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Original Research

Antimicrobial Resistance and the Genomic Epidemiology of Multidrug-Resistant *Salmonella enterica* serovar Enteritidis ST11 in China

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Abstract

Background: With the recent evolution of multidrug-resistant strains, the genetic characteristics of foodborne *Salmonella enterica* serovar Enteritidis and clinical isolates have changed. ST11 is now the most common genotype associated with *S.* Enteritidis isolates. **Methods**: A total of 83 strains of *S.* Enteritidis were collected at the General Hospital of the People's Liberation Army. Of these, 37 were from aseptic sites in patients, 11 were from the feces of patients with diarrhea, and the remaining 35 were of chicken-origin. The minimum inhibitory concentration of *S.* Enteritidis was determined by the broth microdilution method. Genomic DNA was extracted using the QiAamp DNA Mini Kit, and whole-genome sequencing (WGS) was performed using an Illumina X-ten platform. Prokka was used for gene prediction and annotation, and bioinformatic analysis tools included Resfinder, ISFinder, Virulence Factor Database, and PlasmidFinder. IQ-TREE was used to build a maximum likelihood phylogenetic tree. The phylogenetic relationship and distribution of resistance genes was displayed using iTOL. Comparative population genomics was used to analyze the phenotypes and genetic characteristics of antibiotic resistance in clinical and chicken-origin isolates of *S.* Enteritidis. **Results**: The chicken-origin *S.* Enteritidis isolates were more resistant to antibiotics than clinical isolates, and had a broader antibiotic resistance spectrum and higher antibiotic resistance rate. A higher prevalence of antibiotic-resistance genes was observed in chicken-origin *S.* Enteritidis compared to clinical isolates, along with distinct patterns in the contextual characteristics of these genes. Notably, genes such as $bla_{\text{CTX-M}}$ and *dfrA17* were exclusive to plasmids in clinical *S.* Enteritidis, whereas in chicken-origin *S.* Enteritidis they were found in both plasmids and chromosomes. Additionally, *floR* was significantly more prevalent in chicken-origin isolates than in clinical isolates. Careful analysis revealed that the delayed isolation of chicken-origin *S.* Enteritidis contributes to accelerated gene evolution. Of note, certain resistance genes tend to integrate seamlessly and persist steadfastly within the chromosome, thereby expediting the evolution of resistance mechanisms against antibiotics. Our comparative analysis of virulence genes in *S.* Enteritidis strains from various sources found no substantial disparities in the distribution of other virulence factors. In summary, we propose that chicken-origin *S.* Enteritidis has the potential to cause clinical infections. Moreover, the ongoing evolution and dissemination of these drug-resistant genes poses a formidable challenge to clinical treatment. **Conclusions**: Constant vigilance is needed to monitor the dynamic patterns of drug resistance in *S.* Enteritidis strains sourced from diverse origins.

Keywords: *Salmonella enterica* serovar Enteritidis; WGS; antimicrobial resistance; virulence gene; population genomics

1. Introduction

Salmonella is one of four key causes of diarrheal diseases worldwide [1]. Most cases of salmonellosis are mild, but they can sometimes be life-threatening [2]. The severity of the disease depends on host factors and on the serotype of *Salmonella* [3]. *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis), a non-typhoid *Salmonella*, causes substantial morbidity and mortality in humans and animals, and is therefore a major concern for public health [4]. *S.* Enteritidis usually causes acute diarrheal disease after entering the digestive tract with contaminated food. However, it has been widely reported that infection with multidrug-resistant (MDR) *S.* Enteritidis can lead to non-effective control of clinical infection, resulting in extra-intestinal infections such as bacteremia, endocarditis, meningitis, etc. The prevalence of MDR *Salmonella* isolates has increased over the last few decades, and antimicrobial-resistant *Salmonella* isolates have been associated with an increased rate of hospitalization [5,6]. The World Health Organization (WHO) has declared that antibiotic-resistant *S.* Enteritidis is a critical-priority bacterium [7].

