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Antimicrobial Resistance and Genomic Epidemiology of *tet*(X4)-Bearing Bacteria of Pork Origin in Jiangsu, China

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Abstract: The emergence of tigecycline-resistant bacteria in agri-food chains poses a public health concern. Recently, plasmid-mediated *tet*(X4) was found to be resistant to tigecycline. However, genome differences between *tet*(X4)-positive *Escherichia coli* of human and pork origins are still under-investigated. In this study, 53 pork samples were collected from markets in Jiangsu, China, and 23 *tet*(X4)-positive isolates were identified and shown to confer resistance to multiple antibiotics, including tigecycline. *tet*(X4)-positive isolates were mainly distributed in *E. coli* (n = 22), followed by *Klebsiella pneumoniae* (n = 1). More than half of the *tet*(X4) genes were able to be successfully transferred into *E. coli* C600. We downloaded all *tet*(X4)-positive *E. coli* isolates from humans and pork found in China from the NCBI database. A total of 42 known STs were identified, of which ST10 was the dominant ST. The number of ARGs and plasmid replicons carried by *E. coli* of human origin were not significantly different from those carried by *E. coli* of pork origin. However, the numbers of insertion sequences and virulence genes carried by *E. coli* of human origin were significantly higher than those carried by *E. coli* of pork origin. In addition to *E. coli*, we analyzed all 23 *tet*(X4)-positive *K. pneumoniae* strains currently reported. We found that these *tet*(X4)-positive *K. pneumoniae* were mainly distributed in China and had no dominant STs. This study systematically investigated the *tet*(X4)-positive isolates, emphasizing the importance of the continuous surveillance of *tet*(X4) in pork.

Keywords: *tet*(X4); plasmids; food safety; genomics

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1. Introduction

In recent years, multidrug-resistant (MDR) Gram-negative bacteria have posed a serious threat to public health [1,2]. Because of its broad-spectrum antibacterial activity, tigecycline is considered the last resort in the clinical treatment of infection caused by MDR bacteria [3,4]. Tigecycline belongs to a class of drugs called glycylcyclines. Similar to tetracycline, it can reversibly bind to the 30 S subunit of the ribosome, interfering with amino acid translation and inhibiting bacterial growth [5,6]. However, He et al. discovered the plasmid-mediated mobile tigecycline resistance genes *tet*(X3) and *tet*(X4) in Enterobacteriaceae and *Acinetobacter* in 2019 [7]. The *tet*(X4) gene often possesses complex genetic environments and is distributed in plasmids of multiple plasmid replicon types [8]. Notably, previous studies have shown that pork is an important reservoir of *tet*(X4) [9,10]. However, studies on the genomic epidemiology of *tet*(X4) in pork are still lacking.

The *tet*(X4) gene has been identified in a variety of Enterobacteriaceae, such as *E. coli*, *K. pneumoniae*, *Aeromonas caviae* and *Escherichia fergusonii* [10,11]. However, the vast

majority of reported *tet*(X4) are distributed in *E. coli*. Furthermore, the presence of *tet*(X4) usually does not result in a significant fitness cost to *E. coli*, which further exacerbates the spread of *tet*(X4) in *E. coli* [10]. In addition to *E. coli*, the *tet*(X4) gene was sporadically detected in *K. pneumoniae* of different sources, including human sources and pork samples [10,12]. In this study, we analyzed the emerging *tet*(X4)-positive isolates isolated from pork samples in Yangzhou, China, in 2021. Meanwhile, we also compared the genomic differences of all reported *tet*(X4)-positive *E. coli* from human and pork sources in China using genomics methods, providing a genomic landscape of *tet*(X4)-positive isolates from various sources.

2. Materials and Methods

2.1. Bacterial Isolates

The 53 pork samples were randomly collected from markets in Yangzhou, China, in May 2021. Tigecycline-resistant isolates were selected on MacConkey agar plates with tigecycline (4 mg/L). 16S rRNA gene sequencing was used to perform bacterial species identifications of purified isolates. The *tet*(X4) gene was determined by PCR with reported primers [7].

