncFII plasmids from this study (Fig. 1c). A BLASTn search of pPK-2248-tetX4 against the NCBI nr database retrieved other similar IncFII type *tet*(X4)-negative plasmids pGD0503Z13 (KR653209) and pST45-1 (CP050754) of chicken and a patient in China. However, the most similar IncFII *tet*(X4)-bearing plasmid was pRF65_113kb_flye (at 59% coverage in 98.08% identity) discovered in China (Li et al., 2020) (Fig. 1a), indicating widespread global transmission.

Table 1
Characteristics of *tet*(X4)-positive *E. coli* in this study.

Strain IDs	Source	Year of isolation	MIC	MLST	*tet*(X4) plasmid type	*tet*(X4) plasmid size	*mcr-1* plasmid type	Conjugation frequency to J53	Conjugation frequency to N23
PK-2248	Chicken cloacal	2018	16	6726	IncFII	113 kbp	IncI2	3.8×10^{-2}	Negative
PK-2340	Slaughter-house waste water	2018	16	694	IncFII	100 kbp	IncI2	6.1×10^{-5}	4.1×10^{-7}
PK-4114	Crow	2019	16	4388	IncFII	100 kbp	–	9.8×10^{-3}	5.25×10^{-6}
PK-4375	Chicken meat	2019	16	224	IncQ1	12 kbp	IncI2	1.5×10^{-3}	3.6×10^{-8}

Table 2
Antimicrobial resistance and virulence genes profile of *E. coli.*

Strain ID	Phenotypic resistance	Antimicrobial resistance genes	Virulence genes
PK-2248	AMP, C, ENR, GM, SXT, DC, TE, TYL	*aadA1*, *aadA2*, *aph(3′)-lb*, *aph(6)-ld*, bla_{EC-18}, $bla_{TEM-215}$, *cmlA1*, *dfrA12*, *floR*, *fosA4*, *mcr-1.1*, *mph(A)*, *qnrS1*, *sul3*, *tet*(A), *tet*(X4)	*astA*, *fyuA*, *gad*, *hra*, *irp2*, *ompT*, *terC*, *traT*
PK-2340	AMP, C, GM, SXT, DC, TE, TYL	*aadA1*, *aadA2*, bla_{EC-15}, $bla_{TEM-215}$, *cmlA1*, *dfrA12*, *fosA4*, *floR*, *mcr-1.1*, *mph(A)*, *sul3*, *tet* (A), *tet*(X4)	*astA*, *capU*, *gad*, *iss*, *terC*, *traT*
PK-4114	AMP, SXT, C, DC, TE, TYL	bla_{EC-18}, *dfrA12*, *floR*, *mph(A)*, *tet*(X4)	*cvaC*, *etsC*, *gad*, *hlyF*, *iroN*, *iss*, *iucC*, *iutA*, *lpfA*, *mchF*, *ompT*, *sitA*, *terC*, *traT*, *tsh*
PK-4375	AMP, GM, C, SXT, DC, TE	*aac(3)-lle*, *aph(3′)-la*, *aph(3′)-lb*, *aph(6)-ld*, bla_{EC-18}, *floR*, *mcr-1.1*, *sul2*, *tet*(A), *tet*(X4)	*astA*, *cea*, *cma*, *gad*, *hra*, *iss*, *iucC*, *iutA*, *lpfA*, *neuC*, *ompT*, *papA_F19*, *sitA*, *terC*, *traT*

AMP, ampicillin; C, chloramphenicol; DC, doxycycline; ENR, enrofloxacin; GM, gentamicin; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline; TYL, tylosin.

