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Detection and Whole-Genome Analysis of tigecycline resistant *Escherichia coli* in poultry and meat samples in Türkiye

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ABSTRACT

The emergence and dissemination of tigecyline resistant *Enterobacterales* (TRE) in animals is a critical issue. This study aimed to investigate the presence of TRE in the gut of healthy avians as well as meat samples. A total of 940 ceacal samples from 94 commercial poultry flocks were collected at slaughter and a total of 335 meat samples [(chicken (n = 159), turkey (n = 4) and beef/lamb (n = 172)] were collected from supermarkets and butcher shops. Out of 960 samples, 146 (19.21 %) samples from chicken farms and 24 (13.3 %) from turkey farms were positive for TRE. Forty-nine *Escherichia coli* isolates were determined to carry the *tet*(X4) gene by PCR and exhibited multi-drug resistance. Whole-genome short-read sequencing (WGS) on all *tet*(X4) positive *E. coli* isolates and long-read sequencing on a selection of five isolates were carried out. WGS identified four ST types (ST206 being the most dominant, ST609, ST744 and ST189), indicating significant homogeneity among tigecyline resistant *E. coli* strains. In 47 isolates, the *tet*(X4) gene was transferrable to *E. coli* EC600 and it was found to be located on the IncX1 plasmid. Additionally, all *tet*(X4)-positive *E. coli* isolates also harbored other resistance genes, including *floR, aadA2* and *tet*(A). In this study, the identification of *tet*(X4) carrying *E. coli* in healthy chicken and meats suggests the likely source of food-producing animals for humans. Therefore, active surveillance of critical priority lineages of TRE should focus on to contain the potential public health risk.

Introduction

Antibiotic resistance has become one of the most important public health concerns, which has been reported to cause 541,000 deaths in 2019 as estimated by European Antimicrobial Resistance Collaborators (Mestrovic et al., 2022). With available evidence that the continued excessive use and misuse of antibiotics is an escalating phenomenon, in part because of usage in agriculture, trend in antibiotic resistance is increasing to the alarming level that is estimated to cause 10 million annual deaths by 2050 (O'Neill, 2016; Roberts and Zembower, 2021). Tigecycline (9-t-butylglycylamido derivative of minocycline) is a last line antibiotic, which has been used for the treatment of severe infections caused by extensively drug-resistant bacteria in humans since its approval in 2005 (Rose and Rybak, 2006). Not surprisingly, reports of clinical resistance to tigecycline began and apparent since 2007 (Sun et al., 2013).

Several mechanisms have been identified for tigecycline resistance in Gram negative bacteria. Among them two main mechanisms, enzymatic inactivation and efflux from the cell, are mediated mostly by plasmid encoded genes, *tet*(X) variants and *tmexCD1-toprJ*, respectively (Zhang et al., 2022a). The *tet*(X) gene family encoding a flavin-dependent monooxygenase were first discovered in the strain of *Bacteriodes* and shown to hydroxylate tetracycline substrates pathway (Yang et al., 2004). The first report of *tet*(X3) in *Acinetobacter baumannii* and *tet*(X4) carrying *Escherichia coli* was in China, in 2019 (He et al., 2019; Sun et al., 2019). Outside China, the presence of *tet*(X) and variants, especially *tet*

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(X4) were also reported in more than 20 countries worldwide (Wang et al., 2022; Zhang et al., 2022a). Recently, the *tet*(X4) gene has been reported mostly in *Enterobacterales* species, especially in *E. coli*. Most importantly, the isolates carrying the *tet*(X4) gene were also reported to show resistance against eravacycline and omadacycline drugs approved recently for human therapy (Chen et al., 2022; Sun et al., 2019). Evidence also shown that they are resistant to other antibiotics, including beta-lactams, chloramphenicol and sulfamethoxazole (Shao et al., 2024).

tet(X4) carrying bacteria have been frequently detected in a variety of food-producing animals (cattle, poultry and pigs), foods of animal origin, wild animals, humans, and manure worldwide (Zhang et al., 2022b). Tigecycline usage in livestock has not been approved in the past, however, other tetracycline antibiotics including doxycycline and tetracycline has been widely used, explaining the development of resistant strains in animal production. Our previous study revealed the presence of *tet*(X4)-harboring *E. coli* isolates in sewage water in 2022 in Türkiye (Kürekci et al., 2022). However, there is no reports on the gut colonization by tigecycline resistant *Enterobacterales* strains among avian species and other livestock. This study aimed to shed light on the prevalence, types of tigecycline resistant *Enterobacterales* strains in avians at slaughter and meat samples in Türkiye. Molecular identification and whole-genome sequencing are used to determine the mechanisms for tigecycline resistance.

Material and Methods

Sampling Procedure

A total of 940 ceacal specimens from avians were collected at slaughter from four slaughter plants, for which three chicken commercial companies (A, B and C) and one turkey company (D) were participated in this study. The participation of companies in this study was on a voluntary basis. Between June 2022 and December 2022, a total of 94 randomly selected flocks (n=28, A; n=25, B; n=23, C and n=18, D) were routinely sampled every fortnight and ten ceacal samples per flock were collected on each sampling day.

A total of 159 samples of chicken meat, turkey meat (n=4) and beef/lamb meat (n=172) samples were purchased randomly from supermarkets and butcher's shops between August and December 2022 in Ankara, Sivas and Hatay provinces in Türkiye. Chicken meat samples came from 14 major poultry companies, presented by letters A-O to preserve anonymity, that produce and sell all over the country.