2.2. Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) of *tet*(X4)-positive isolate strains were conducted against nine antibiotics and antimicrobials, including chloramphenicol, ciprofloxacin, meropenem, florfenicol, streptomycin, colistin, cefoperazone, tigecycline and tetracycline. *E. coli* ATCC 25922 was used as the quality control strain. The resistance breakpoint was interpreted according to the EUCAST criteria (>0.5 mg/L, V12.0) for tigecycline and CLSI guidelines for other antimicrobials [13].

2.3. Conjugation Experiments

The assessment of the transferability of the *tet*(X4) gene was conducted by conjugation experiments using *tet*(X4)-positive isolates as the donor strains and rifampicin-resistant *E. coli* C600 (Rif^R) as the recipient strain (1:1) at 37 °C [14]. The transconjugants were recovered on LB agar plates containing rifampicin (300 mg/L) and tigecycline (4 mg/L). PCR was used to further confirm the transconjugants. The plasmid replicon types carried in the original isolates and corresponding transconjugants were identified by PCR (Table S1).

2.4. Whole Genome Sequencing

According to the results of bacterial species identification and resistance phenotypes, six representative isolates were selected for WGS. The genomes of tigecycline-resistant strains were extracted with the FastPure bacteria DNA isolation Minikit (Vazyme, China) and quantified by a Qubit 4 Fluorometer. The genomic DNA samples were sequenced using the Illumina Hiseq 2500 platform with a 2 × 150 bp paired-end library. The paired-end reads were de novo assembled using SPAdes version 3.14.0 with the default parameters.

2.5. Bioinformatics Analysis

The assembled sequences were annotated through the RAST online server (https://rast.nmpdr.org/, accessed on 1 August 2022) automatically. ResFinder, PlasmidFinder and ISfinder with the default parameters were used to detect the antibiotic resistance genes (ARGs), plasmid replicon types and insertion sequences [15–17]. For *tet*(X4)-carrying *K. pneumoniae* that was only sequenced with short-read sequencing, the contigs acquired by de novo assembly were aligned with *tet*(X4)-positive circular plasmids carrying different replicons to obtain the *tet*(X4)-positive plasmid types [18]. Virulence genes were determined using ABRicate (https://github.com/tseemann/abricate,

accessed on 1 August 2022) and Kleborate (https://github.com/katholt/Kleborate, accessed on 1 August 2022). The multi-locus sequence types (MLST) of all *tet*(X4)-positive isolates were assigned using the mlst software (https://github.com/tseemann/mlst, accessed on 1 August 2022). Phylogenetic trees of *E. coli* and *K. pneumoniae* were constructed using Roary and FastTree based on single nucleotide polymorphisms (SNPs) of core genomes [19,20]. The phylogeny analysis was visualized and retouched using iTOL (https://itol.embl.de, accessed on 18 August 2022).

2.6. Data Availability

The sequences obtained in this paper have been deposited in the GenBank database under the BioProject number PRJNA900003.

3. Results

3.1. Characterization of tet(X4)-Bearing Isolates among Pork

A total of 23 tigecycline-resistant isolates were collected from 53 pork samples. The 16S rRNA gene analysis showed that they were all *E. coli* (95.65%), except one that belonged to *K. pneumoniae* (4.35%). Antimicrobial susceptibility testing showed that these isolates all belonged to MDR isolates. Except for tigecycline (8–128 mg/L), these isolates were also resistant to other antibiotics such as florfenicol, chloramphenicol, streptomycin and tetracycline. However, all these isolates were susceptible to colistin and meropenem (Table S2).

