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Emergence of transferable tigecycline and eravacycline resistance gene *tet*(X4) in *Escherichia coli* isolates from Iran

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Tigecycline (TGC) and eravacycline (ERV) are critical last-resort antibiotics used to treat complicated infections caused by extensively drug-resistant Gram-negative bacteria particularly carbapenem- and colistin-resistant Enterobacterales. The recent emergence of plasmid-mediated TGC resistance gene *tet*(X) in Enterobacterales of both animal and human origins represents a significant public health threat. In this study, we characterized *tet*(X4)-bearing *Escherichia coli* isolates recovered from cattle fecal samples in Iran. A total of 395 fecal samples obtained from calves were screened for tigecycline-resistant (TGC-R) *E. coli* by inoculating in to selective culture media containing tigecycline. The presence of *tet*(X) gene among the recovered TGC-R enteric bacteria was assessed using PCR. Genetic relatedness of the *tet*(X)-bearing strains was analyzed via ERIC-PCR. Three *tet*(X)-bearing strains were further characterized by whole genome sequencing (WGS) using Illumina platform. The transferability and stability of *tet*(X)-bearing elements were evaluated by conjugation assay and successive subculturing on antibiotic-free culture media respectively. A total of five *tet*(X)-positive *E. coli* isolates exhibiting high-level resistance to tigecycline (MIC = 64 mg/L) and eravacycline (MIC > 8 mg/L) were recovered and categorized in to two groups ($n = 4$, $n = 1$) based on ERIC-PCR and antimicrobial susceptibility patterns. WGS analysis identified *tet*(X4) variant in three isolates, which belonged to sequence types ST224 ($n = 2$) and ST10 ($n = 1$). ResFinder database analysis revealed coexistence of *tet*(X4) with multiple antibiotic resistance genes including *aadA*, *aph*, $bla_{CTX-M-15}$ and / or bla_{TEM-1B}, *floR*, *cmlA*, *dfrA*, *sul* and *qnrS*/mutations in *gyrA* and *parC* genes. The *tet*(X4)-positive *E. coli* isolates contained the IncX1 and p0111 (strains B52 and R37) or IncX1, IncQ1, IncI1-I(α) and IncFII/IncFIA/IncFIB (strain M55) replicon types according to PlasmidFinder analysis. The *tet*(X4) gene was successfully mobilized to tigecycline -susceptible recipient *E. coli* isolates through conjugation assay and demonstrated high stability persisting over 10 consecutive passages in antibiotic-free media in both transconjugants and their donors. This study reports, for the first time in Iran, the emergence of transferrable high-level tigecycline/eravacycline resistance gene *tet*(X4) in *E. coli* isolates. Given the public health implications, control measures should be implemented to regulate the use of tetracyclines and potentially phenicols in food animals to prevent emergence and further transmission of such superbugs along the animal- environment -human chain.

Keywords Tigecycline resistance, *tet*(X4), *Escherichia coli*, Plasmid, Fecal samples

Tigecycline and eravacycline, are third-generation tetracyclines that serve as last-line broad-spectrum antibiotics for treating complicated infections caused by newly emerged multidrug-resistant (MDR) Gram-positive and Gram-negative bacterial strains. These new generation tetracyclines were approved to overcome classical tetracycline resistance mechanisms such as Tet-type efflux pumps and ribosomal protection proteins[1–3]. However, tigecycline-resistant bacteria have been increasingly reported following the widespread clinical use of this agent, particularly against carbapenem-resistant Enterobacterales[4].

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While chromosomal mechanisms, such as the overproduction of resistance-nodulation-division (RND) efflux pumps (e.g., AcrAB-TolC)[5,6] followed by *rpsJ* alterations[7], remain the most common drivers of tigecycline resistance, recent studies have reported the emergence of novel transferrable plasmid-encoded tigecycline resistance genes in Enterobacterales from animals and humans. These include the RND-type efflux pump gene cluster, *tmexCD-toprJ*[8], and the tigecycline inactivating enzyme-encoding *tet*(X) gene[9]. Tet(X), is a flavin-dependent monooxygenase that hydroxylate and inactivate all tetracyclines, including tigecycline as well as other newerFDA-approved derivatives such as eravacycline, and omadacycline[10,11]. The first report of mobile tigecycline resistant genes *tet*(X3), and *tet*(X4) occurred in animal- and human- derived Enterobacterales from China in 2019[9]. Notably these *tet*(X) variants demonstrate significantly higher enzymatic activity compared to the prototypical Tet(X) enzyme[12] conferring high-level tigecycline resistance (minimum inhibitory concentration (MIC) values of 16–32 mg/L) and posing, a significant threat to tigecycline efficacy. The emergence and dissemination of *tet*(X) genes among animal bacteria continue while tigecycline is not authorized for use in veterinary medicine. This spread has been attributed to the extensive use of older tetracyclines and phenicols in farm animals notably in livestock[13,14]which provides selective pressure for development of tigecycline resistant/non-susceptible bacterial isolates[14]. In addition to *tet(X)*, the mobilized RND efflux pump gene cluster *tmexCD1-toprJ1*has been identified as a critical resistance determinant, reducing susceptibility to tigecycline as well as other antibiotics, including quinolones, cephalosporins, phenicols, and aminoglycosides[8]. Antibiotics such as tetracyclines, cephalosporins, quinolones, sulfonamides and to a lesser extent colistin are used in Iran in livestock production for the treatment of bacterial diseases such as diarrhea. The overexpression of chromosomal AcrAB-TolC efflux pump has been identified as a major tigecycline resistance mechanism among animal- and human-derived Enterobacterales in Iran[6]. However, transferrable tigecycline resistance mechanisms have not yet been reported from this region. Given the global dissemination of *tet*(X4) in diverse environments[15], this study aimed to identify and characterize tigecycline-resistant *E. coli* isolates from the enteric bacteria of livestock and investigate the possible involvement of mobile tetracycline destructase-encoding genes among the detected bacterial isolates.