Isolation of tigecycline resistant isolates

Meat samples and the ceacal samples were used for isolation of *Enterobacterales* with selective enrichment for tigecycline resistance (Li et al., 2021a). 25 g of meat was weighed aseptically and placed into a stomacher bag with 250 mL of LB broth with 1 μg/mL tigecycline. Fresh cecal content of each animal was also suspended in 5 mL of LB broth with 1 μg/mL tigecycline. After overnight incubation at 37 °C, a loopful of suspension was streaked on MacConkey agar with 1 μg/mL tigecycline and were incubated at 37 °C for 24 h. Up to five colonies (red/-pink-colored as well as non-lactose fermenting ones) from each positive plate were transferred into Blood agar plates and incubated at 37 °C for 24 h. Non-lactose fermenting Gram negative bacteria and their results will be reported elsewhere. All isolated bacteria were identified by MALDI-TOF MS (Bruker, Germany). All isolates were preserved in Brain Heart Infusion broth (Oxoid) with 20 % glycerol and stored at -80 °C for further analysis.

Molecular typing of *tet*(X4) carrying *E. coli* was initially analyzed with pulsed-field gel electrophoresis (PFGE) as outlined by PulseNet (www.cdc.gov/pulsenet). Genomic DNA was prepared out of the bacteria in agarose blocks and digested using the restriction enzyme *Xba*I (Thermo Fisher Scientific, Vilnius, Lithuania) to produce distinct DNA fragment patterns. These fragments were resolved in a 1 % agarose gel using CHEF-DR II unit (Bio-Rad, Hercules, CA, USA). Band patterns were analyzed with the *Salmonella* serotype Braenderup strain as reference marker using Bionumerics software (version 7.0; Applied Maths, USA), with genetic similarity assessed using the Dice coefficient, and clustering performed using the unweighted pair group method with arithmetic mean (UPGMA).

Antimicrobial susceptibility testing of isolates

All isolates obtained were tested for susceptibility to tigecycline by use of broth microdilution assay at concentration of 0.125-128 μg/mL as described by Babaeli and Haeili (2021). Tigecyline-resistant strains (n=240), determined by use of the EUCAST epidemiological cut-off value (>0.5 μg/mL), were further tested against a panel of 15 antibiotics (ampicillin, meropenem, ciprofloxacin, azithromycin, amikacin, gentamicin, tigecycline, ceftazidime, cefotaxime, chloramphenicol, colistin, nalidixic acid, tetracycline, trimethoprim and sulfamethoxazole) by use of Sensititre™ plates (EUVSEC3; Thermo Fisher Scientific). *E. coli* ATCC 25922 and previously isolated tigecycline resistant *E. coli* strain (Kürekci et al., 2022) were used for quality control. The results of susceptibility testing in this study were interpreted according to CLSI clinical breakpoints (CLSI, 2020).

Molecular detection of tigecycline resistance genes

All isolates were screened for plasmid-mediated tigecycline resistance genes (*tet*(X), *tet*(X2), *tet*(X3), *tet*(X4) and *tmexCD-toprJ*) by PCR assay (Bartha et al., 2011; He et al., 2019; Li et al., 2021b). DNA extraction was performed by a commercially available DNA purification kit (Invitrogen, PureLink™ Genomic DNA mini kit) according to the manufacturers' protocol. The primers used for each gene are given in supplementary Table 1S. Amplicons were further confirmed by Sanger sequencing through comparison with these deposited in the GenBank database with the Basic Local Alignment Search Tool (BLAST) program.

Conjugation experiments

To investigate the transferability of *tet*(X4), conjugation assays were performed as described by Li et al. (2021b). The *tet*(X4)-positive isolates were used as donors and *E. coli* C600 (rifampicin resistant) was used as the recipient. Bacterial isolates were streaked onto LB agar plates, followed by inoculation in LB broth overnight. Overnight cultures were 100 times diluted and cultivated for 3 h at 37 °C. Then, donors and the recipient were mixed at a ratio of 1:1, and 100 μL of the mixed culture was applied onto LB agar plates, followed by incubation at 37°C for 16 to 20 h. After incubation, we subsequently collected conjugation mixture recovered from LB agar plates in sterile saline. LB agar plates containing tigecycline (2 μg/mL) and rifampin (300 μg/mL) were used to recover *tet*(X4)-bearing transconjugants. The presence of *tet*(X4) in transconjugants was confirmed by PCR as described previously. We also used PCR to validate the plasmid type harboured by the transconjugants by PBRT 2 kit system (DIATHEVA, Italy).

Whole-genome sequencing and bioinformatics analysis

Total genomic DNA were extracted from all *tet*(X4)-carrying isolates as mentioned above. DNA concentrations were measured using Qubit (Invitrogen). Then, samples were used for the library construction and sequencing using the Illumina HiSeq 2500 platform to generate 150 bp paried-end reads. SPAdes was used to assemble the short-read raw sequences to acquire draft genomes (Bankevich et al., 2012). The sequence assembly was curated using prokka (Seemann, 2014), and then applied for phylogenetic analysis using Roary (Page et al., 2015). A pairwise nucleotide polymorphisms (SNPs) distance matrix was generated using snp-dists 0.6.3 (https://github.com/tseemann/snp-dists). The