3.2. Transferability of the tet(X4) Gene

To evaluate the transferability of *tet*(X4) in these isolates, conjugation assays were performed for these *tet*(X4)-positive isolates with *E. coli* C600 as the recipient. The *tet*(X4) gene in 14 isolates, including 13 *E. coli* isolates and 1 *K. pneumoniae* isolate, was successfully transferred to C600. The results of plasmid replicon typing showed that the *tet*(X4) gene was mainly located on IncX1-IncHI2A hybrid plasmids (35.71 %), followed by IncX1 plasmids (21.43 %) (Table S3).

3.3. Phylogenetic Analysis of tet(X)-Positive E. coli

To further investigate the evolutionary relationship of the *E. coli* isolated from pork samples, we downloaded all genomes of *tet*(X)-positive *E. coli* isolated from humans (n = 48) and pork (n = 69) in the NCBI database and constructed a phylogenetic tree based on SNPs of the core genomes (Figure 1, Table S4). We noted that some *tet*(X)-positive *E. coli* isolated from pork samples share high similarity (1–68 SNPs) with *tet*(X)-positive *E. coli* collected from a human source, and there is a possibility of clonal transmission. The MLST analysis showed that these *tet*(X4)-positive *E. coli* were divided into 42 known STs, of which ST10 was predominant. In addition, we noticed that these isolates all carried multiple ARGs [6–23].