Results

Bacterial isolation and *tet*(X) gene detection

A total of 395 samples were obtained from five unrelated and geographically distant cattle farms (total number of cattle per farm ranged from 30 to 2500 (total ~ 3180) and were screened for the presence of tigecycline resistant enteric bacteria by inoculating the samples into tigecycline containing selective culture media. A total of five tigecycline/eravacycline resistant *E. coli* isolates designated as M55, R37, B52, R34 and S91 were obtained from two different farms. All five isolates were characterized with high level tigecycline (MIC = 64 mg/L) and eravacycline resistance (MIC > 8 mg/L, Figure S1) and were found to carry the *tet*(X) gene as determined by PCR (Figure S2 and Table 1). In contrast to isolate M55, the remining 4 isolates showed almost the same Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR banding patterns (Fig. 1) and antimicrobial susceptibility profile being assumed to belong to the same lineage. Therefore, only three strains including R37 and B52 (with similar ERIC-PCR pattern, but obtained from two different farms) and M55 were sent to IRCCS San Raffaele Institute, Italy for whole genome sequencing (WGS) under collaborative agreement.

Bacterial genotyping and antimicrobial susceptibility testing

WGS analysis revealed that the tigecycline and eravacycline-resistant isolates belonged to sequence types ST224 (R37 and B52) and ST10 (M55) with all isolates carrying *tet*(X4) variant. Phenotypic antimicrobial susceptibility testing demonstrated distinct resistance patterns among the identified isolates. The R37 group, which included strains R37, B52, R34 and S91, showed resistance to aminoglycosides (gentamicin, tobramycin and kanamycin), fluoroquinolones (ciprofloxacin), phenicols (chloramphenicol), cephalosporins (ceftriaxone, ceftazidime, and cefepime), sulfonamides/trimethoprim (sulfamethoxazole-trimethoprim), and all tested tetracyclines (Table 1).

Strain	Farm	Broth dilution MIC (mg/L)			Disk diffusion		
		TGC	ERV	COL	S	I	R
M55	A	64 R	> 8 R	0.25 S	CRO, CAZ, FEP, ATM, IMP, NIT, AN, GM, TOB	CIP	AMX, DOX, TE, C, K, SXT
R37	A	64 R	> 32 R	0.5 S	NIT, AN, IMP		AMX, CRO, CAZ, FEP, ATM, DOX, TE, GM, K, TOB, CIP, C, SXT
B52	B	64 R	> 32 R	0.5 S	NIT, AN, IMP		AMX, CRO, CAZ, FEP, ATM, DOX, TE, GM, K, TOB, CIP, C, SXT
R34	B	64 R	> 32 R	0.5 S	NIT, AN, IMP		AMX, CRO, CAZ, FEP, ATM, DOX, TE, GM, K, TOB, CIP, C, SXT
S91	B	64 R	> 32 R	0.5 S	NIT, AN, IMP		AMX, CRO, CAZ, FEP, ATM, DOX, TE, GM, K, TOB, CIP, C, SXT

Table 1. Antimicrobial susceptibility testing results of *tet*(X4)-positive *E. coli* isolates. TGC, tigecycline; ERV, eravacycline; COL, colistin; CRO, ceftriaxone; CAZ, ceftazidime, AMX, amoxicillin; NIT, nitrofurantoin; AN, amikacin; DOX, doxycycline; GM, gentamicin; FEP, cefepime; ATM, aztreonam; TE, tetracycline; CIP, ciprofloxacin; C, chloramphenicol; IMP, imipenem; TOB; Tobramycin, K, Kanamycin; SXT, sulfamethoxazole/trimethoprim; S, susceptible; I, intermediate; R, resistant. Interpretation of results was performed based on breakpoints issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for Enterobacterales (TGC, ERV and COL) and Clinical and Laboratory Standards Institute (CLSI) guidelines (all agents tested by disk diffusion assay).

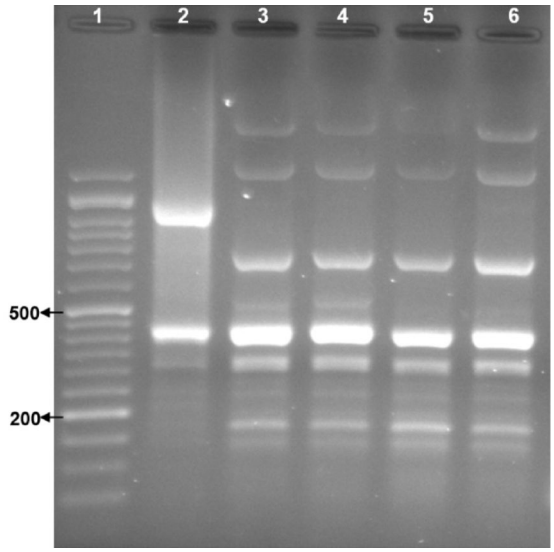

Fig. 1. ERIC-PCR fingerprint of *tet*(X)-positive *E. coli* isolates on 2% agarose gel. Lane 1, 50 bp DNA marker (GeneDireX); lane 2 to 6, Isolates M55, R34, R37, S91, B52.