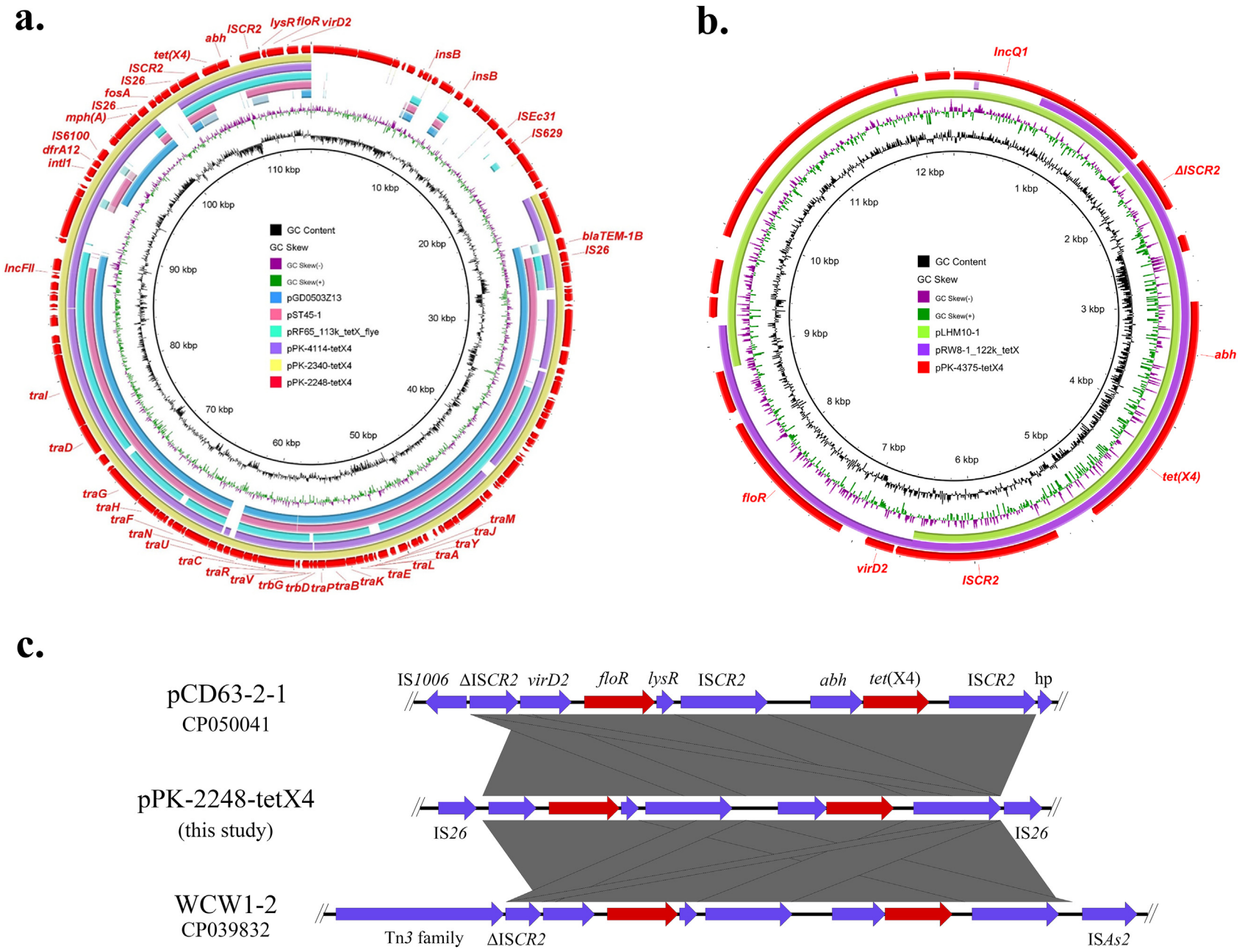

Fig. 1. Comparative genomics and genetic environments of *tet*(X4) plasmids in this study (a) Structures of *tet*(X4) carrying IncFII plasmids pPK-2248-tetX4, pPK-2340-tetX4 and pPK-4114-tetX4 and comparison to other similar plasmids. (b) Structure of *tet*(X4)-carrying IncQ1 plasmid and comparison to another similar plasmid. (c) Genetic context of *tet*(X4) regions identified in IncFII plasmid visualized using Easyfig. The arrows represent the positions and transcriptional directions of ORFs. Regions of homology are marked by grey shading and antibiotic resistance genes are coded in red.