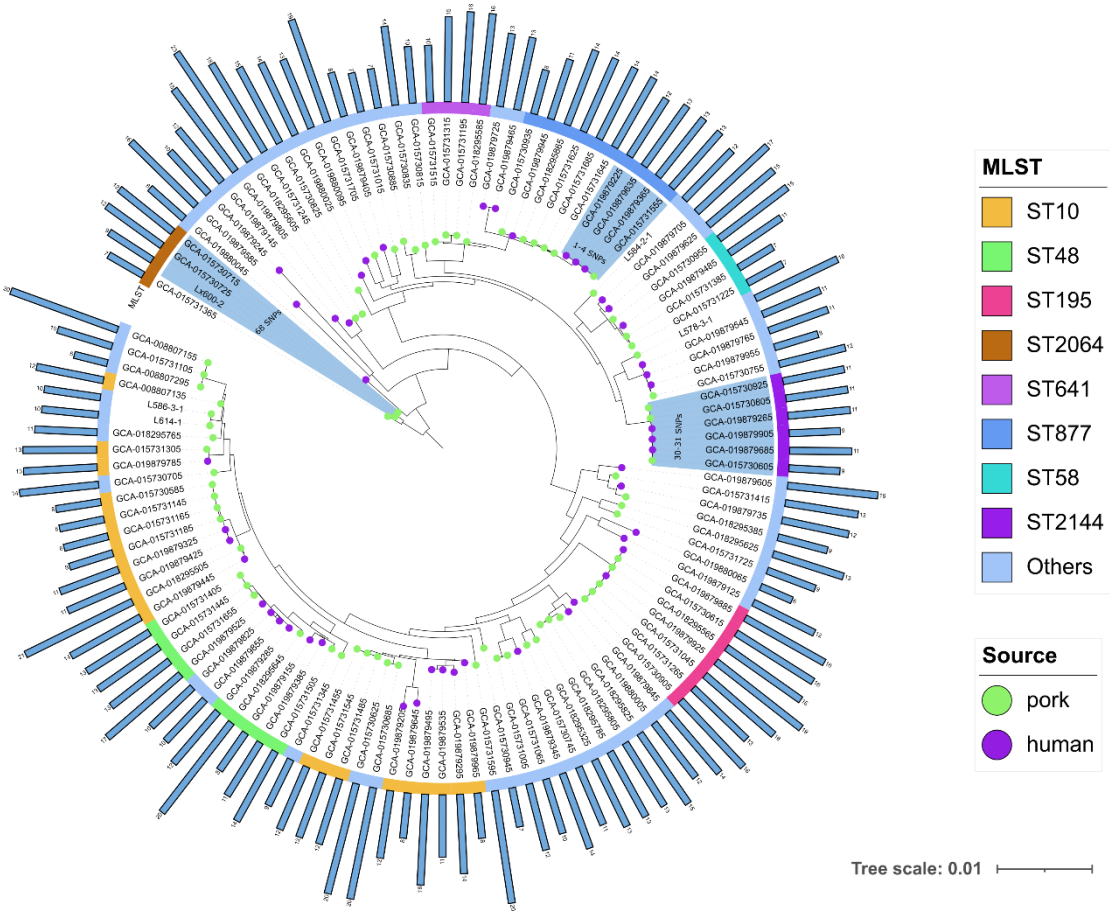

Figure 1. Phylogenetic analysis of 122 *tet*(X4)-positive *E. coli* isolates from pork and human samples. Blue-shaded areas represent strains with minor SNP differences. Histograms represent the number of resistance genes carried in the isolates.

3.4. Genome Sequence Features of tet(X)-Positive E. coli

In order to further elucidate the genomic characteristics of *tet*(X4)-positive *E. coli* isolated from pork and humans, we counted the ARGs, virulence genes, plasmid replicons and insertion sequences carried by these *E. coli* isolates. As shown in Figure 2, the number of ARGs carried by *E. coli* of human origin was close to that carried by *E. coli* of pork origin, with no significant difference ($p > 0.5$). Similar to the results of ARGs, there was also no significant difference in the number of plasmid replicons carried by *E. coli* from two different sources ($p > 0.5$). However, *E. coli* of a human source carries far more virulence genes ($p < 0.5$) and insertion sequences ($p < 0.001$) than *E. coli* of a pork source.