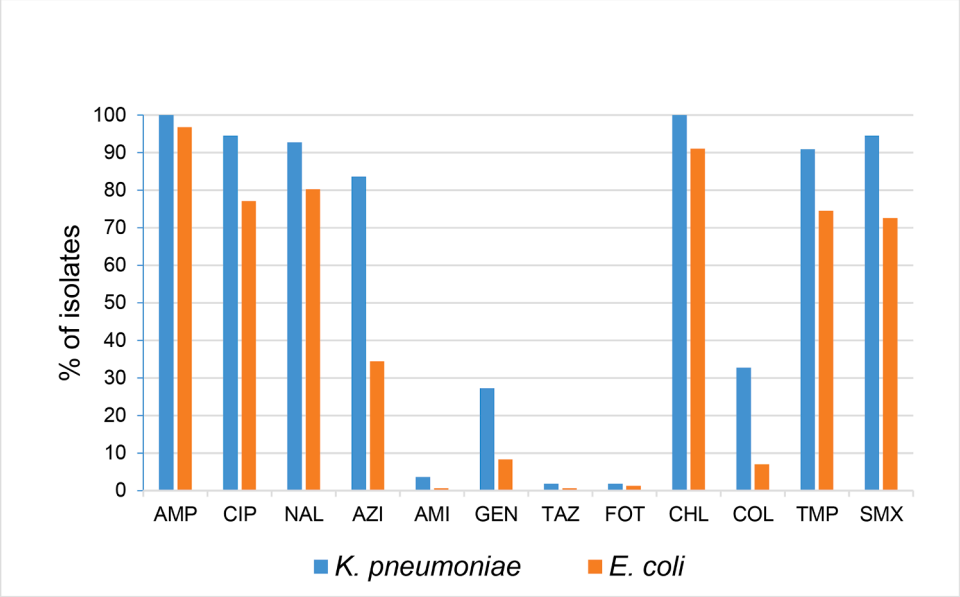

Fig 1. The prevalence (%) of resistance to antimicrobials among *E. coli* and *K. pneumoniae*. AMP, ampicillin; CIP, ciprofloxacin; NAL, nalidixic acid; AZI, azithromycin; AMI, amikacin; GEN, gentamicin; TAZ, ceftazidime; FOT, cefotaxime; CHL, chloramphenicol; COL, colistin; TMP, trimethoprim; SMX, sulfamethoxazole.

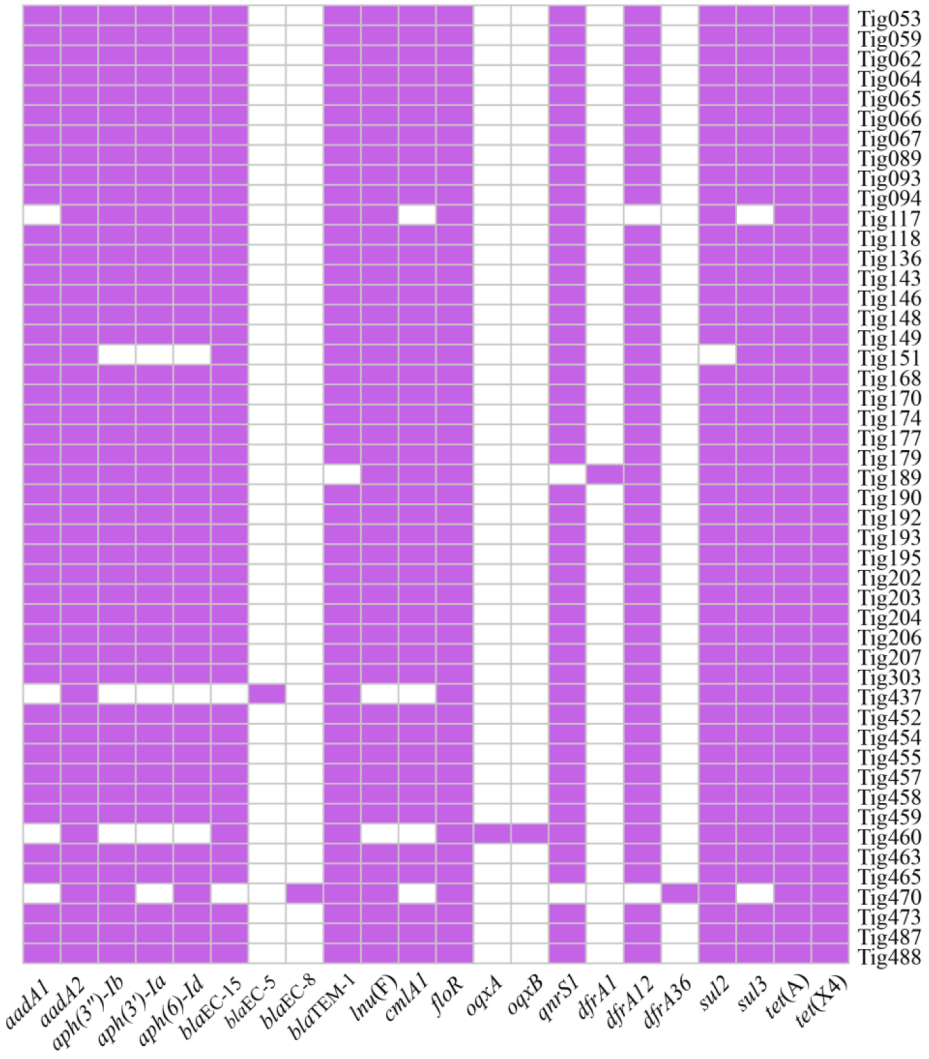

Fig 2. Antimicrobial resistance gene profile of *tet*(X4) carrying *E. coli* isolates.

multilocus sequence typing (MLST), plasmid replicons, and antimicrobial resistance genes were analyzed using tools MLST (Larsen et al., 2012), PlasmidFinder (Carattoli and Hasman, 2020) and ResFinder (Bortolaia et al., 2020) (https://www.genomicepidemiology.org/services/). TBtools was used to visualize antimicrobial resistance genes (Chen et al.,2023). Five representative isolates were selected based on their antimicrobial resistance profile and sources and further sequenced with Oxford nanopore single-molecule long-read MinION sequencing. To acquire the complete sequence of chromosome and plasmids, Unicycler was used to perform *de novo* assembly with the hybrid strategy based on the Illumina short-read data and Oxford nanopore long-read data (Wick et al., 2017). The complete genome sequences were then annotated using Rapid Annotation using the Subsystems Technology annotation website server (https://rast.nmpdr.org/rast.cgi) (Overbeek et al., 2014). Circular comparisons between *tet*(X4)-harboring plasmids were performed using the BRIG (Alikhan et al., 2011). Comparisons between plasmids and draft genome sequences were performed using the website server (https://server.gview.ca/) (Stothard et al., 2019).

