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Original Research

Epidemiology and genetic characterization of *tet*(X4)-positive *Klebsiella pneumoniae* and *Klebsiella quasipneumoniae* isolated from raw meat in Chengdu City, China

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ABSTRACT

The rapid spread of mobile tigecycline resistance presents a significant public health threat, particularly with the increasing prevalence of *tet*(X4)-positive Enterobacterales across various species. This study aimed to investigate the epidemic features and transmission dynamics of *tet*(X4)-positive *Klebsiella pneumoniae* (*K. pneumoniae*) through the analysis of 206 raw meats, including pork (n = 182), beef (n = 16), duck (n = 5), and chicken (n = 3). These samples were collected from schools, markets, and restaurants in Chengdu City, China. A total of 25 isolates were obtained from 13 administrative regions. All isolates exhibited resistance to tetracycline, tigecycline, ampicillin, chloramphenicol, and florfenicol. Over half of the isolates also demonstrated resistance to streptomycin (80 %), sulfamethoxazole/trimethoprim (72 %), ciprofloxacin (64 %), and ampicillin/sulbactam (56 %). Among these strains, 14 distinct sequence types (STs) were identified, revealing evidence of inter-regional clonal spread, notably among 9 *K. pneumoniae* ST3393. Phylogenetic analysis revealed the presence of two *K. pneumoniae* ST5 closely resembling hypervirulent *K. pneumoniae* from Jiangsu. Importantly, 12 isolates were capable of transferring tigecycline resistance to *Escherichia coli* J53. Further plasmid analysis showed that the *tet*(X4)-harboring plasmids in *K. pneumoniae* could be classified into four types, primarily belonging to the IncFIA(HI1)/HI1A/HI1B hybrid plasmid (n = 16) and IncFII plasmid (n = 7), which significantly contributed to the cross-species dissemination of *tet*(X4). In summary, this study highlights the prevalence of MDR *tet*(X4)-positive *K. pneumoniae* in Chengdu, driven predominantly by clonal expansion and plasmid-mediated horizontal gene transfer. These findings emphasize the importance of continuous surveillance of *tet*(X4)-positive *K. pneumoniae* in raw meat and the implementation of effective measures to control their spread.

1. Introduction

In recent years, the global surge in multidrug resistance (MDR) pathogens, coupled with limited advancements in developing effective antibiotics, has led to a critical public health crisis. Tigecycline, the first member of glycylcycline class antibacterial agents, is the 9-t-butylglycylamido derivative of minocycline [1]. Specifically designed to combat major tetracycline resistance mechanisms, including efflux and ribosomal-type resistance, tigecycline demonstrates broad-spectrum *in vitro* activity against both Gram-positive and Gram-negative bacteria [2]. It is increasingly employed, particularly as the last line of defense antibiotic, for treating MDR Gram-negative bacterial infections [3,4]. Despite the emergence of resistance, tigecycline remains among the most effective drugs in clinical practice. According to the China Antimicrobial Surveillance Network (CHINET) report in 2023, examining resistance rates in clinically significant bacteria from 2018 to 2022, *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and *Klebsiella pneumoniae* (*K. pneumoniae*) exhibited resistance rates to tigecycline at 0.1 %, 0.3 % and 4.0 %, respectively [5]. However, the landscape may shift due to the rapid emergence and dissemination of

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HIGHLIGHTS

Scientific question

The widespread dissemination of the mobile tigecycline resistance gene *tet*(X4) poses a significant threat to public health, as it has been detected in multiple species of Enterobacterales across various sources, including animals, the environment, animal-derived food, and humans. However, the prevalence and spread of *tet*(X4) in the clinically important pathogen *Klebsiella pneumonia* (*K. pneumonia*) have not been well understood.

Evidence before this study

Previous research on *tet*(X4) primarily focused on its presence in *Escherichia coli*, with only sporadic reports of its detection in *K. pneumoniae* from different sources. However, in our previous study, we have already identified the presence of *tet*(X4) in clinical *K. pneumoniae* obtained from the feces of hospitalized patients.

New findings

In this study, we have made new findings regarding *tet*(X4)-positive *K. pneumoniae* isolates. We discovered a relatively high prevalence of multidrug-resistant *tet*(X4)-positive *K. pneumoniae* isolates in schools and markets. In addition, our investigation revealed that both clonal spread, indicating transmission within specific *K. pneumoniae*, and horizontal transfer, referring to the transfer of the *tet*(X4) gene between different strains, contribute to the spread of tigecycline resistance. These findings highlight the urgent need for appropriate surveillance and control measures to prevent further dissemination.

Significance of the study

This study underscores the importance of continuous surveillance of *tet*(X4)-positive *K. pneumoniae*, particularly in raw meat, as it can serve as a potential source of transmission. These findings emphasize the necessity of implementing effective measures to prevent the spread of *tet*(X4)-positive *K. pneumoniae* and to address the associated multidrug resistance.

mobile tigecycline resistance genes among animals, animal-derived food, environments, and humans. Presently, several resistance mechanisms, including *tet*(A) variants, *tmexCD1-toprJ1*, and novel *tet*(X) variants, have been identified in conjugative plasmids [6–9]. While *tet*(A) variants have been relatively infrequent, *tmexCD1-toprJ1* and *tet*(X) variants are more commonly documented. However, *tmexCD1-toprJ1* exhibits specificity towards certain species, primarily *K. pneumoniae* in Enterobacterales and *Pseudomonas* spp. [7,10].