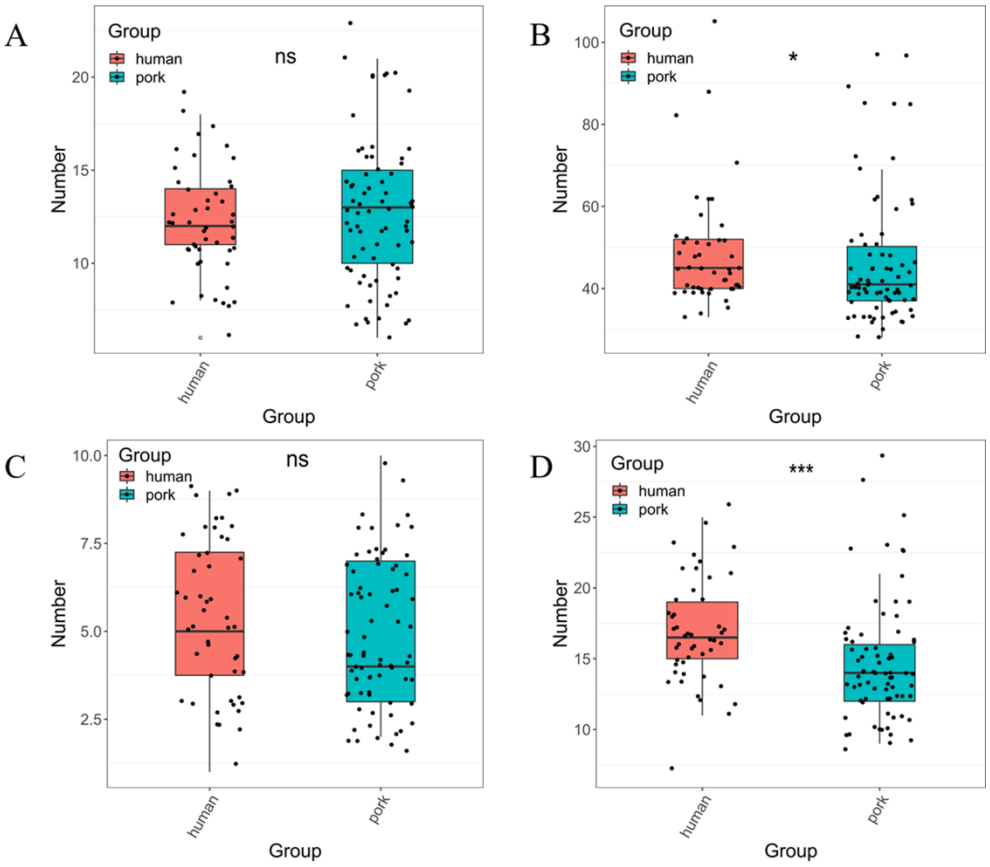

Figure 2. Genome analysis of 122 *tet*(X4)-positive *E. coli* collected from this study and NCBI database. (**A**) Number of ARGs carried by *E. coli* from different sources. (**B**) Number of virulence genes carried by *E. coli* from different sources. (**C**) Number of plasmid replicon types carried by *E. coli* from different sources. (**D**) Number of insertion sequences carried by *E. coli* from different sources. A dot represents an isolate. *: $p < 0.05$; ***: $p < 0.001$; ns: $p > 0.05$.

3.5. Phylogenetic Analysis of tet(X)-Positive K. Pneumoniae

In addition to *E. coli*, a *tet*(X4)-positive *K. pneumoniae* isolate X585-1 was isolated in this study. We downloaded all *tet*(X)-positive *K. pneumoniae* (n = 29) from the NCBI database and constructed a phylogenetic tree based on SNPs of the core genomes (Figure 3, Table S5). We found that ST types and serotypes of the *tet*(X)-positive *K. pneumoniae* were diverse, and there were no dominant *tet*(X)-positive clones. These isolates were found in multiple countries but were mainly distributed in China (n = 18). Except for *tet*(X), these *K. pneumoniae* also carry multiple ARGs, including genes conferring resistance to β-lactams (bla_{TEM-1}, n = 14), sulfonamides (*sul1*, n = 18), aminoglycosides (*aadA2*, n = 14), tetracyclines (*tetA*, n = 25) and trimethoprims (*drfA12*, n = 10). The *tet*(X)-positive *K. pneumoniae* carried only a small number of the virulence genes compared to the ARGs.

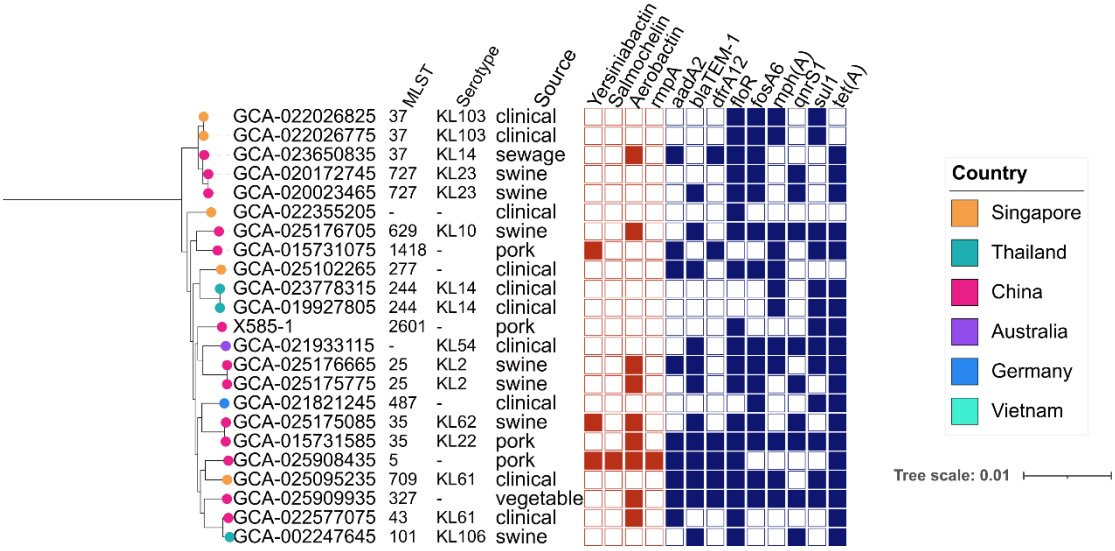

Figure 3. Phylogenetic relationship of 23 *tet*(X)-positive *K. pneumoniae* isolates. Resistance genes and virulence genes are indicated by squares; solid graphics indicate yes, and hollow graphics indicate no.