The WGS data of the *tet*(X4) carrying *E. coli* were deposited at DDBJ/ENA/GenBank under the BioProject ID PRJNA11955358.

Results

Of the 76 chicken flocks examined, the proportions of farms with at least one TRE positive sample were 86.95 %, 71.42 %, and 48 % for C, A and B, respectively. From 760 ceacal samples of chicken collected, 19.21 % (n=146) were positive for TRE, whereas out of 180 turkey ceacal samples, 13.3 % specimens were positive for presumptive TRE. According to MALDI-TOF-MS results, the bacteria isolated from ceacal samples of chicken (n=152) were identified as 86.92 % (n=132) *E. coli*, 11.76 % (n=18) as *K. pneumoniae* and 1.30 % (n=2) as *K. oxytoca*. On the other hand, all isolates (n=24) from turkey ceacal samples were identified as *K. pneumoniae*.

Out of a total of 159 chicken meat samples, TRE was observed in 31 samples (19.49 %) belonging to eight companies, but no TRE was detected in the four turkey meat samples examined. A total of 33 *Enterobacterales* strains were isolated from positive chicken meat samples, 20 of which were identified as *E. coli* and 13 as *K. pneumoniae*. A total of 4.06 % of the beef/lamb meat samples were positive, and the bacteria detected were *E. coli* (n=5), *Enterobacter cloacae* (n=1) and *Serratia marcescens* (n=1).

MIC values of TRE isolates are given in Supplementary Table S2. The antimicrobial resistance testing results confirmed *E. coli* isolates as tigecycline resistant with MICs of ≥1 μg/mL (1 μg/mL for 51 isolates, 2 μg/mL for 52 isolates, 4 μg/mL for 20 isolates, 8 μg/mL for 20 isolates and 16 μg/mL for 14 isolates). For *K. pneumoniae* it is 1 μg/mL for 3 isolates, 2 μg/mL for 20 isolates, 4 μg/mL for 29 isolates and 8 μg/mL for 3 isolates, while for *K. oxytoca* it is 1 μg/mL for 1 isolate and 4 μg/mL for 1 isolate. As expected, all isolates were resistant to tetracycline with an MIC value of ≥32 μg/mL. When the antibiotic sensitivities were examined, *E. coli* and *K. pneumoniae* isolates were found to be highly resistant to ampicillin (96.8 % and 100 %, respectively), chloramphenicol (91.1 % and 100 %, respectively), ciprofloxacin (77.1 % and 94.5 %, respectively), nalidixic acid (80.3 % and 92.7 %, respectively), trimethoprim (74.5 % and 90.9 %, respectively), sulfomethaxazole (72.6 % and 94.5 %, respectively) and azithromycin (34.4 % and 83.6 %, respectively). While all isolates were found to be susceptible to meropenem, they were found to be resistant to amikacin, gentamicin, colistin, ceftazidime and cefotaxime at a very low rate (Fig 1).

According to PCR amplification results of a total of 240 isolates isolated during the study, 243 bp DNA bands specific to the *tet*(X) gene were obtained in 20.41 % (49/240). All positive isolates are *E. coli*, and their distribution according to the sources they were obtained from is as follows; 13 were found to be from chicken meat samples, one from red meat sample and 35 from chicken ceacal samples. All of the isolates with *tet*(X) were found to be *tet*(X4) positive according to PCR results, and subsequently sequence analysis.

Table 1
Completed whole-genome sequences of five *tet*(X4)-positive *E. coli* isolates.

Strains	MLST	Component	Replicon types	Size (bp)	Resistance gene
Tig089	ST206	Tig089-chromosome	-	4,895,093	*sul3, sul2, aadA1, aadA2, cmlA1, aph(3′)-Ia, tet*(A), *strA, strB, dfrA12*
		pTig089-168kb	IncFIA, IncFIB, IncFIC	168,740	*tet*(A), *qnrS1*
		pTig089-tetX	IncX1	46,845	*tet*(X4), *tet*(A), *lnu*(F), *floR, aadA2*
		pTig089-4kb	ColRNAI	4,982	-
Tig168	ST206	Tig168-chromosome	-	4,856,059	*sul3, sul2, aadA1, aadA2, cmlA1, aph(3′)-Ia, tet*(A), *strA, strB, dfrA12*
		pTig168-167kb	IncFIA, IncFIB, IncFIC	167,912	*tet*(A), *qnrS1*
		pTig168-tetX	IncX1	46,845	*tet*(X4), *tet*(A), *lnu*(F), *floR, aadA1*
		pTig168-4kb	ColRNAI	4,982	-
Tig488	ST206	Tig488-chromosome	-	4,856,835	*sul3, sul2, aadA2, cmlA1, aph(3′)-Ia, tet*(A), *strA, strB, dfrA12*
		pTig488-170kb	IncFIA, IncFIB, IncFIC	170,619	*tet*(A), *qnrS1*
		pTig488-tetX	IncX1	46,848	*tet*(X4), *tet*(A), *lnu*(F), *floR, aadA1*
		pTig488-4kb	ColRNAI	4,982	-
Tig460	ST609	Tig460-chromosome	-	4,672,068	-
		pTig460-tetX	IncFIA (HI1), IncFIB(K)	94,353	*aadA2,* bla_{TEM-1B}, *floR, oqxB, oqxA, qnrS1, sul2, sul3, tet*(A), *tet*(X4), *dfrA12, bleO*
		pTig460-80kb	IncFII (pCoo)	80,025	bla_{TEM-1C}, *floR*
Tig437	ST189	Tig437-chromosome	-	4,659,316	*tet*(A)
		pTig437-tetX	IncFIA (HI1), IncFIB(K)	86,037	*aadA2,* bla_{TEM-1B}, *floR, qnrS1, sul2, sul3, tet*(X4), *dfrA12*
		pTig437-6kb	IncQ1	6,477	-
		pTig437-5kb	Col156	5,146	-