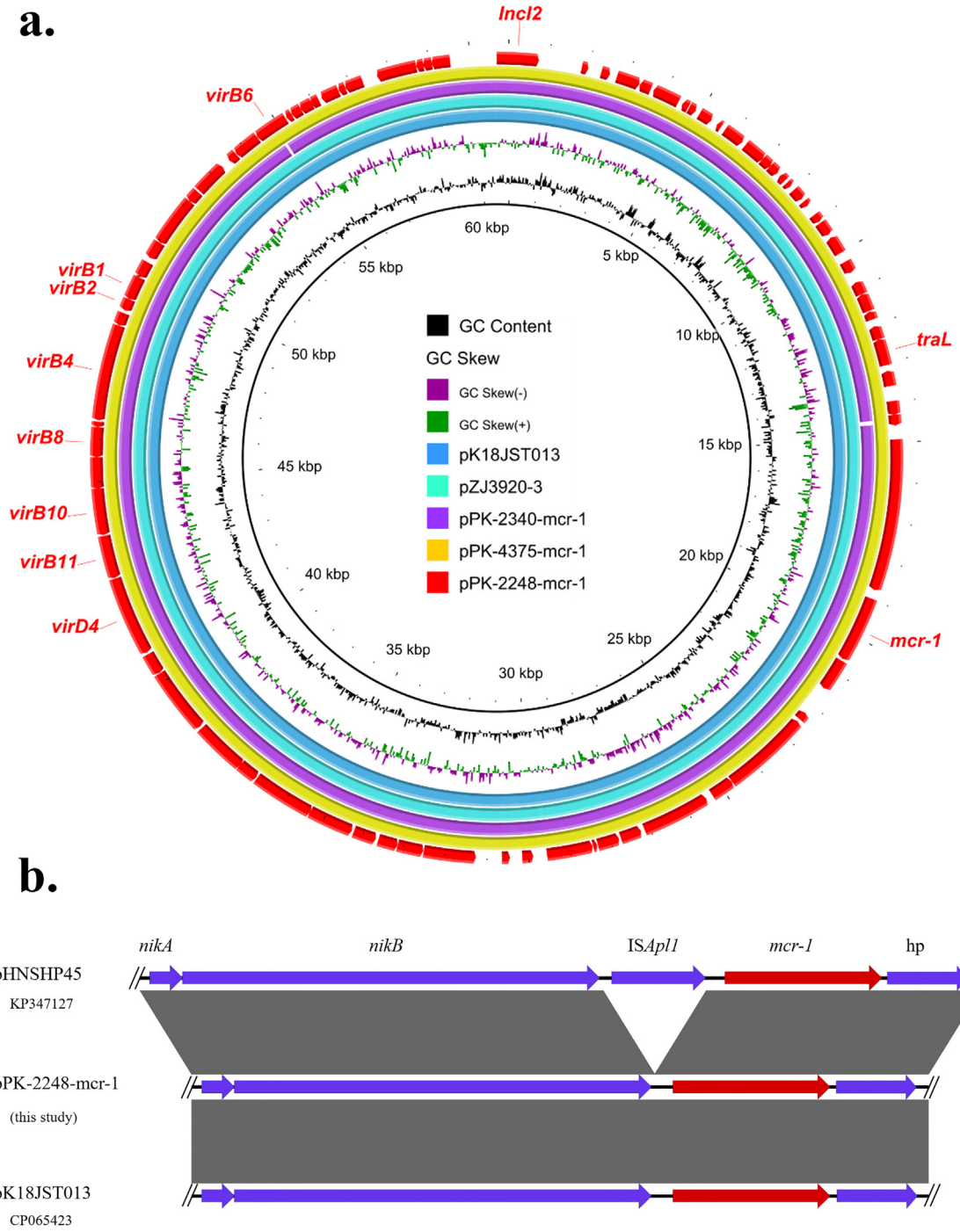

Fig. 2. Comparative genomics and genetic environments of *mcr-1* plasmid in this study (a) Structures of *mcr-1* bearing IncI2 plasmids pPK-2248-mcr-1, pPK-2340-mcr-1 and pPK-4375-mcr-1 and comparison to other similar plasmids. (b) Genetic context of *mcr-1* region identified in IncI2 plasmid was visualized using Easyfig. The arrows represent the positions and transcriptional directions of ORFs. Regions of homology are marked by grey shading and *mcr-1* gene is colored in red.