3.6. The Genetic Context of tet(X4) Carried by K. Pneumoniae

The BLAST comparison results indicated that the sequence of *K. pneumoniae* X585-1 exhibited high similarity to the online IncFII (pCRY) plasmid pSDP9R-tetX4 (NZ_MW940621) found in *K. pneumoniae* (Figure 4). This result implies that the *tet*(X4) gene was also located on the pSDP9R-tetX4-like plasmid. In addition to *tet*(X4), the *tet*(X4)-positive plasmid in X585-1 does not carry other ARGs. The core genetic environment of *tet*(X4) (IS*CR2*-*abh*-*tet*(X4)-IS*CR2*) carried by plasmid pMX581-tetX was the same as the plasmid pSDP9R-tetX4.

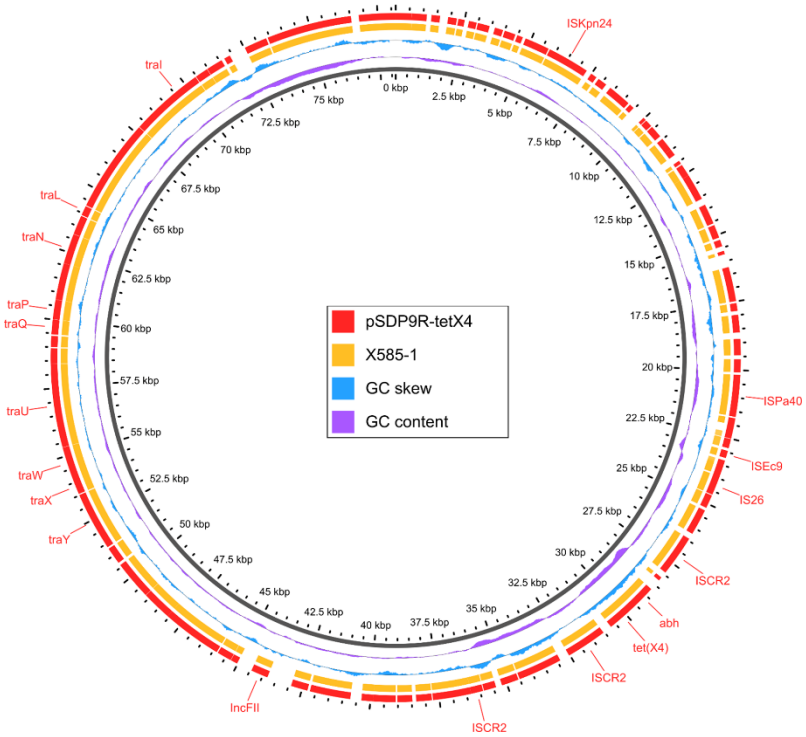

Figure 4. Circular comparison of the *tet*(X4)-bearing plasmid pSDP9R-tetX4 (NZ_MW940621) available in NCBI database and draft genome sequences of X585-1. The outermost circle with arrows denotes the reference plasmid pSDP9R-tetX4.