Since 2019, a series of novel *tet*(X) variants have been consistently discovered in various sources globally [11,12]. Among these variants, *tet*(X4) has emerged as the most prevalent variant across multiple species of Enterobacterales, with *E. coli* being particularly dominant [13]. In numerous studies, the *tet*(X4) gene has been consistently found on transferable plasmids, facilitating its horizontal gene transfer between different organisms [14]. This observation is further supported by the diverse sequence types (STs) of *tet*(X4)-positive *E. coli* reported in multiple studies [15,16]. However, limited research has explored the emergence of *tet*(X4) in other Enterobacterales species, such as *Citrobacter freundii* (*C. freundii*), *K. pneumonia,* and *Salmonella enterica*, with only a limited number of strains identified [13,17].

K. pneumoniae is a prominent etiological agent for nosocomial and community-acquired infections, widely distributed not only in clinical settings but also in diverse environmental niches [18–20]. Moreover, it is frequently detected in animal-derived foods [21]. Unlike other opportunistic pathogens, *K. pneumoniae* often carries a greater number of acquired antimicrobial resistance genes (ARGs) and plasmids, with the identification of hundreds of ARGs within this species to date. Many ARGs, such as bla_{SHV}, bla_{TEM}, and bla_{CMY}, were first discovered in *K. pneumoniae* before spreading to other pathogens [22]. Consequently, *K. pneumoniae* is considered an "amplifier" for transmitting environmental resistance genes to clinical pathogens. It has gained significant attention in bacterial resistance research, as an "ESKAPE" pathogen alongside *Enterococcus faecium*, *S. aureus*, and *Acinetobacter baumannii* and *Enterobacter* spp. [23]. Despite being observed in meats [24,25], vegetables [26], and inpatients [27], the spread of *tet*(X4)-positive *K. pneumoniae* between different sources remain unclear. This study collected *tet*(X4)-positive *K. pneumoniae* from raw meat in Chengdu City, China, aiming to comprehensively investigate their epidemiological and genetic features, shedding light on potential hazards from a "One Health" standpoint.

2. Materials and methods

2.1. Strain collection, isolation, and identification

From June to August 2020, a comprehensive collection of 206 raw meat samples was conducted across various establishments in Chengdu City. These establishments included 90 schools, 72 farmers' markets, 33 supermarkets, and 11 restaurants, spread across 19 administrative regions. The majority of the samples consisted of pork (n = 182), followed by beef (n = 16), duck (n = 5), and chicken (n = 3). Immediate processing of all samples was carried out upon collection, involving incubation with 100 mL of buffered peptone water (BPW) at a temperature of 37 °C. After a duration of 6 h, the precultures were transferred to LB broth supplemented with 30 mg/L vancomycin and 1 mg/L tigecycline, and subsequently cultivated for a period of 12 h. Following that, the cultures were inoculated onto CHROMagar™ Orientation (CHROMagar, France) supplemented with 4 mg/L tigecycline and incubated for a duration of 24 h. The blue colonies observed on the plates were subsequently selected and subjected to purification on LB agar, a process that was repeated three times. The purified colonies were then subjected to identification using MALDI-TOF-MS (Autof ms1000, Antu, China), with a score above 9.0 indicating a reliable outcome. To screen for *tet*(X4)-positive isolates, polymerase chain reaction (PCR) and Sanger sequencing techniques were employed, utilizing the previously described primer [8].

2.2. Antimicrobial susceptibility test

The broth microdilution method recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) was employed to conduct antimicrobial susceptibility testing on all *K. pneumoniae* isolates. Antimicrobial agents tested included ampicillin, ampicillin/sulbactam, amikacin, aztreonam, cefotaxime, ceftazidime-avibactam, tetracycline, tigecycline, meropenem, colistin, streptomycin, ciprofloxacin, azithromycin, chloramphenicol, florfenicol, and sulfamethoxazole/trimethoprim. The minimum inhibitory concentration (MICs) of most antimicrobials were interpreted under the EUCAST guidance and Clinical and Laboratory Standards Institute (CLSI) standard (M100). However, the MIC of florfenicol was interpreted in accordance with the CLSI standard VET01S. *E. coli* ATCC25922 was used to control the quality.

2.3. Conjugation assay

All *K. pneumoniae* isolates were utilized as donors, while *E. coli* J53 was employed as the recipient. The screening of the corresponding

Fig. 1. Geographical distribution of 25 *tet*(X4)-positive *K. pneumoniae* isolates. These samples were obtained from 7 markets and 15 schools located in 13 administrative regions (highlighted in green) within Chengdu, China. The highest number of *tet*(X4)-positive *K. pneumoniae* was observed in Dujiangyan. Strains isolated from schools are denoted by red markings, while those from markets are represented by black markings. Abbreviations: *K. pneumoniae*, *Klebsiella pneumoniae*; ST, sequence type.

transconjugants was conducted on LB agar supplemented with 100 mg/L NaN_3 and 2 mg/L tigecycline. The verification of the *tet*(X4) gene in the transconjugants was accomplished through PCR and gel electrophoresis, alongside the determination of the species character using MALDI-TOF-MS. Additionally, the susceptibility of these transconjugants to tigecycline was assessed following the aforementioned methodology.

2.4. Whole genetic sequence and bioinformatic analysis

Genomic DNA of *tet*(X4)-positive *K. pneumoniae* isolates were extracted by QIAcube automated nucleic acid extraction system in conjunction with the QIAamp DNA minikit, following the instructions provided by the manufacturer (QIAGEN, Germany). Subsequently, whole-genome sequencing (WGS) was conducted using the Illumina NovaSeq 6000 platform (Personal Biotechnology, Shanghai, China). Following quality control measures, the genome was assembled using SKESA v2.4.0 [28] and annotated using the RAST server (https://rast.nmpdr.org/) and Prokka v.1.14.6 [29]. The plasmid replicons and ARGs were predicted using Staramr v.0.9.1 while the insertions sequence (IS) was identified by ISfinder (https://www-is.biotoul.fr/blast.php) [30]. The phylogenetic tree was constructed using Parsnp v.1.7.4 and visualized by iTOL (https://itol.embl.de/itol.cgi). STs were determined through Kleborate v.2.3.1 and the minimum spanning tree of Multilocus sequence typing (MLST) was constructed using BioNumerics 7.6 (Applied Maths, Belgium). Brig v.0.95 was employed to compare the differences between each *tet*(X4)-harboring plasmid [31]. Online-available datasets of *tet*(X4)-positive *K. pneumoniae* were downloaded from National Center for Biotechnology Information (NCBI) public databases (https://www.ncbi.nlm.nih.gov/, data were accessed in December 2022).