Strain	Farm	MLST	Antimicrobial resistance genes							Virulence genes
			aminoglycoside	β-lactam	sulfonamide	trimethoprim	chloramphenicol	tetracycline	quinolone	
B52	B	ST224	*aph(6)-Id* *aph(3")-Ib* *aadA24* *aac(3)-IIa*, *aph(3')-Ia* *aadA2b*	$bla_{CTX-M-15}$ bla_{TEM-1B}	*sul2*, *sul3*	*dfrA14*	*floR* *cmlA1*	*tet*(X4) *tet*(A)	*gyrA* (S83L, D87 N) *parC* (S80I) *parE* (S458 A)	*csgA*, *fdeC*, *fimH*, *lpfA*, *yehABCD*, *hlyE*, *astA*, *cma*, *cvaC*, *terC*, *nlpI*, *gad*, *hrA*, *hha*
R37	A	ST224	*aph(6)-Id* *aph(3")-Ib* *aadA24*, *aac(3)-IIa*, *aph(3')-Ia* *aadA2b*	$bla_{CTX-M-15}$ bla_{TEM-1B}	*sul2*, *sul3*	*dfrA14*	*floR* *cmlA1*	*tet*(X4) *tet*(A)	*gyrA* (S83L, D87 N) *parC* (S80I) *parE* (S458 A)	*csgA*, *fdeC*, *fimH*, *lpfA*, *yehABCD*, *hlyE*, *astA*, *cma*, *cvaC*, *terC*, *nlpI*, *gad*, *hrA*, *hha*
M55	A	ST10	*aph(3')-Ia* *aadA1*, *aadA2b*, *aph(3")-Ib*, *aph(6)-Id*	bla_{TEM-1B}	*Sul1*, *sul2*, *sul3*	*dfrA14* *dfrA7*	*cmlA1* *floR*	*tet(X4)* *tet(A)*	*qnrS1*	*csgA*, *fimH*, *iha* *yehABCD*, *hlyA*, *hlyE*, *iroN*, *fyuA*, *cib*, *AslA*, *traJ*, *traT*, *terC*, *nlpI*, *capU*, *anr*, *hha*, *irp2*, *gad*

Table 2. Genomic features of *tet*(X4)-bearing *E. coli* isolates based on WGS data.

WGS-based resistance gene analysis of strains R37 and B52 identified multiple antimicrobial resistance genes (ARGs) including *aph*, *aadA* and *aac* variants (resistance to aminoglycosides), $bla_{CTX-M-15,}$ bla_{TEM-1B} (resistance to β-lactams), *floR* and *cmlA* (resistance to phenicols), *sul* and *dfrA* (resistance to sulfonamide /trimethoprim), *gyrA* and *parC* mutations (resistance to quinolones), *tetA* and *tet*(X4) (Table 2). In contrast, the M55 isolate displayed resistance to amoxicillin, kanamycin, chloramphenicol, sulfamethoxazole-trimethoprim, and tetracyclines, with reduced susceptibility to quinolones. This resistance pattern was attributed to the presence of bla_{TEM-1B}, *aph(3')-Ia*, *floR/cmlA*, *sul* + *dfrA*, *tet(X4)* and *qnrS1* genes (Table 2). All *tet*(X4)-carrying isolates remained susceptible to amikacin, imipenem, nitrofurantoin and colistin.

WGS-based characterization of virulence genes and plasmid replicons

Both R37 and B52 contained several virulence genes coding for adhesins (*fdeC*, *fimH*, *lpfA* and *yeh* fimbrial operon), hemolysin (*hlyE*), enterotoxin EAST-1(*astA*), colicin M (*cma*), microcin V (*cvaC*), tellurium resistance (*terC*) and several other genes as indicated in Table 2. Strain M55 also carried almost similar set of genes coding for adhesins (*iha*, *fimH*, and *yeh* operon*)* as well as genes coding for colicin (*cib*), siderophore receptor (*iroN*, *fyuA*), hemolysins (*hlyA*/*E*), tellurium resistance (*terC*), *traJ* and etc. (Table 2). According to PlasmidFinder analysis strains R37 and B52 harboured IncX1 and p0111-type plasmids. The plasmid replicon types identified in strain M55 included IncX1, IncQ1, IncI1-I(α) and IncFII/IncFIA/IncFIB(AP001918), (multiple replicons found on the same contig indicating fusion between different plasmid types). The *tet*(X4)-bearing elements from *E. coli* B52 and M55 showed a high degree of similarity to the backbone of the reference plasmid pF45S (GenBank accession PP854070.1) which belonged to IncX1 incompatibility group indicating possible carriage of *tet*(X4) gene on IncX1 plasmid, in both isolates. Comparative genomic analysis revealed the presence of multiple

insertion sequence (IS) elements, which are associated with genetic mobility and structural rearrangement of resistance determinants[9,16]. Both plasmids contained multiple antimicrobial resistance genes, including *floR*, and *tetA* confirming their multidrug resistance potential. Further analysis identified conjugation-associated genes, including *virB*, suggesting the presence of a Type IV Secretion System (T4SS) that may facilitate conjugative transfer[17] (Fig. 2).