Draft genome sequences of all isolates were acquired in 48 *tet*(X4) positive *E. coli* isolates, were analyzed for acquired antibiotic resistance genes. One isolate failed to meet WGS quality control standards, and therefore excluded from further analysis. Apart from *tet*(X4), 21 other resistance genes were identified in these 48 *E. coli* isolates, conferring resistance to diverse antibiotics, such as aminoglycosides, β-lactams, phenicol, lincosamides, fluoroquinolones, sulfonamides, and tetracyclines (Fig 2). Aminoglycoside resistance gene *aadA2*, florfenicol resistance gene *floR*, and tetracyclines resistance gene *tet*(A) were found in all 48 isolates. Apart from *floR* and *aadA2*, other resistance genes responsible for phenicol resistance *cmlA1* (n=44), and for aminoglycosides resistance, including *aadA1* (n=44), *aph(3″)-Ib* (n=45), *aph(3′)-Ia* (n=44), and *aph(6)-Id* (n=45), were also observed. A total of four genes conferring resistance to β-lactams were detected: bla_{EC-15} (n=46), bla_{EC-5} (n=1), bla_{EC-8} (n=1) and bla_{TEM-1} (n=47). Additionally, three trimethoprim resistance genes *dfrA1* (n=1), *dfrA12* (n=46), *dfrA36* (n=1), and two sulfonamides resistance genes *sul2* (n=47) and *sul3* (n=46) were identified. Furthermore, three quinolone resistance genes, *oqxA, oqxB*

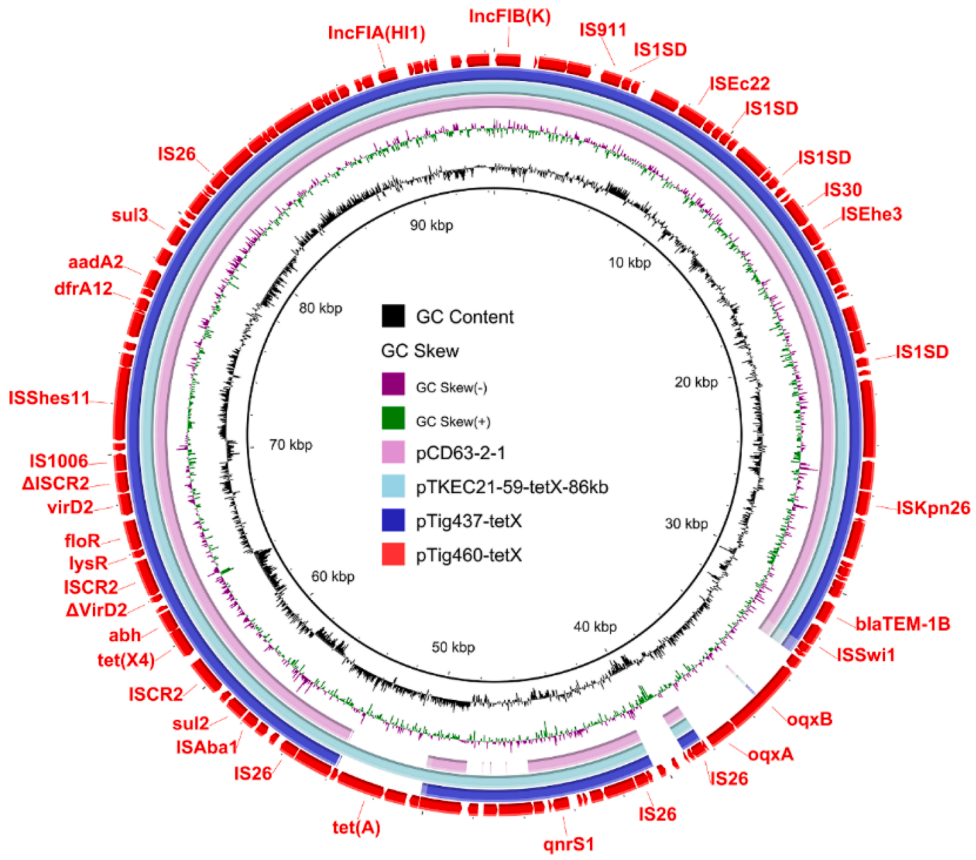

Fig 3. Comparative plasmid map of the IncFIA(HI1)-IncFIB(K) plasmids (pTig437-tetX and pTig460-tetX) harbouring *tet*(X4) with other published plasmids; pCD63-2-1 and pTKEC21-59-tetX-86kb.

and *qnrS1*, were found in one, one and 46 isolates, respectively. Lincosamide resistance gene *lnu(F)* was found in 46 isolates.