3. Results

3.1. Prevalence and characteristics of tet(X4)-positive K. pneumoniae

A total of 25 *tet*(X4)-positive *K. pneumoniae* isolates were obtained from 206 raw meat samples. Of these, 24 were isolated from pork, and one from duck meat, no strains were identified in chicken or beef samples. Geographically, these isolates were distributed across 13 out of 19 surveyed administrative regions that were surveyed. The highest number of isolates (8) was found in Dujiangyan, including three farmers' markets, a supermarket, and a school. Qionglai followed, with 4 isolates originating from three schools and a farmers' market. The remaining 11 regions each had only 1–2 isolates, involving 11 schools and 2 farmers' markets (Fig. 1). All isolates exhibited resistance to at least three antibiotic groups, resulting in 12 unique resistance profiles (Table S1). The highest resistance rates were observed for tetracycline, tigecycline, ampicillin, chloramphenicol, and florfenicol (each 100 %). Subsequently, resistance rates were documented for streptomycin

Fig. 2. MLST analysis of 54 *tet*(X4)-positive *Klebsiella pneumoniae*. ST3393, exclusively reported in this study, owned the largest number of strains. Only ST5 and ST25 were shared between this study and the NCBI database. Abbreviations: ST, sequence type; MLST, multilocus sequence typing; NCBI, National Center for Biotechnology Information.

(80 %), sulfamethoxazole/trimethoprim (72 %), ciprofloxacin (64 %), ampicillin/sulbactam (56 %), cefotaxime (24 %), and aztreonam (4 %). All isolates remained susceptible to amikacin, azithromycin, ceftazidime-avibactam, meropenem, and colistin.

3.2. MLST of 25 local isolates and 29 online strains

MLST analysis of the 25 *K. pneumoniae* isolates revealed 13 STs, with ST3393 being the most prevalent (n = 9), followed by ST873 (n = 3). *K. pneumoniae* ST3393 were widespread, spanning seven administrative regions, including eight schools and a farmers' market. Three *K. pneumoniae* ST873 were identified in farmers' market (Dujiangyan, n = 2) and a school (Pujiang). Furthermore, both *K. pneumoniae* ST5 and ST45 were found in farmers' markets, with two ST5 isolates originating from Dujiangyan and Pengzhou, and two *K. pneumoniae* ST45 from Dujiangyan and Longquanyi. These findings suggested the potential clonal spread of specific *tet*(X4)-positive *K. pneumoniae* isolates across Chengdu, despite ST diversity among all isolates. In addition to our collection, we obtained 29 *tet*(X4)-positive *K. pneumoniae* genomes from the NCBI database (Table S2). The majority of these strains (n = 22) originated from eight provinces in China, namely Jiangsu, Sichuan, Guangxi, Guangdong, Shandong, Anhui, Hebei, and Beijing. The remaining strains were obtained from Singapore (n = 5), Australia (n = 1), and Thailand (n = 1). These strains also exhibited a diverse range of STs, totaling 24 distinct STs. However, our study and the NCBI database only shared two common STs (ST5 and ST25), suggesting that the prevalence of *tet*(X4)-positive *K. pneumoniae* may vary across different geographical regions (Fig. 2).

3.3. Phylogenetic analysis of 54 tet(X4)-positive K. pneumoniae

To further explore the evolutionary relationship between these isolates, a core genome-based phylogeny was conducted on the 25 local isolates and 29 NCBI strains. The analysis revealed three species, including 43 *K. pneumoniae*, 6 *Klebsiella quasipneumoniae* (*K. quasipneumoniae*) subsp. similipneumoniae, and 5 *K. quasipneumoniae* subsp. quasipneumoniae, according to the latest *K. pneumoniae* taxonomy (Fig. 3A). Notably, despite the WGS analysis confirming the affiliation of isolate SWJC4150Bt with *K. quasipneumoniae* subsp. similipneumoniae, it exhibited a distant relationship with the three species, indicating this isolate may be a novel species of this genus. Consequently, after the redescription of the species, three separate phylogenetic trees were constructed. Among the 43 *K. pneumoniae* strains, 120,491 single nucleotide polymorphisms (SNPs) were shared. The resulting phylogenetic tree exhibited two distinct clades as determined by the hierarchical Bayesian clustering (BHC) algorithm (Fig. 3B). Nine *K. pneumoniae* ST3393 isolates formed a closely related independent clade, with a genetic distance of 0 - 44 SNPs. Among these, eight *K. pneumoniae* ST3393 isolates from pork exhibited a closer genetic relationship (0 - 3 SNPs), while being relatively distant from another isolate (SWJC3585Bt) obtained from duck meat (41 - 44 SNPs). The remaining 34 strains exhibited dispersed distribution in another clade, and the clonal spread of *K. pneumoniae* among diverse sources was not observed. Notably, two *K. pneumoniae* ST5 isolates from pork in Sichuan Province displayed a close genetic relationship to a hypervirulent *K. pneumoniae* ST5 strain from Jiangsu Province, suggesting a potential inter-provincial spread of *K. pneumoniae* ST5 through pork transportation. Furthermore, analysis of two phylogenetic trees for *K. quasipneumoniae* revealed significant genetic divergence among these strains, each sharing over 20,000 SNPs (Fig. 3C&D).