Transmissibility and stability of *tet*(X4)

The conjugation assay was performed to evaluate the ability of *tet*(X4)-harbouring *E. coli* isolates R37 and M55 belonging to different STs (ST224 and ST10, respectively), to transfer tigecycline resistance to two different *mcr*-negative colistin resistant *E. coli* strains used as the recipients. The *tet*(X4)-bearing element in each donor isolate could be successfully transferred to only one of the two used recipient strains, with conjugation frequencies of approximately 1.0×10^{-9} (M55) and 5.6×10^{-10} (R37). All the *tet*(X4)-carrying transconjugants exhibited increased MICs of tigecycline (MIC = 64 mg/L) and tested positive for the presence of *tet*(X4) by PCR (Fig. 3). Notably, the recipient cell ECrec2 (Fig. 3A, plate No. 6) —initially resistant to cephalosporins, amikacin, gentamicin, tetracycline and ciprofloxacin but susceptible to tigecycline (MIC = 0.5 mg/L) and nitrofurantoin—acquired resistance to tigecycline (MIC = 64 mg/L) and eravacycline (MIC > 32 mg/L) following conjugation (named transconjugant V1, Fig. 3A, plate No.5). The F1 transconjugant (ECrec1 recipient cell which acquired *tet*(X4)-bearing element from R37 donor cell) acquired resistance to chloramphenicol in addition to tigecycline, eravacycline and tetracycline (Fig. 3, plate No. 2), indicating that the *tet*(X4)-bearing plasmid carried various antibiotic resistance genes. According to Illumina sequencing, *tet*(X4) and *floR* genes were found to be located on the same contig in strains B52 and M55, explaining the co-transmission of these two genes following conjugation.

The stability of *tet*(X4)-mediated resistance was assessed by serial passages of *tet*(X4)-positive donor isolates and their transconjugants in antibiotic-free media. Overall, all four types of *tet*(X4)-positive isolates (R37, M55, V1, and F1) retained the *tet*(X4)-bearing elements at rates exceeding 70%. The M55 donor strain and its corresponding transconjugants (V1) exhibited the highest plasmid stability, with a retention rate exceeding 96% after 10 consecutive passages in antibiotic-free media (Fig. 4).

Discussion

The dissemination of the newly emerged tigecycline/eravacycline-inactivating enzyme Tet(X4) which confers pan-tetracycline-resistance, poses a significant threat to both human and animal health. While *E. coli* has been identified as the primary host and reservoir of the *tet*(X4) gene, recent studies have also reported its detection in other Enterobacterales species, such as *K. pneumoniae*[18] and *Citrobacter freundii*[19]. Currently, *tet*(X4) is predominantly detected in *E. coli* isolates originating from food-producing animals[20]and food products[19,21]. However, its overall prevalence in humans remains lower[22], although a recent increasing trend has been