According to the PFGE analysis performed for 48 *E. coli* isolates carrying the *tet*(X4) gene, it was determined that they were divided into five different pulsotypes according to the 80 % similarity rate (Supplementary Figure S1). It was determined that 46 isolates from flocks of different chicken companies and from chicken meat samples offered for sale had similar band pattern and were therefore clonally related. Two isolates (Tig470 and Tig460) isolated from chicken meat belonging to two different companies and one isolate (Tig437) from a beef sample were found not to be clonal to the other isolates as they showed different band patterns. Consistent with PFGE analysis, WGS data also showed the close relationship of the majority of isolates, as measured by SNP distance and MLST profile. The *tet*(X4)-carrying E. coli isolates were predominantly found to be ST206 (n=46). The MLST of two chicken meat isolates were ST609 for Tig460 (company E) and ST744 for Tig470 (company A). The MLST of isolate Tig437 obtained from red meat sample was ST189 (Supplementary Table S3).

47 *E. coli* isolates carrying the *tet*(X4) gene were found to be conjugative and PCR experiments provided confirmation that the transconjugants carry the IncX1 plasmid. WGS results revealed the presence of 20 replicon types among *tet*(X4) positive *E. coli* isolates (Supplementary Table S4). Completed sequences of five isolates (Tig089, Tig168, Tig488, Tig460 and Tig437) were acquired. Analysis revealed that *tet*(X4) genes in 48 isolates were located in two kinds of plasmids, including two (Tig460 and Tig437) *tet*(X4)-bearing IncFIA(HI1)-IncFIB (K) plasmids (pTig460-tetX and pTig437-tetX) and 46 *tet*(X4)-harboring IncX1 plasmids (Table 1). pTig460-tetX and pTig437-tetX plasmids shared a high degree of genetic identity and were MDR plasmids carrying *aadA2,* $bla_{\text{TEM-1B}}$, *floR, qnrS1, sul2, sul3, tet*(X4), and *dfrA12* (Table 1). pTig460-tetX also carried *oqxB, oqxA*, and *tet*(A). Sequence analysis showed that pTig460-tetX shared 99.97 % identity at 92 % coverage with plasmid pTKEC21-59-tetX-86kb (CP092453) in *E. coli* and shared 99.97 % identity at 85 % coverage with plasmid pCD63-2-1 (CP050041) in *E. coli* (Fig 3). The genetic structure of *tet*(X4) was IS*CR2-tet*(X4)-*abh*-Δ*VirD2*-IS*CR2*, which may form the *tet*(X4)-bearing circular intermediate to further mediate the transmission of *tet*(X4).

In isolates Tig089, Tig168, and Tig488, the *tet*(X4) genes were located on the IncX1 plasmids, that were MDR plasmids co-harboring various resistance genes, including *tet*(X4), *tet*(A), *lnu*(F), *floR*, and *aadA2* (Table 1). Sequence analysis revealed that these three *tet*(X4)-bearing IncX1 plasmids (pTig089-tetX, pTig168-tetX, and pTig488-tetX) shared 100 % identity at 100 % coverage with plasmid p663Rt_52k_tetX (CP080077) in *E. coli* and plasmid pJZ18-tet(X4) (ON390805) in *K. pneumonia* (Fig 4). Comparisons between pTig089-tetX and draft genome sequences of remaining 43 *tet*(X4) positive isolates revealed that *tet*(X4) in these isolates were located on IncX1 plasmids (Fig 5). The genetic structure of *tet*(X4) was IS*26-abh-tet*(X4)-IS*CR2*.

Discussion

Tigecycline is considered as the last-line antibiotic in clinical treatment, particularly for MDR Gram negative bacteria. Low frequency of tigecycline resistance has been reported in clinical samples (Dong et al., 2022), yet the use of tetracycline group of antibiotics, especially doxycycline, in livestock production faces the challenge of resistance emergence, which might lead to reduced therapeutic efficacy in humans. There have been many studies regarding the isolation and characterization of TRE in different samples from livestock and environments worldwide, however there is no data regarding the frequency and characteristics of TRE isolates in animals in Türkiye. This study