Additionally, strain PK-4375 carried *tet*(X4) gene on a IncQ1 type plasmid, pPK-4375-*tet*X4. The *tet*(X4) gene was on a 6,698 bp region on pPK-4375-*tet*X4 with the genetic structure ΔIS*CR2*-*abh*-*tet*(X4)-IS*CR2*-*virD2*-*floR* (Fig. 1b). This 7 kb region was the same as the G3-2 type *tet*(X4) region previously reported (Li et al., 2020), which illustrates that this genetic structure/plasmid may transfer between different source strains. Moreover, the plasmid backbone of pPK-4375-*tet*X4 was highly similar to other reported plasmids including pig *E. coli* pLHM10-1 (CP037909) and wastewater *E. coli* pRW8-1_122k_tetX (MT219826) (Fig. 1b). The 12,331 bp plasmid pPK-4375-*tet*X4 only contained *tet*(X4) and *floR* resistance gene differing from other reported IncQ plasmids (Li et al., 2017a, 2017b; Loftie-Eaton and Rawlings, 2012). IncQ type plasmids have a broad host-range and are thought to disseminate through bacterial population found in sewage and other environments (Stalder et al., 2019). The arise of *tet*(X4)-positive IncQ-type plasmids *E. coli* strains form different sectors greatly enhances the risk of *tet*(X4) horizontal transfer.

The co-resistance of tigecycline with colistin resistance is worrisome. All three *mcr-1*-positive plasmids belonged to IncI2 type and shared >99% nucleotide identity with each other. Our IncI2-type

plasmids showed high similarity to those recovered from NCBI nr database plasmids pK18JST013 (CP065423, *Salmonella*) and pZJ3920-3 (CP020548, *E. coli*) (Fig. 2a). Compared with the originally reported IncI2 *mcr-1* plasmid pHNSP45, these three plasmids all lacked the IS*Apl1* element flanking the *mcr-1* gene (Fig. 2b), which may stabilize the *mcr-1* gene. This structure was also found in strains from other countries including Pakistan (Ji et al., 2019; Li et al., 2017a, 2017b; Mohsin et al., 2019b), which imply this type of plasmids is globally widespread. Beside *mcr-1*, no other antimicrobial resistance gene was found on *mcr-1* bearing plasmids.

3.3. Conjugation and fitness cost

All *tet*(X4) carrying strains of this study displayed conjugation with *E. coli* J53. In addition, PK-2340, PK-4114 and Pk-4375 also transfer *tet* (X4) to *E. coli* N23 (a wild-type clinical strain from Indian origin) which also carries bla_{NDM-1} (Table 1). No conjugation to other wild-type *E. coli* strains (CX351 and CX17) was observed. Furthermore, growth curves of the transconjugates and control strains demonstrated no fitness cost with wild-type *E. coli* strain, N23 (transconjugate Tc2340. N1; p-value = 0.0291). In contrast, *tet*(X4) plasmid acquisition by *E. coli* J53 displayed a significant fitness cost to its transconjugate, Tc2258.J1 (p-value ≤ 0.0001), Tc4114.J1 (p-value = 0.0014) and Tc2340.J1 (p-value ≤ 0.0001) (Fig. 3).

Tetracyclines are the most commonly used antibiotics in food producing animals in Pakistan (Mohsin et al., 2019a; Umair et al., 2020). Selective pressure due to tetracyclines may well be linked to the emergence of tigecycline resistance in Pakistan. Carbapenem resistance among Gram-negative bacteria is already a serious challenge in clinical settings in Indian subcontinent (Stewardson et al., 2019). The possibility of co-resistance to colistin, tigecycline and carbapenem could pose a catastrophic scenario.

4. Conclusions

To the best of our knowledge, this is the first report on the detection of *tet*(X4)-mediated tigecycline resistance from multiple non-clinical sources from South Asia and our data, if extrapolated across the continent has immense ramifications for the future treatment of XDR infections. With the burden carbapenem and colistin resistance already high in South Asia, these findings underscore the importance and urgency to counter the emergence and co-existence of mobile tigecycline and colistin resistant bacteria.