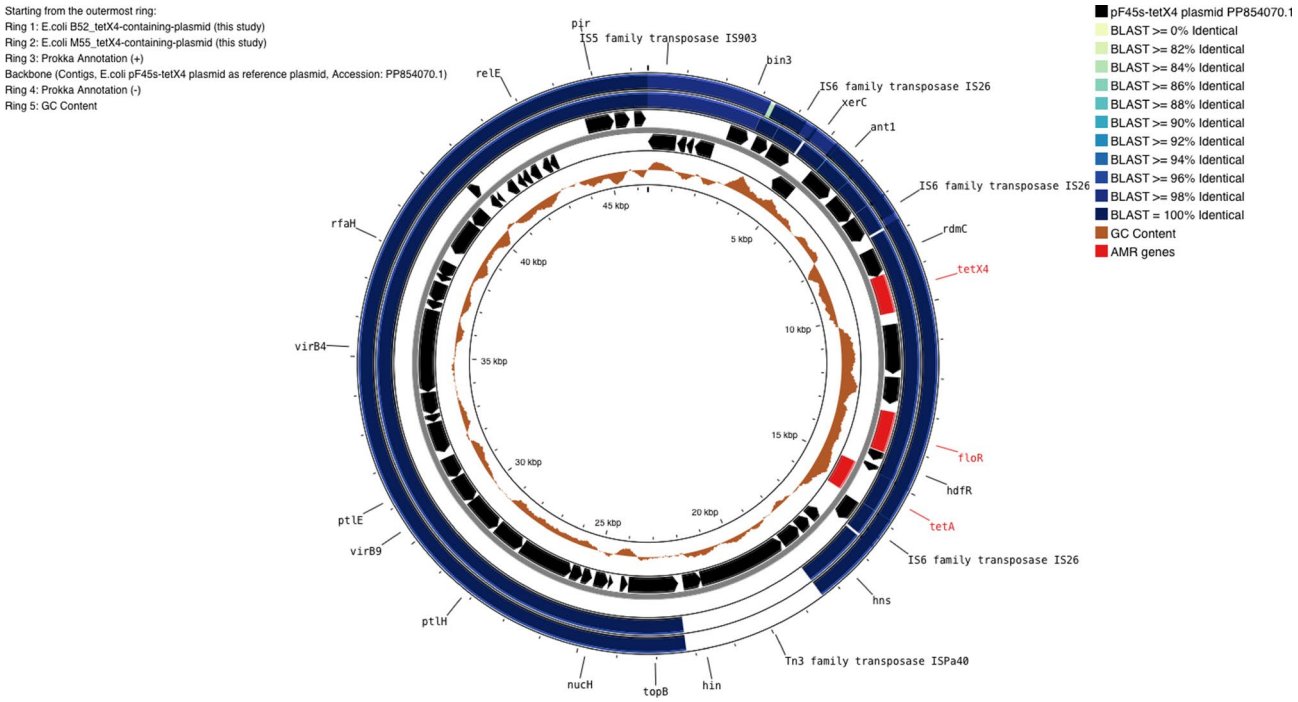

Fig. 2. Comparative genomic analysis of the *tet*(X4)-harboring plasmids detected in *E. coli* isolates B52 (Ring 1) and M55 (Ring 2) both identified in this study, with the reference plasmid pF45S (*E. coli*, Accession: PP854070.1). Color Gradient in Outer Rings: BLAST identity comparisons showing the percentage of sequence similarity between the study plasmids and the reference plasmid.

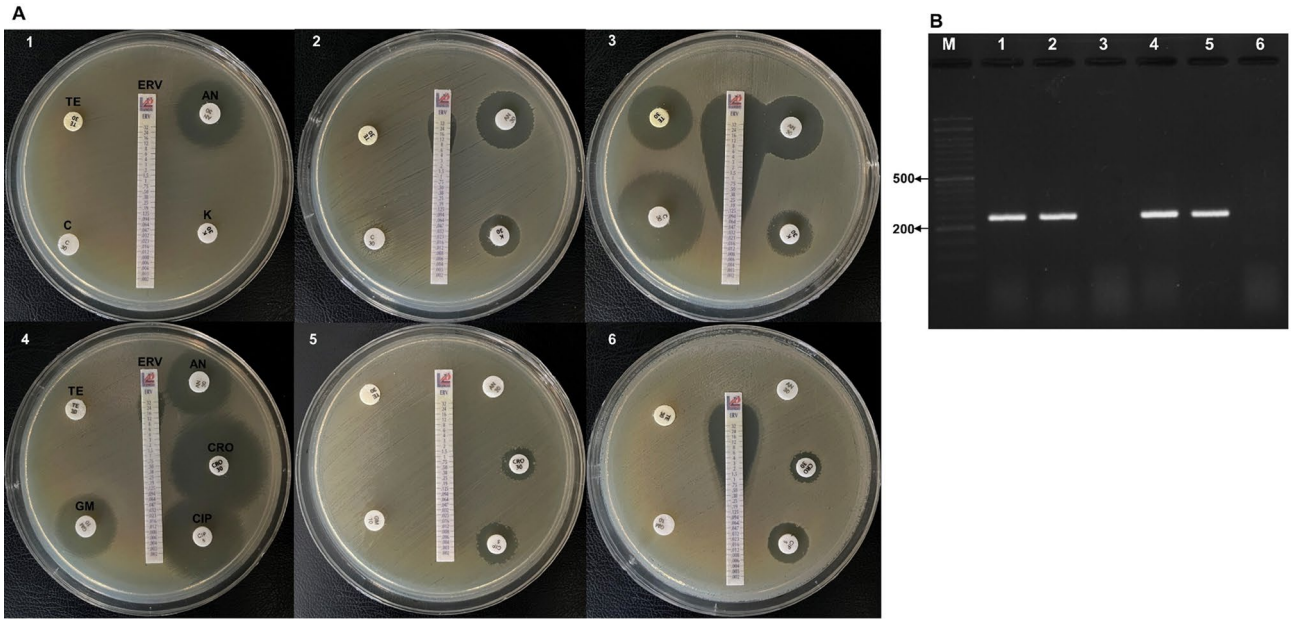

Fig. 3. Comparison of antibiotic susceptibility testing results obtained for eravacycline and other agents in donor cells (1, R37;4, M55, *tet*(X4)-positive cells), their transconjugants (2, F1; 5, V1, after conjugation) and recipient cells (3, ECrec1;6, ECrec2, before conjugation) (ERV, eravacycline; CRO, ceftriaxone; AN, amikacin; GM, gentamicin; TE, tetracycline; CIP, ciprofloxacin; C, chloramphenicol; K, Kanamycin) (**A**); Agarose gel electrophoresis of PCR-amplified product of *tet*(X) gene on 2% agarose gel, lane 1: R37 (donor), lane 2: F1 (transconjugant), lane 3, ECrec1 (recipient cell before conjugation), lane 4: M55 (donor), lane 5: V1 (transconjugant), lane 6: ECrec2 (recipient cell before conjugation), M: 50 bp DNA marker (GeneDireX) (**B**).