3.4. Distribution of ARGs and plasmid replicons

The ARGs within 54 *K. pneumoniae* isolates exhibited complex diversification. Among these isolates, we identified a total of 99 acquired ARGs conferring resistance to various antimicrobials. Apart from *tet*(X4), the most prevalent ARGs was the quinolone resistance gene *oqxB* (92.59 %), followed by tetracycline resistance gene *tet*(A) (81.48 %). Additionally, 11 resistance genes were detected in over half of these strains, including quinolones [*oqxA* (79.63 %) and *qnrS1* (74.07 %)], phenicol [*floR* (75.93 %)], sulfonamide [*sul1* (55.56 %) and *sul2* (72.22 %)], trimethoprim [*dfrA12*, (50.00 %)], β-lactamase [$bla_{\text{TEM-1B}}$ (57.41 %)], aminoglycosides [*aadA2* (57.41 %), *aph*(3″)-*Ib* (51.85 %), and *aph*(6)-*Id* (51.85 %)] ARGs, and the disinfectant resistance gene [*qacE* (53.70 %)] (Fig. 4). Among the 25 *tet*(X4)-positive *K. pneumoniae* isolates in this study, the number of resistant genes ranged from 4 to 28, with an average of 16.28 per strain. Statistical analysis revealed no significant difference ($P > 0.05$) in the number of ARGs compared to *tet*(X4)-positive *K. pneumoniae* isolates in the NCBI database (17.24 replicons).

Plasmid replicon analysis revealed considerable diversity, with a total of 30 known replicons identified among the 54 strains. Among the 25 local isolates, it was observed that they carried 4 - 28 replicons, with an average of 4.88 replicons per isolate. Statistical analysis indicated no significant difference ($P > 0.05$) between these *tet*(X4)-positive *K. pneumoniae* isolates and those present in the NCBI database (4.66 replicons). However, distinctions persisted between the two groups. The three most frequently observed replicons were IncFIA (HI1) (n = 20), IncHI1A (n = 16), and IncHI1B(R27) (n = 16). Con-

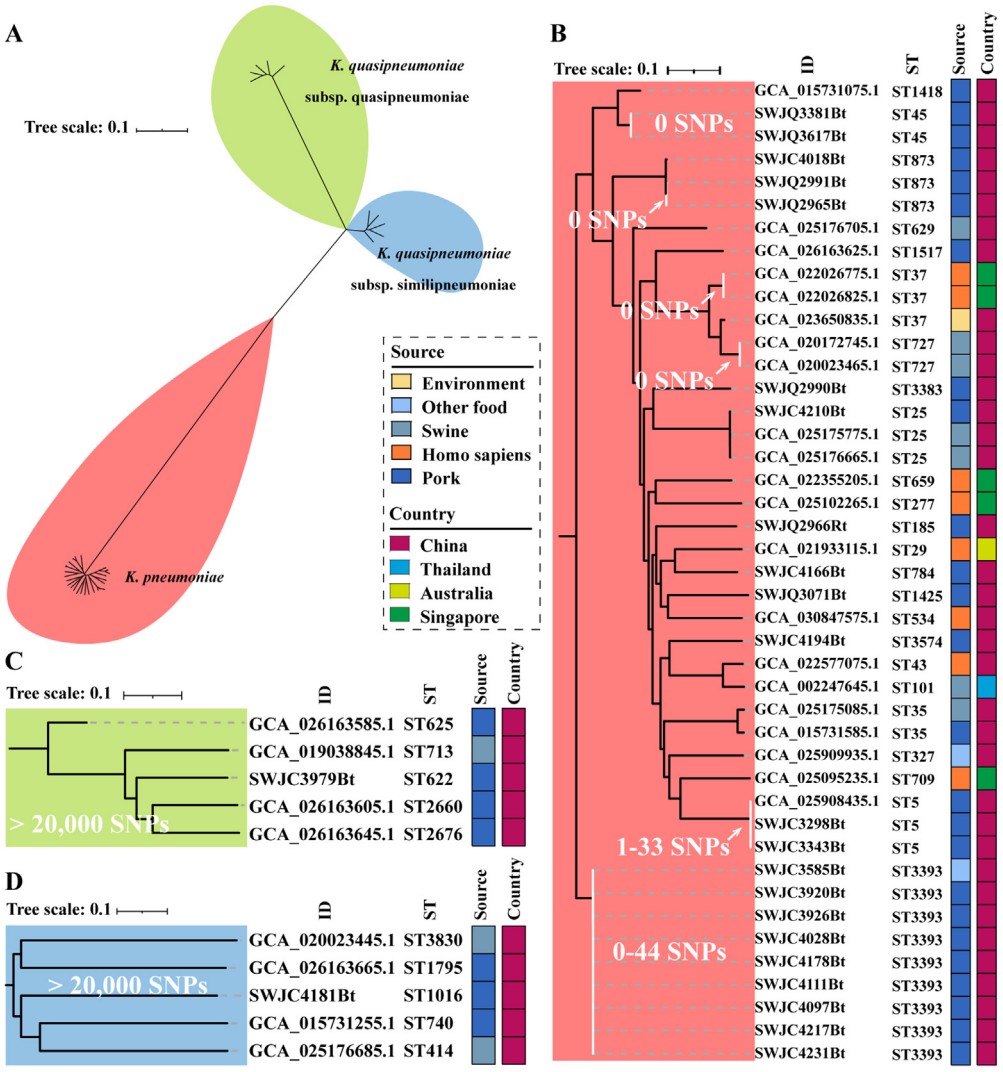

Fig. 3. The core genome phylogeny of the *tet*(X)-positive *K. pneumoniae* strains. A) The phylogenetic tree of 54 *tet*(X)-positive *K. pneumoniae* strains, divided into three clades. Re-constructed phylogenetic tree according to three species: *K. pneumoniae* (B), *Klebsiella quasipneumoniae* (*K. quasipneumoniae*) subsp. quasipneumoniae (C), and *K. quasipneumoniae* subsp. similipneumoniae (D). Abbreviations: *K. pneumoniae, Klebsiella pneumoniae*; SNPs, single nucleotide polymorphisms; ST, sequence type.; ID, identification numbers.

versely, *tet*(X4)-positive *K. pneumoniae* strains from the online database exhibited IncFIB(K) (n = 19), IncFIA(HI1) (n = 15), and IncFII(K) (n = 12) as the most prevalent replicons.