4. Discussion

Our previous investigation suggests that pork is an important reservoir of the *tet*(X4) gene [10]. However, there is still a lack of research on whether the *tet*(X4) gene carried in pork can spread to humans and the genome differences between *tet*(X4)-positive *E. coli* of human and pork origins. In this study, we use genomics to answer the above questions and provide some theoretical basis for subsequent research. A total of 23 *tet*(X4)-positive isolates were isolated from 53 pork samples, mainly *E. coli*, demonstrating that *E. coli* is an important host of *tet*(X4) among pork samples, which is consistent with the previous study [9]. The *tet*(X4) gene is usually located on different plasmid Inc types and can spread to the same or different bacterial species [8]. The tet(X4) gene isolated from pork samples was mainly located on the IncX1-IncHI2 and IncX1 plasmids. In addition, the IncX1 plasmid carrying tet(X4) usually has no significant fitness cost to the host, suggesting that the IncX1 plasmid is an important vector of the tet(X4) gene [10]. More than half of these *tet*(X4) genes were able to be successfully transferred into C600, indicating that these *tet*(X4) genes are located on mobile elements, such as plasmids. Most of these transferable plasmids were IncX1-type plasmids, highlighting that this type of plasmid may be more easily transferable to other strains [21].

Although the *tet*(X4) gene is mainly present in animal-derived samples, it has also been detected in human clinics in recent years [19]. Comprehensive genomic analysis proved that there is a possibility of clonal transmission of *tet*(X4)-positive isolates between pork samples and clinical samples. This phenomenon will greatly limit the choice of clinical medication and pose great challenges to public health. We noticed that these *tet*(X4)-positive *E. coli* isolated from pork and clinical samples all belonged to MDR isolates and carried a variety of ARGs. However, there was no significant difference in the number of ARGs carried by these two different sources of *E. coli*. In addition, we found that clinical samples carried significantly more virulence genes than pork samples. *E. coli* isolated from clinical samples carry more mobile elements. Mobile elements such as IS*CR2* and IS*26* play an important role in the spread and transfer of *tet*(X4), further exacerbating the spread of *tet*(X4) between different pathogens [23,24].

At present, *K. pneumoniae* has become the most important pathogen of nosocomial infections in China [25]. Some *K. pneumoniae*-evolved carbapenem-resistant *K. pneumoniae* and carbapenem-resistant hypervirulent *K. pneumoniae* have emerged, and tigecycline is regarded as the last choice for clinical treatment [26]. Although only a small number of *tet*(X)-positive *K. pneumoniae* are currently detected [12], they are detected in animal, environmental, as well as human-derived samples and require global vigilance. In addition, we found that *tet*(X)-positive *K. pneumoniae* had no dominant clones, indicating that mobile elements such as plasmids as well as insertion sequences play a key role in the spread of *tet*(X) genes. In addition to the *tet*(X) gene, we found that these *K. pneumoniae* also carry multiple ARGs, which are at risk of co-transmission. This phenomenon suggests that we need to revisit the importance of mobile elements in mediating the spread of ARGs.

5. Conclusions

In conclusion, *tet*(X4)-positive *E. coli* and *K. pneumoniae* in pork samples were systematically analyzed in this study. *tet*(X4)-positive *E. coli* isolates in pork samples were all MDR isolates. There is a possibility of the clonal transmission of *tet*(X4)-positive isolates between pork samples, as well as between pork and clinical samples. Notably, mobile elements may play a key role in the spread of *tet*(X) genes, which suggests that we should pay more attention to the role of these mobile genetic elements in the spread of ARGs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/genes14010036/s1, Table S1: The primers for detecting different plasmid replicons, Table S2: Antimicrobial susceptibility testing (MICs, mg/L) of 23 tet(X4)-positive strains, Table S3: Plasmid replicons of transconjugants, Table S4: Basic information of 117 tet(X4)-positive E. coli collected from the NCBI database, Table S5: Basic information of 22 tet(X)-positive K. pneumoniae genomes collected from the NCBI database.

Author Contributions: Conceptualization, R.L. and Z.W.; methodology, Y.L.; software, Y.H.L.; validation, K.B., M.W. and R.L.; data curation, X.X.; writing—original draft preparation, Y.H.L, and Y.L.; writing—review and editing, Y.L. and R.L.; supervision, R.L. and Z.W.; funding acquisition, Z.W. and R.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interes.

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