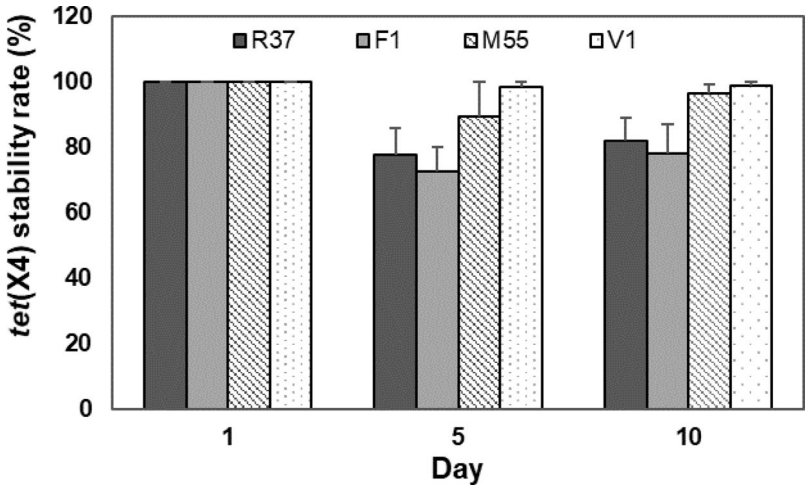

Fig. 4. Stability of plasmid-encoded *tet*(X4) gene in *E. coli* donor strains (R37, M55) and their transconjugants (F1, V1), over 10 days in the absence of tigecycline.

observed[23]. Recent studies have documented correlations between *tet*(X4)-bearing *E. coli*isolates from clinical settings and those from animal and environmental sources, suggesting active cross-sectoral dissemination of these pan-tetracycline-resistant isolates among humans, animals, and farm environments[24]. In this study, the recovery rate of *tet*(X4)-positive *E. coli* was 1.26%, which is relatively higher than rates reported by other studies (0.22%[25], and 0.65%[26], both from China). In contrast, significantly higher detection rates of *tet*(X4) -positive isolates have been reported in retail pork samples (58 out of 139 (41.7%)[19]and 7 out of 34 samples (20.6%)[27]) in China, as well as wastewater samples in Türkiye (4 out of 20 samples (20%))[15].

According to MLST analysis, isolates M55 and R37 which were collected from the same farm (Farm A) belonged to two different sequence types, ST10 and ST224 respectively. In contrast, isolate B52 was obtained from a different site (Farm B) and characterized as ST224. The remaining two *tet*(X4) positive isolates (R34 and S91) were obtained from farm B. To date, the *tet*(X4) has been detected in a variety of phylogenetically unrelated

strains with diverse STs including ST10, ST609[15]and ST195[18,28]. However, some certain sequence types, such as ST10[26,29]and ST761[30–32] have been identified as the most prevalent clones among *tet*(X)-positive *E. coli* isolates from different geographical locations and hosts.

The *tet*(X4) gene can be disseminated horizontally through plasmids with varied replicon types including IncX1, IncQ1, IncFII, IncA/C, as well as hybrid plasmids such as IncFIA-HI1 A-HI1B, and IncFIB(K)-IncFIA(HI1)-IncX1[33]. Among these, the IncX1 and IncFIA-HI1A-HI1B plasmids are recognized as the dominant vectors for the *tet*(X4) gene[18,30,34]. IncX1-type plasmid has been detected in animal-derived strains from various species across diverse regions demonstrating its strong transmission ability and broad host range[18,24].

A recent study demonstrated that an IncX1 plasmid harboring *tet*(X4) in *E. coli* from cattle, could be successfully mobilized and stabilized in clinically important *Enterobacteriaceae*. This finding suggests that *tet*(X4)-bearing IncX1 plasmid play a critical role in the widespread dissemination of *tet*(X4) in clinical settings[35]. Furthermore, hybrid plasmids harboring multiple replicons may exhibit broader host ranges by avoiding plasmid incompatibility, thereby facilitating the widespread dissemination of the *tet*(X4) gene in various clinical settings and farming environments[36].

According to comparative genomic analysis between the *tet*(X4)- bearing elements in isolates M55 and B52 and their nearest neighbor plasmid pF45S as reference, IncX1 plasmid was found as the possible vector mediating dissemination of *tet*(X4) among the studied isolates. Conjugation assays confirmed the transferability of *tet*(X4)-harboring elements from donor isolates to recipient strains. The observed conjugation frequencies were approximately 10^{-9} to 10^{-10} transconjugants per recipient cell. These findings, together with results obtained from the plasmid stability assay, indicate that *tet*(X4)-carrying plasmids are conjugative and exhibit high stability in both transconjugants and donor cells, even in the absence of tigecycline selective pressure.

The transfer frequency of *tet*(X4) gene through conjugation has been reported to vary from 10^{-2} to 10^{-7}[37] or 10^{-9} to 10^{-11}transconjugants per recipient cell[38]under optimal laboratory conditions. In addition to the plasmid type, several other factors influence this variability, including the characteristics of recipient cells, growth phase, cell density, donor-to-recipient ratio, conjugation method, carbon and metal ion concentrations, temperature, pH, and mating duration[39,40].

WGS-based analysis of ARGs revealed the co-existence of *tet*(X4) with genes conferring resistance to several clinically important antimicrobials including aminoglycosides, quinolones, β-lactams, and phenicols. This finding suggests that tigecycline resistance may be co-selected by the presence of other antimicrobial resistance determinants. In our study, tetracyclines (oxytetracycline), quinolones (enrofloxacin), sulfonamides and cephalosporins (ceftiofur) were among the commonly used antibiotics for disease treatment in farms where *tet*(X4) bearing strains were isolated. While tigecycline is approved exclusively for human clinical use, plasmid-borne tigecycline resistance genes have been reported at significantly higher prevalence in bacteria isolated from animals compared to humans. A recent study reported a link between overuse of phenicols in animals and accumulation of tigecycline resistance conferring genes[13]. Liu et al. demonstrated that the presence of tetracycline enhances the stability of *tet*(X4)-bearing plasmids particularly in *E. coli* isolated from poultry[41]. Furthermore, we recently observed that excessive exposure of *E. coli* and *K. pneumoniae* isolates to tetracycline and chloramphenicol can lead to development of tigecycline resistance or decreased susceptibility, possibly through alterations in the major regulators of the AcrAB efflux pump[14]. These findings, suggested that selective pressure exerted by tigecycline, which is a human-restricted antimicrobial may not be exclusively required for the development of tigecycline resistant isolates[42]. Instead, the extensive use of older generation tetracyclines or phenicols in veterinary practice can promote the accumulation of tigecycline resistance determinants among animal's gut microbiome with subsequent potential for dissemination between herds or into human hosts[16,42]. This potential tigecycline-independent mechanism of resistance development poses a serious threat for the therapeutic utility of tigecycline and other newer-generation tetracyclines, such as eravacycline and omadacycline, as the last-resort agents for the treatment of infections caused by notorious extensively drug-resistant Gram-negative bacteria.