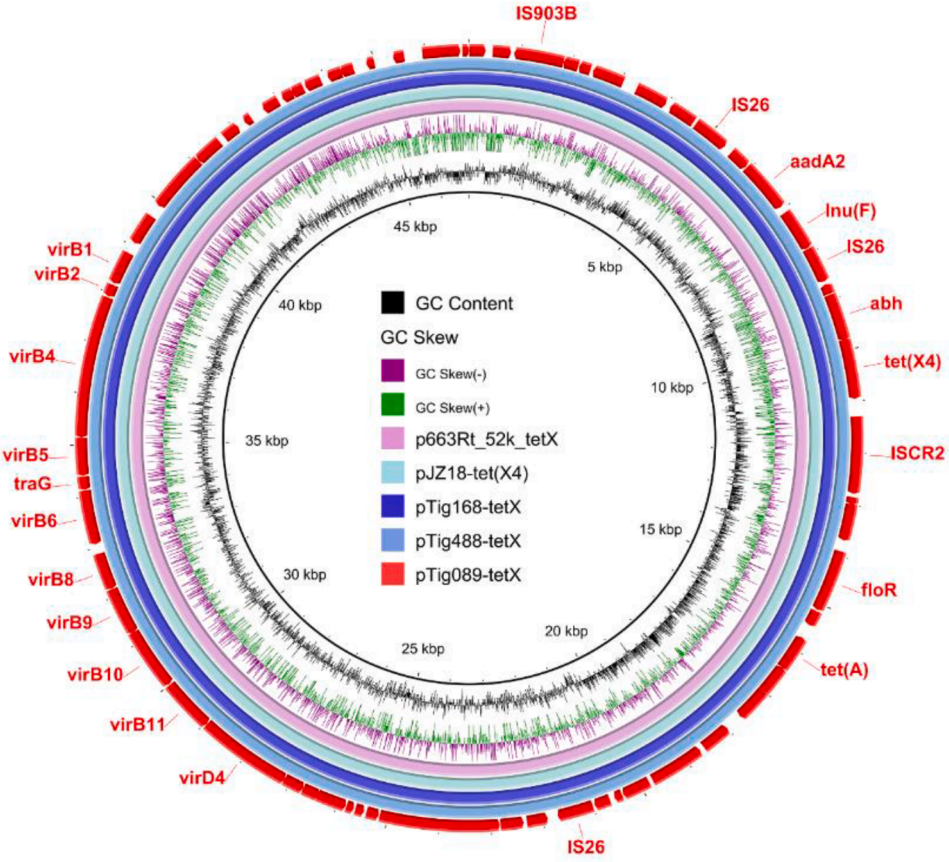

Fig 4. Comparative plasmid map of the IncX1 plasmids (pTig168-tetX, pTig488-tetX and pTig089-tetX) harbouring *tet*(X4) with other published plasmid; p663Tt_52k_tetX.

investigated the occurrence of TRE isolates in healthy avians and meat samples, which was the first study, to our knowledge, in Türkiye.

In the initial phase of this study, the screening of TRE strains revealed that *E. coli* and *K. pneumoniae* were the most commonly isolated tigecycline resistant organisms. While tigecycline resistance *E. coli* was exclusively found in chicken samples (ceacal and meat), *K. pneumoniae* was detected in the ceacal samples of turkey species. Even tough detection frequency of tigecycline resistant bacterial species in different samples could vary by isolation method and study design, the most commonly isolated TRE include *E. coli* and *K. pneumoniae* in livestock environment, especially in poultry and pig (Chen et al., 2022; Feng et al., 2022; Li et al., 2021b; Mohsin et al., 2021). Other TRE species were also observed, including *K. oxytoca, E. cloacae* and *S. marcescens* in the current study. Results of this study also showed that the frequency of TRE occurrence varied based on the poultry company. This may indicate disparity in the use of antibiotics in farms. Considering that no tigecycline resistant *E. coli* was detected in turkey ceacal samples, one can also attribute this to the antibiotic use in these livestock species too. Because of improper use of other tetracycline antibiotics, such as doxycycline in poultry production has been reported to create a selection of tigecycline resistant bacteria (Chen et al., 2022; Fu et al., 2020), tigecycline resistance could be linked with tetracycline antibiotics use in livestock, even though the use of tigecycline in livestock has not been approved in Türkiye.

High level tigecycline resistance in clinical, environmental and animal related isolates of *Enterobacterales*, particularly *E. coli*, has commonly been linked the *tet*(X4) gene (Li et al., 2023; Mohsin et al., 2021; Zhang et al., 2022a; Yang et al., 2023). The current study reaffirmed this concept, as 49 isolates of *E. coli* were identified to be *tet*(X4) carriers, which were mainly from healthy chicken ceacal content (4.6 %) and chicken meat (8.17 %) and one was recovered from red meat sample (0.6 %). Most of the studies related to the prevalence of *tet*(X4)-positive TRE are from China and these studies reported that *tet*(X4)-positive *E. coli* in 8.9 % of cloacal swaps from healthy chicken and up to 8 % of retail meat samples (Dong et al., 2022; Sun et al., 2021; Li et al., 2021b). Additionally, *tet*(X4)-positive *E. coli* rate was reported at 11 % in chicken meat samples and 24.4 % in cloacal swaps of healthy chicken in Pakistan (Li et al., 2022).

Many TRE strains in our study were found to be multiresistant to chloramphenicol, quinolone antibiotics, ampicillin and sulfamethoxazole. Several studies have also determined the high level of resistance to these antibiotics in *tet*(X4)-positive *E. coli* strains found in animals (Shao et al., 2024; Sun et al., 2021). This has been attributed to the coexistence of multiple resistance genes concurrently such as *floR, qnrS, aadA, dfrA* and *sul* (Li et al., 2022; Wang et al., 2022). WGS data obtained herein are consistent with previous findings and revealed 21 other antimicrobial resistance-related genes, among which the *floR, aadA2* and *tet*(A) genes were found in each isolate. Noteworthy is the high presence (more than 90 % of isolates) of the *aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id,* $bla_{\text{EC-15}}$, $bla_{\text{TEM-1}}$, *qnrS1, cmlA1, dfrA12, lnu*(F), *sul2* and *sul3* genes.

Using PFGE, *tet*(X4) positive isolates were initially found to be not genetically distinct. WGS analysis identified four MLST types, among which ST206 emerged as the predominant sequence type. Recently, *tet*(X4) carrying ST206 was also isolated predominantly from avian sources (geese, ducks, and migrotary birds) (Zhang et al., 2023). Notably, one isolate was found to be belong to ST609, whereas another one isolate belonged to ST189. Feng et al., (2022) also discovered ST189 as the commonest type in China (Feng et al., 2022). In our previous study, ST609, associated with *tet*(X4)-positive *E. coli*, was identified in sewage samples in Türkiye (Kürekci et al., 2022). Additionally, the phylogenetic