CRediT authorship contribution statement

Mashkoor Mohsin: Conceptualization & Writing- original draft. BH: Methodology, Conjugation & Writing - review & editing. Ruichao Li: Genomic analysis & Writing - review & editing. Willames M. B. S. Martins: Conjugation & Fitness cost. Sabahat Abdullah: Collected samples & PCR. Kirsty Sands: Bioinformatics. Timothy Walsh: Conceptualization & Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

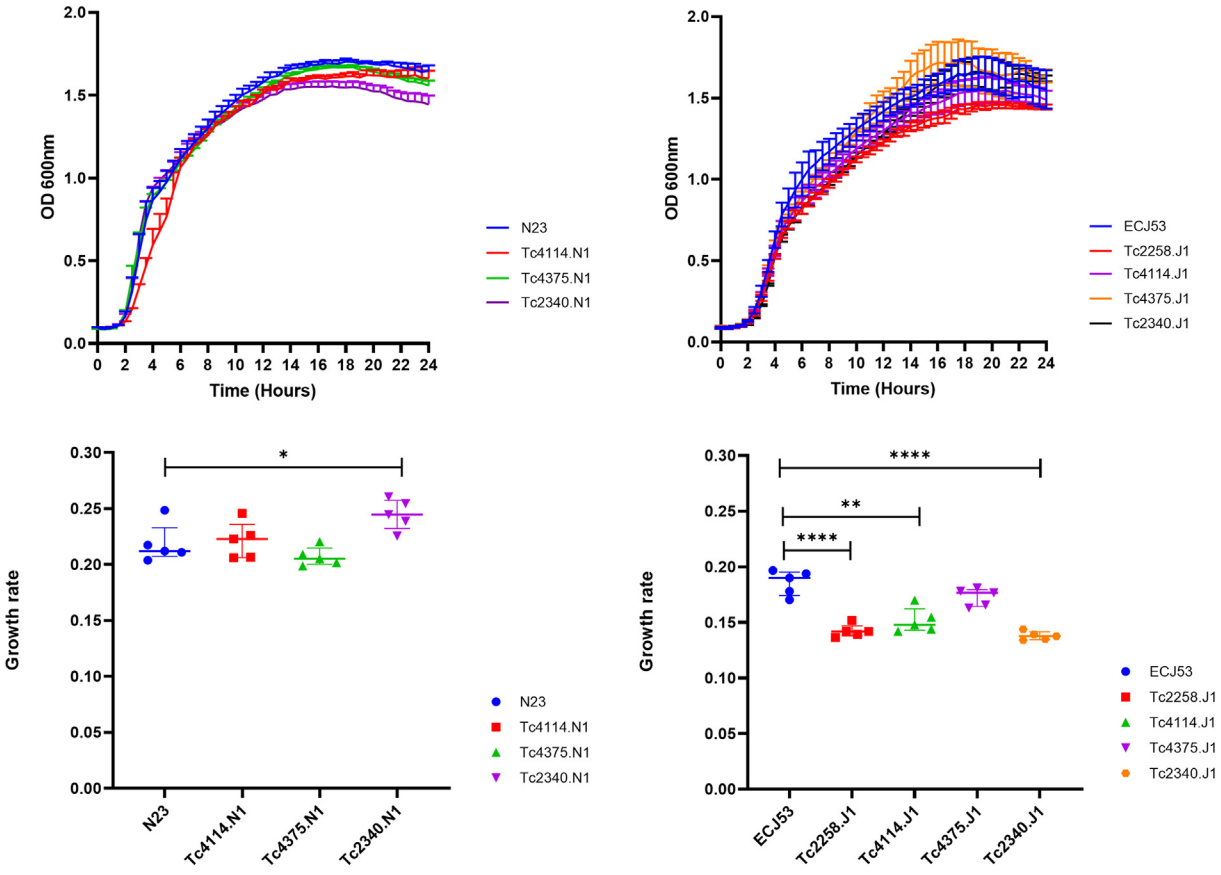

Fig. 3. Growth rate of conjugated *tet*(X4) *E. coli* in comparison with control groups Conjugates resulting with wild-type *E. coli* strain N23 (Tc2340.N1, Tc4114.N1 and Tc4375.N1) are shown on the left and conjugates resulting with *E. coli* J53 (Tc2248.J1, Tc2340.J1, Tc4114.J1 and Tc4375.J1) are shown on the right side. Significant difference between growth rates was observed for N23 vs Tc2340.N1 (p-value = 0.0291), EJ53 vs Tc2258.J1 (p-value ≤ 0.0001), EJ53 vs Tc4114.J1 (p-value = 0.0014) and EJ53 vs Tc2340.J1 (p-value ≤ 0.0001).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147613.

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