Conclusion

This study represents the first published report of MDR *tet*(X4)-positive *E. coli* isolates with high level resistance to tigecycline and eravacycline, from animal sources in Iran. This finding poses a significant threat to human health and food safety. While, no human cases of *tet*(X4)-positive bacteria have been reported from Iran to date, there is growing concern that these animal-originated *tet*(X4)-positive strains may reach to clinical settings or, spread their MDR-*tet*(X4)-bearing plasmids to human pathogens and therefore, contribute to difficult-to-treat human infections in future. Enhanced surveillance of *tet*(X4)-harboring pathogens is urgently required in both clinical settings and animal/farming environments. Such measures are essential to curb the dissemination of pan-tetracycline-resistant MDR strains across environmental, agricultural, and human sectors ensuring both public health and the safety of food products.

Materials and methods

Bacterial isolation and identification

Fresh fecal samples (n= 395) were collected from calves in farm (aged 1 to 12 months) between November 2022 and June 2023. Samples were obtained by swabbing the rectum with a sterile cotton swab. The collected samples were inoculated on Eosin Methylene Blue (EMB) agar plates and incubated at 37 °C for 24 h. Preliminary screening for tigecycline resistant isolates was performed following a previously described method[43]. Briefly, bacterial suspensions (~ 5×10^5 CFU/mL) were prepared in Mueller Hinton broth (MHB) supplemented with 3 mg/L tigecycline and incubated aerobically at 37 °C for 16 to 24 h. A 10-µL sample from tigecycline-containing MHB cultures showing visible bacterial growth, was transferred to EMB agar plates supplemented with 3 mg/L

tigecycline followed by overnight incubation at 37 °C. Colonies growing on the selective media were identified using conventional biochemical methods (including IMViC (indole test, methyl red test, Voges-Proskauer reaction, citrate utilization test), urease, motility and ONPG (O-nitrophenyl-beta-D-galactopyranoside) tests and reactions observed on Triple Sugar Iron (TSI) agar (carbohydrate utilization pattern, H_2S and gas production))[44] and subjected to tigecycline susceptibility testing using the broth dilution method.

Antimicrobial susceptibility testing

Testing susceptibility to tigecycline and colistin was performed by standard broth dilution method using colistin sulfate and tigecycline hydrate powders (Glentham Life Sciences, Corsham, United Kingdom) and freshly prepared (less than 12-h-old) MHB from Difco (BD Diagnostic Systems, Sparks, MD, United States). Moreover, the MIC of eravacycline was determined using MIC test strips (Liofilchem, Roseto degli Abruzzi, Italy) containing concertation gradient range of 0.002 − 32 mg/L. The obtained MIC results were interpreted according to breakpoints issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, V14.0) for Enterobacterales. The Kirby-Bauer disk diffusion method was used for testing susceptibility to other antibiotics including amoxicillin, ceftriaxone, ceftazidime, cefepime, aztreonam, imipenem, tetracycline, doxycycline, ciprofloxacin, nitrofurantoin, chloramphenicol, amikacin, gentamicin, tobramycin, kanamycin and sulfamethoxazole-trimethoprim. The inhibition zone diameter around each antibiotic disk was measured and results were interpreted according to CLSI guidelines (M100-ED34, 2024)[45]. *Escherichia coli* ATCC 25922 was used as a quality-control strain for antimicrobial susceptibility testing.

Detection of *tet*(X) gene by polymerase chain reaction (PCR)

To screen for the presence of *tet*(X) gene, the chromosomal DNA of identified tigecycline resistant bacterial isolates was extracted by boiling method as described previously[46]. Detection of *tet*(X) variants was performed by PCR using gene specific primers (F:5'-TTAGCCTTACCAATGGGTGT and R:5'-CAAATCTGCTGTTTCA CTCG[47]) under the following condition: 1 cycle of denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 35 s, annealing at 54 °C for 35 s and elongation at 72 °C for 35 s, and a final cycle of elongation at 72 °C for 10 min. The amplified products were resolved on 2% (w/v) agarose containing Safe DNA Gel Stain by electrophoresis at constant voltage of 100 V for 40 min and were visualized under the UV light.

Enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis

ERIC-PCR was performed to assess the genetic relatedness of tigecycline resistant *tet*(X)-positive isolates. To this end, bacterial DNA was obtained by the boiling method followed by measuring the quantity (ng/µl) and quality of extracted DNA samples by spectrophotometer. PCR was performed using primers ERIC-1R (5'- A TGTAAGCTCCTGGGGATTCAC) and ERIC-2 (5'- AAGTAAGTGACTGGGGTGAGCG)[48]according to protocol described previously[49]. The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel containing safe DNA gel stain and at constant voltage of 100 V for 1 h. The obtained banding patterns were visualized under UV radiation.