3.5. Plasmid classification and comparative analysis of their potential structures

To explore the possible plasmid structure and genetic feature of these isolates, we conducted multiple plasmid sequence alignments using Brig software. Our results revealed that 25 local *K. pneumoniae* isolates can be matched to four known incompatibility group plasmids from other *K. pneumoniae* strains.

Firstly, pRDZ41 (accession: CP139495.1), obtained from a human-derived *K. pneumoniae* in the NCBI database, is a 190 kb IncFIA(HI1)/HI1A/HI1B hybrid plasmid carrying six ARGs, including *tet*(X4), *aadA22*, bla_{TEM-1B}, *lnu*(G), *qnrS1*, and *floR*. By comparison, 16 *K. pneumoniae* isolates in this study and 9 from the NCBI database carried similar plasmid sequences. Of these, 17 were isolated from pork, 6 from pigs, and the remainder from the environment (n = 1) and duck meat (n = 1) (Fig. 5A). A conjunction experiment with 17 local strains showed that six isolates transformed their tigecycline resistance to the recipient strain J53, with a frequency in the range of (7.34 ± 3.56) × 10^{-9} - (1.59 ± 0.12) × 10^{-6}. Subsequently, using pRDZ41 as the reference sequence for BLAST comparison revealed over 10 highly similar 190 kb plasmids (99 % coverage and 99 % similarity) from *E. coli* isolated from pork, chicken, human, pets, and livestock manure samples, such as pYPE10-190 k-tetX4 (accession: CP041449.1), pT16R-1(accession: CP046717.1), and pE-T306-tetX4 (accession: CP090284.1), etc. Furthermore, this type of plasmid is also present in *Morganella morganii* (pXY36-tet(X4), accession: NZ_ON390820.1), *Enterobacter cloacae* (pTECL_2-190 k-tetX4, accession: MZ773210.1), among others (Fig. S1A). This observation suggests a wide host range, indicating its ability to horizontally spread across diverse species.

Secondly, pYZ-58-tetX (accession: CP109771.1) is a 79,225 bp IncFII plasmid retrieved from the NCBI database, originating from *K. pneumoniae* isolated from pork and carrying only the *tet*(X4) gene. In comparison, 15 *K. pneumoniae* strains shared similar plasmid sequences, with 7 from this study and 8 from the NCBI database. These strains were sourced from pork (n = 13), pigs (n = 1), and vegetables (n = 1) (Fig. 5B). The conjunction experiment of seven local strains showed that four isolates transformed their tigecycline resistance to