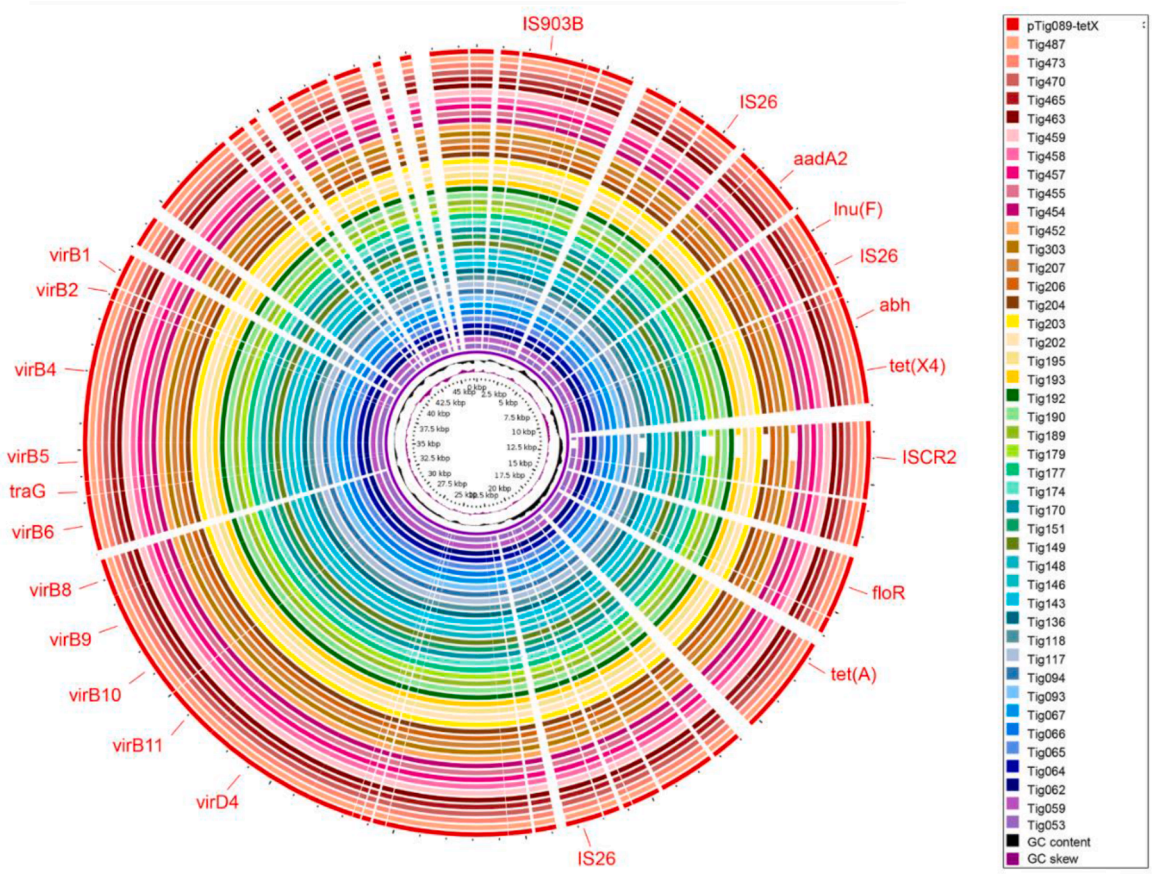

Fig 5. Comparative plasmid map of the IncX1 plasmids found in this study. The draft genome sequences were used here for comparison. The out ring indicates the complete sequence of plasmid pTig089-tetX constructed by nanopore long-read data.

analysis of these isolates revealed high sequence similarity with minimal SNP variations. Hence, there is enough evidence showing the clonal expansion of *tet*(X4) carrying *E. coli*. The observed overlap in both ceacal samples and chicken meat also suggests the occurrence of transmission.

Analysis of 48 *E. coli* strains containing *tet*(X4) found that *tet*(X4) was most commonly located on IncX1 plasmid, that determined to be mobilizable and carried other resistance genes (*tet*(A), *lnu*(F), *floR* and *aadA*). Conversely, *tet*(X4) was present on non-conjugative plasmids [IncFIA(HI1)-IncFIB(K)] in two isolates. Numerous studies have shown that the plasmid is an important factor in the horizontal transfer of the *tet*(X4) gene, which has been typically situated on plasmids, among which the IncX1 has been reported commonly in many *E. coli* strains (Cai et al., 2021; Cui et al., 2022; Feng et al., 2022). Notably, two genetic structures of *tet*(X4) were found, including IS*26*-*abh*-*tet*(X4)-IS*CR2* and IS*CR2*-*tet*(X4)-*abh*-Δ*VirD2*-IS*CR2*. This is in line with previous findings showing the importance of IS*CR2* in the transfer of this gene through a rolling-circle transposition process (Bai et al., 2019; Zhang et al., 2022b). Globally, four basic structures were previously described as carrying *tet*(X4) gene. IncFIA(HI1)-IncFIB(K) was found to be identical to an element, that was found in *E. coli* strain of sewage in our previous study in Türkiye, implying the spread of this environmentally from poultry farms (Kürekci et al., 2022).

In conclusion, tigecycline resistance has been of great interest because of the importance and extensive use of this group of antibiotics and this study is the first finding of *tet*(X4) carrying *E. coli* in healthy chicken and meats, indicating the importance of these as potential vehicles for transmission of these bacteria. The ST206 *E. coli* carrying *tet* (X4)-harboring IncX1 plasmids expressed the same antibiotic resistance phenotype and genotype, implying the clonal distribution in the poultry production in Türkiye. Consistent surveillance of *tet*(X4)-bearing bacteria and other tigecycline resistant bacteria among the animal farming industry should be performed to avoid the widespread transmission of such high-risk clones.

Disclosures

The authors state that they have no known competing financial interests or personal ties that could have influenced the work reported in this study.

Declaration of competing interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.104707.

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