Whole genome sequencing and bioinformatic analyses

Whole genome sequencing was performed as described previously[5]. Briefly, genomic DNA samples of three *tet(X)*-bearing *E. coli* strains were extracted using the Maxwell 16 Cell DNA Purification kit (Promega, US). The quality of the extracted DNA was determined using Qubit quantification system (Thermo Fisher Scientific; Waltham, MA, USA). The DNA library was generated using the Nextera XT DNA Library Prep Kit (Illumina, CA, US) according to manufacturer's instruction. Sequencing was performed on an Illumina NextSeq 500 platform with 150-bp paired-end reading. The sequenced raw reads were trimmed with Trimmomatic and reads shorter than 20 bp were discarded. The high quality cleaned read files were then ensured using FASTQC, and *de novo* assembly was conducted using Unicycler v0.4.8 (https://github.com/rrwick/unicycler, accessed in December 2024). Plasmid sequences were annotated using Prokka v1.14.6 (https://github.com/tseemann/prokka, accessed in December 2024) with default parameters[50]. Comparative genomic analysis was performed using BLASTn (NCBI) and BRIG v0.95 to assess sequence homology between the identified plasmids and the reference *tet*(X4)-bearing plasmid pF45S (*Escherichia coli*, Accession: PP854070) which belonged to IncX1 incompatibility group. A circular genome alignment was generated, displaying sequence identity across different regions. Functional annotations included antimicrobial resistance genes (*tet*(X4), *tet*(A), *floR*), insertion sequences, and genes associated with plasmid conjugation (*virB*, Type IV secretion system). The in silico whole genome-based analysis was performed using the online tools available from Center for Genomic Epidemiology (http://www.genomicepidemiology.org/services/, accessed on March 2025) to determine: (a) sequence types using MLST 2.0[51] (https://cge.food.dtu.dk/services/MLST/), (b) acquired resistance genes and chromosomal mutations associated with antimicrobial resistance using ResFinder 4.6.0[52] (http://genepi.food.dtu.dk/resfinder), (c) plasmid replicon types using the PlasmidFinder 2.1[53] (https://cge.food.dtu.dk/services/PlasmidFinder/) and (d) virulence genes using VirulenceFinder 2.0[54] (https://cge.food.dtu.dk/services/VirulenceFinder/).

The FASTQ sequences of all strains were deposited in the NCBI sequence read archive with BioProject number PRJNA1209696.

Assessing *tet*(X4) transmissibility via conjugation assays

The transferability of *tet*(X4)-carrying elements was assessed by conjugation assay using *tet*(X4)-harboring *E. coli* strains (R37 and M55) as donor and two different *mcr*-negative colistin resistant- *E. coli* strains as recipient (with tigecycline MICs of ≤ 0.5 mg/L). Briefly, 10 µL of overnight cultures of all donor and recipient isolates were inoculated in 10 mL antibiotic-free Luria–Bertani (LB) broth (1:1000 ratio) and incubated at 37 °C with shaking at 200 rpm until OD600 reached to a value of 0.3 ± 0.05. Afterwards, a 1:5 ratios of donor to recipient cell were

mixed in 6 ml of LB broth and incubated overnight at 37 °C in a shaking incubator with gentle shaking (80 rpm). Transconjugants were selected on MHA agar supplemented with 16 mg/L tigecycline and 4 mg/L colistin and incubated at 37 °C overnight. PCR detection of *tet*(X4) gene, tigecycline, eravacycline and colistin MIC determination and ERIC-PCR pattern comparison were performed to confirm the transconjugants. Conjugation frequency was determined as the number of transconjugants divided by the number of recipients[55].

Evaluation of stability of *tet*(X4)-harboring elements

Plasmid stability testing was performed by continuously culturing the *tet*(X4)-positive isolates in antibiotic-free culture media. To this end, 10 µL of overnight cultures of transconjugants and their donors (R37 and M55) were inoculated in 10 mL antibiotic-free LB broth and after 24 h incubation at 37 °C, were continuously sub-cultured in daily refreshed antibiotic-free LB broth for 10 consecutive days. A sample from daily bacterial cultures was taken, diluted and inoculated both onto antibiotic-free and tigecycline containing (8 mg/L) MacConkey agar plates for bacterial enumeration. The retention rate of *tet*(X4)-bearing plasmid was calculated at days 1, 5 and 10 by dividing the number of colonies that grew on tigecycline-containing MacConkey agar plates by the number of colonies on antibiotic-free media[56].

Data availability

The FASTQ sequences of all strains were deposited in the NCBI sequence read archive with BioProject number PRJNA1209696 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1209696).

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Author contributions

M.H designed the study and supervised the project. M.H., D.M.C. and A.G., conceptualized and analyzed the data, and drafted the manuscript. M.H., M.A., K.M., and M.O. generated the data, conducted analyses, contributed to the manuscript text, and created the figures. All authors reviewed and approved the final version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

The study was approved by the Research Ethics Committee of University of Tabriz (Approved IRB No. IR.TABRIZU.REC.1402.121). All methods were performed in accordance with the relevant guidelines and regulations and reported in accordance with ARRIVE guidelines.

Additional information

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