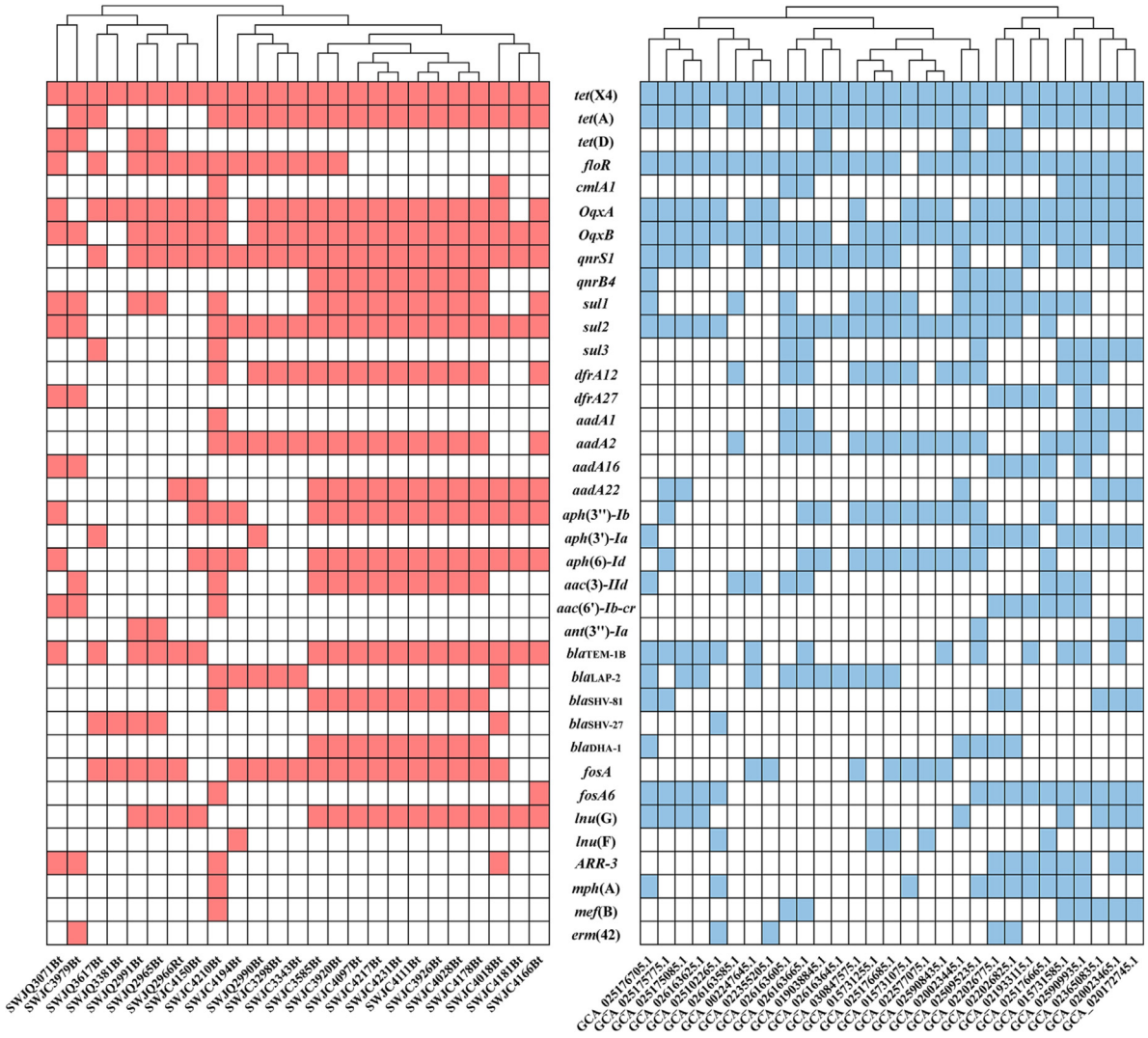

Fig. 4. Distribution of ARGs in *tet*(X4)-positive *Klebsiella pneumoniae*. Red represents the isolates collected in this study, and blue indicates those from the NCBI database. Abbreviations: NCBI, National Center for Biotechnology Information; ARGs, antimicrobial resistance genes.

the recipient strain J53, with a frequency ranging from (2.15 ± 0.76) × 10^{-9} - (1.25 ± 0.60) × 10^{-6}. Subsequently, using pYZ-58-tetX as the reference for BLAST comparison, two highly similar plasmids were identified (98 % coverage and 100 % similarity), namely pNTT31XS-tetX4 (accession: CP077430.1) from porcine-derived *K. pneumoniae* in Jiangsu Province and pSDP9R-tetX4 (accession: MW940621.1) from pork-derived *K. pneumoniae* in Shandong Province, both approximately 79 kb in length (Fig. S1B).

Thirdly, pJZ18-tet(X4) (accession: ON390805.1) is a 57 kb IncX1 plasmid from the NCBI database, carrying six ARGs, including *tet*(X4), *aadA2*, bla_{SHV-12}, *lnu*(F), *tet*(A) and *floR*. In comparison, this plasmid was identified in four strains, including one isolate SWJC4194Bt from this study (Fig. 5C). The conjunction experiment involving SWJC4194Bt demonstrated that this isolate could transfer its tigecycline resistance to the recipient strain J53, with a frequency of (1.59 ± 0.12) × 10^{-6}. A BLAST comparison of pJZ18-*tet*(X4) revealed 3 highly similar plasmids (100 % coverage, 99.9 % similarity), all 57 kb in length, obtained from *E. coli* and *C. freundii* (Fig. S1C).

Finally, p20SC1-3AZ6BT (accession: CP139498.1) is a 229 kb IncFIA(HI1)/C/R hybrid plasmid obtained from *K. pneumoniae* isolated from pig anal swabs in Sichuan, China. This plasmid carries 17 ARGs, including *tet*(X4), *oqxB*, *tet*(A), *floR*, *aac*(3)-*IId*, *aac*(3)-*VIa*, *aac*(6′)-*Ib-cr*, *aadA16*, *ant*(3″)-*Ia*, *ARR-3*, bla_{CMY-2}, bla_{TEM-1A}, *dfrA27*, *erm*(42), *qacE*, *sul1*, and *tet*(D). Sequence comparison revealed a similar plasmid structure in a local pork-derived *K. pneumoniae* isolate SWJC3979Bt from Sichuan Province in this study (Fig. 5D). The conjunction experiment of SWJC3979Bt showed that this isolate also could transfer its tigecycline resistance to the recipient strain J53. Moreover, only two *tet*(X4)-negative plasmids from *Proteus vulgaris* and *Proteus mirabilis* in the NCBI database showed similarity to p20SC1-3AZ6BT (80 % coverage, 99.9 % similarity), namely pJZ30-tet(X4) (accession: ON390809.1) and pJZ49-tet(X4) (accession: ON390810.1), both isolated from pig slaughterhouses in Sichuan Province, China (Fig. S1D).

Overall, these findings indicate that the presence of the *tet*(X4) in *K. pneumoniae* may be associated with the horizontal transform of conjugative *tet*(X4)-harboring plasmid among Enterobacterales from multiple sources.

4. Discussion

Since the discovery of *tet*(X4) in 2019, numerous studies have explored the presence of *tet*(X4)-positive bacteria in animals, the environment, food, and humans, investigating the transmission dynamics of tigecycline resistance across various sources [16,24,32]. However, research specifically focusing on *tet*(X4)-positive *K. pneumoniae* has been limited, and the relationship between *tet*(X4)-positive *K. pneumoniae* from different sources remains unclear. Thus, this study aimed to

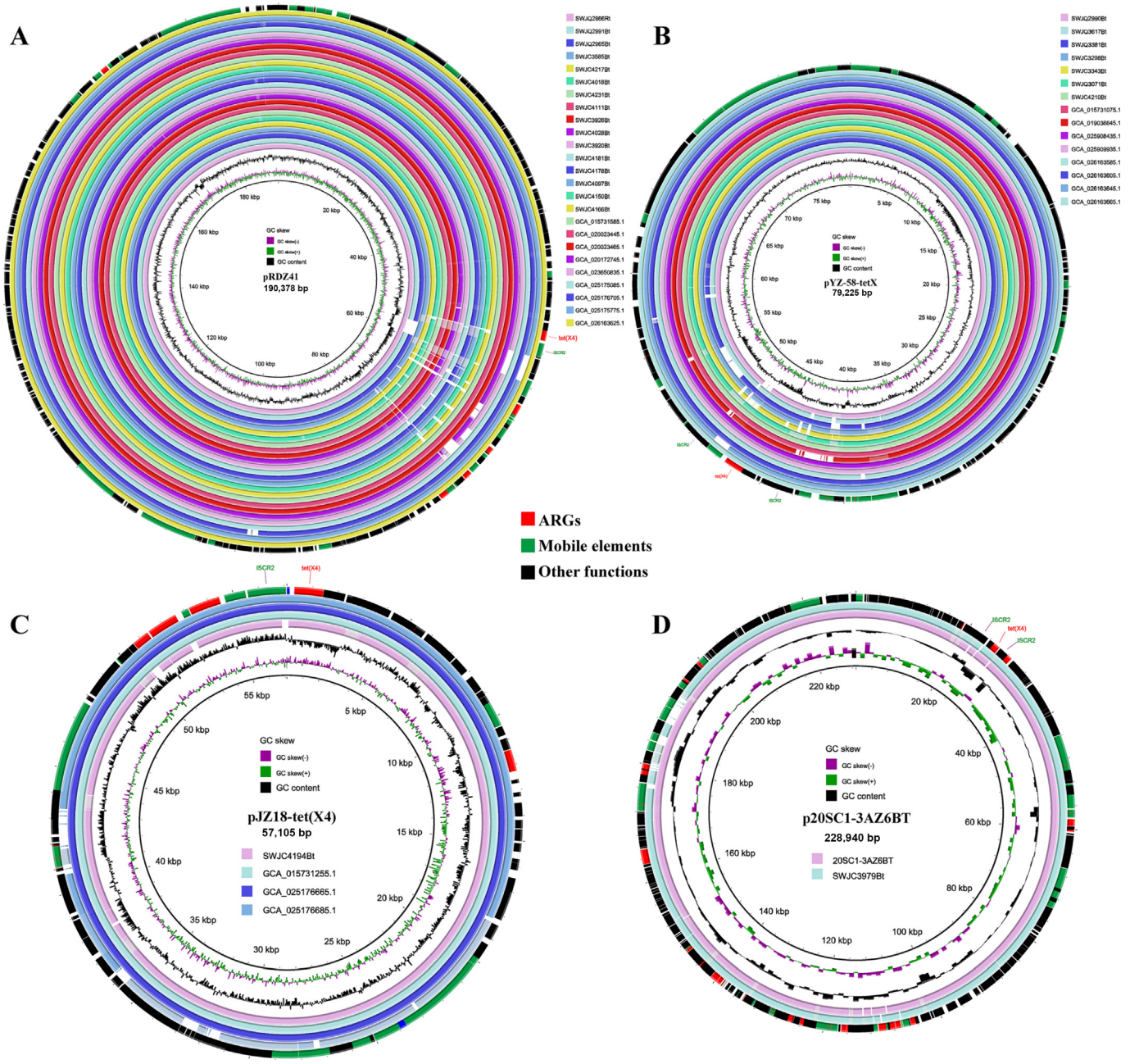

Fig. 5. Comparative sequence analysis of *tet*(X4)-positive *Klebsiella pneumoniae* genome to four online plasmids. A) IncFIA(HI1)/HI1A/HI1B hybrid plasmid; B) IncFII plasmid; C) IncX1 plasmid; D) IncFIA(HI1)/C/R hybrid plasmid. Abbreviation: ARGs, antimicrobial resistance genes.

investigate the prevalence of *tet*(X4)-positive *K. pneumoniae* in schools, supermarkets, farmers' markets, and restaurants across multiple administrative regions of Chengdu, to assess any potential connection between *tet*(X4)-positive *K. pneumoniae* strains from different sources. In limited known studies, although *tet*(X4)-positive *K. pneumoniae* has been sporadically observed in meat or vegetables in certain provinces in China, its detection in clinical *K. pneumoniae* highlights the potential impact on human health [27]. Our study revealed a relatively high prevalence of this strain from schools and farmers' markets, with rates of 16.7 % and 12.5 %, respectively, surpassing those in previous studies conducted in other provinces [24,25]. Moreover, these rates were also higher than the prevalence of *tmexCD1-toprJ1*-positive Enterobacteriaceae (3.4 %) and *tet*(X4)-positive *E. coli* (8.0 %) found in retail meat samples [7,33]. These findings serve as an early warning for the risk of food contamination associated with *tet*(X4)-positive *K. pneumoniae*, posing a significant threat to human health, particularly among students.

In previous studies, the presence of *tet*(X4) alongside other AGRs has often been observed, resulting in *tet*(X4)-positive strains exhibiting resistance to multiple clinical antimicrobial agents [9,15,32]. Consistent with these findings, all *tet*(X4)-positive *K. pneumoniae* isolates in this study were found to be MDR. In addition to exhibiting high-level resistance to tetracyclines, a majority of these isolates also demonstrated resistance to ampicillin, chloramphenicol, streptomycin, ciprofloxacin, and sulfonamides. Therefore, it is crucial to exercise caution when administering these drugs in clinical settings in the presence of *tet*(X4)-positive *K. pneumoniae* infections. Fortunately, all isolates remained susceptible to the other two types of last-resort

antibiotics, including colistin and carbapenems, along with commonly used clinical drugs such as azithromycin and amikacin, suggesting potential treatment options. However, it is important to recognize that resistance mechanisms to these antibiotics have been extensively observed in *K. pneumoniae* strains [22], such as the plasmid-mediated colistin resistance gene *mcr-8* [34] and carbapenemase gene bla_{NDM-5} [35]. Given the notable capacity of *K. pneumoniae* to acquire drug-resistant genes from neighboring sources [22], ongoing surveillance of MDR *tet*(X4)-positive *K. pneumoniae* is imperative to mitigate its impact on human health.

The sequence types of *tet*(X4)-positive *K. pneumoniae* exhibit considerable diversity, similar to *tet*(X4)-positive *E. coli* [16,36]. In this study, we identified 13 distinct STs, most of which have not been previously described in *tet*(X4)-positive *K. pneumoniae*. This finding strongly suggests that the *tet*(X4) gene has undergone extensive dissemination among various STs of *K. pneumoniae*. It is worth noting that clonal spread was commonly associated with the inter-regional transmission of significant ARGs, such as bla_{KPC}-positive *K. pneumoniae* ST11 [37] and *mcr-1*-positive *E. coli* ST10 [38], but has not been frequently observed in *tet*(X4)-positive strains previously [39]. However, our study identified *K. pneumoniae* ST3393 as the predominant sequence type, demonstrating clonal spread capabilities across various educational institutions and a farmers' market spanning seven administrative regions in Chengdu. This discovery raises concerns as it suggests a potential association with the same distribution company, similar to previous instances of foodborne outbreaks in schools resulting from a unified distribution system. For instance, a significant foodborne outbreak in Korea involved more than a thousand students from several schools, linked to the consumption of contaminated chocolate cakes provided by the same company [40]. A thorough examination of the distribution system employed by these schools is therefore crucial. Furthermore, this study identified two *tet*(X4)-positive *K. pneumoniae* ST5 that exhibited a high degree of similarity to a hypervirulent *K. pneumoniae* found in Jiangsu Province, further confirming the potential for inter-regional clonal spread of *tet*(X4)-positive *K. pneumoniae*. Overall, the clonal spread of *tet*(X4)-positive *K. pneumoniae* may represent an overlooked mechanism for the transmission of the *tet*(X4) gene.

Plasmid-mediated horizontal gene transfer (HGT) plays a significant role in the dissemination of various important ARGs, including ESBLs, *mcr-1*, and bla_{NDM} [41,42]. Previous research has also demonstrated the horizontal transfer of *tet*(X4) among diverse Enterobacterales through multiple conjugative plasmids, such as IncX1 and IncX1-containing hybrid plasmids [16,24,43]. In this study, nearly half of the *tet*(X4)-positive *K. pneumoniae* isolates transferred their tigecycline resistance to *E. coli* J53, indicating a high likelihood of the emergence of the *tet*(X4) gene in *K. pneumoniae* being associated with the horizontal transfer of conjugative plasmids between different species. To further support this notion, an analysis was conducted on the potential plasmid structure in all *tet*(X4)-positive *K. pneumoniae*, with two plasmids being predominantly prevalent. Among these, a ~ 190 kb IncFIA(HI1)/HI1A/HI1B hybrid plasmid emerged as one of the most frequently encountered *tet*(X4)-harboring plasmids. This particular plasmid, classified as a high-risk plasmid, has been associated with the dissemination of various clinically significant ARGs, including *tet*(X4), *mcr*, bla_{NDM}, and bla_{OXA} [44]. Significantly, this plasmid has been extensively identified in Enterobacterales derived from both human and nonhuman sources, thereby presenting a potential risk to public health. IncFII plasmids, the other major plasmid type discovered in some provinces in China, are predominantly found in *K. pneumoniae*, serving as a major vehicle for bla_{KPC} dissemination worldwide [45]. Although the plasmid sequence alignment could not provide complete plasmid structure, these results confirm that transferable plasmids play an important role in the spread of tigecycline resistance in *tet*(X4)-positive *K. pneumoniae*.

Given the successful clonal spread of certain dominant STs of *K. pneumoniae* and the wide horizontal transfer of MDR plasmids, it is crucial to develop effective strategies to mitigate the spread of the mobile tigecycline resistance gene *tet*(X4). Continued monitoring of *tet*(X4)-carrying plasmids is essential to understand their transmission dynamics in both clinical and foodborne pathogens. Research on the prevalence and distribution of clinically-associated clones is necessary to elucidate the transmission routes of *tet*(X4)-positive *K. pneumoniae* among animals and humans. Moreover, adopting a comprehensive "One Health" approach encompassing humans, animals, and the environment is imperative to effectively develop infection prevention measures and antibiotic management practices tailored to the specific needs of each sector. Implementation of these strategies will enhance clinical prevention and control measures against antimicrobial resistance, leading to a reduction in the burden caused by *tet*(X4)-positive *K. pneumoniae* infections.

In conclusion, this study offers valuable insights into the prevalence, antibiotic resistance profiles, sequence types, and potential transmission routes of *tet*(X4)-positive *K. pneumoniae* in schools and other public settings in Chengdu. The high rates of tigecycline resistance and MDR profiles in these isolates highlight the urgent need for enhanced surveillance, implementation of infection control measures, and prudent use of antibiotics to mitigate the impact of *tet*(X4)-positive *K. pneumoniae* on public health. Additionally, the HGT and inter-regional transmission of *tet*(X4)-positive *K. pneumoniae* strains via clonal spread emphasizes the importance of adopting "One Health" approach to address this emerging threat. Continued research and collaborative efforts are imperative to further elucidate the epidemiology, mechanisms of resistance, and potential interventions for *tet*(X4)-positive *K. pneumoniae* infections.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Weishuai Zhai: Investigation, Visualization, Writing – original draft. **Yiqing Wang:** Software, Validation, Writing – original draft. **Honghu Sun:** Investigation. **Bo Fu:** Investigation, Software. **Qidi Zhang:** Resources, Supervision. **Congming Wu:** Resources, Supervision. **Jianzhong Shen:** Conceptualization, Project administration, Supervision. **Dejun Liu:** Funding acquisition, Project administration, Writing – review & editing. **Yang Wang:** Funding acquisition, Writing – review & editing.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bsheal.2024.02.004.

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