Since the discovery of penicillin, antibiotics have gradually been used more widely to treat various bacterial diseases in humans and animals, and to promote weight gain in animals [8]. However, the serious negative effects and consequences of antibiotic abuse are now reflected in their residual accumulation in livestock and poultry. These cause public harm through the emergence of antibiotic resistance and "super bacteria" in food that threaten human health [9,10]. Currently, the fluoroquinolone ciprofloxacin and the third-generation cephalosporin ceftriaxone are the recommended antibiotics for treating patients who are at risk of invasive *S*. Enteritidis infection, with the macrolide azithromycin as a possible alternative [11]. Most of the severe antibiotic resistant *S*. Enteritidis strains are susceptible to carbapenems, making them the last line of defense in the treatment of *Salmonella* infections. However, strains that are resistant to these antibiotics have now emerged. For example, Abdel-Kader *et al*. [12] reported the sale of chicken in retail shops that carried $bla_{\text{CMY-2}}$ and ESBL-genes with a high proportion of carbapenems-resistance [13]. The emergence of antibiotic resistance often results from the modification and transfer of antibiotic resistance genes to humans or opportunistic pathogenic microorganisms through horizontal gene transfer. Exactly how antibiotic resistance genes spread rapidly is still unclear. Advances in bioinformatics and sequencing technology have allowed investigation of the mechanism by which the transfer of resistance genes speeds up development of drug-resistant *Salmonella* through examination of the entire genetic makeup [14].

The level of *S*. Enteritidis resistance is related to the use of antibiotics in humans and animals, geographical differences in epidemiology, and serotypes [15]. ST11 is the most common genotype associated with *S*. Enteritidis isolates from humans and food animals across the world [16]. The goal of this study was therefore to utilize bioinformatics techniques to clarify the role of horizontal gene transfer in shaping the genome-wide evolution of MDR *Salmonella*. This should help to reveal the underlying mechanisms that facilitate the swift dissemination of antibiotic resistance genes. For this study, a total of 83 *S*. Enteritidis strains were collected from different regions in China, including chicken-origin isolates and clinical isolates. We evaluated the prevalence and mechanisms of *S*. Enteritidis resistance from the different sources by using phenotypic susceptibility data and whole-genome sequencing (WGS) analysis.

2. Materials and Methods

2.1 Collection of S. Enteritidis Isolates

A total of 83 *S*. Enteritidis strains were collected. Clinical isolates originated from samples collected by the clinical laboratory of the First Medical Center, Chinese People's Liberation Army (PLA) General Hospital, Beijing, China. All strains were isolated from different patients. Thirty-seven strains were isolated from aseptic sites in the human body that were devoid of local inflammation and damage, and were unconnected to the respiratory system, digestive system, urogenital system, or other external environments. These included locations such as venous blood, joint cavities, and cerebrospinal fluid. A further 11 strains were isolated from the feces of diarrheal patients. The other 35 isolates were of chicken-origin obtained from six provinces in China and cultured by the Chinese Institute for Food and Drug Control. The strains were isolated and cultured according to the National Clinical Laboratory Operation Procedures. The genus was identified by a VITEK MS RUO system v3.4 (BioMérieux, Lyon, France), and the serotype by the *Salmonella* serum agglutination test (Lot: 20210101, Statens Serum Institut, Copenhagen, Denmark).

2.2 Whole-Genome Sequencing

Genomic DNA was extracted using the QiAamp DNA Mini Kit (Lot: 172030683, Qiagen, Dusseldorf, North Rhine-Westphalia, Germany) and then sequenced using an Illumina X-ten platform to generate 150-bp paired-end reads from a library with an average insert size of 500 bp. Raw reads were first filtered by fastQC v0.11.9 (Babraham Institute, Babraham, Cambridgeshire, England) to remove low-quality reads, and then assembled using SPAdes v3.13 (Saint Petersburg Academic University, Saint Petersburg, Leningrad Oblast, Russia) using the default parameters [17,18]. The assembled genomes were evaluated by Quast v5.0.2 (Saint Petersburg State University, Saint Petersburg, Leningrad Oblast, Russia). Gene prediction was performed with Prokka v1.14.6 (University of Melbourne, Melbourne, Victoria, Australia), and BLAST v2.2.18 (National Center for Biotechnology Information, Bethesda, MD, USA) was used to compare the virulence genes carried by the strains with the virulence gene database VFDB. The parameters were set to a minimum coverage of 60% and a minimum similarity of 80%. The ResFinder v4.1.11 (Technical University of Denmark, Copenhagen, Denmark) was used to assemble strain sequences related to antibiotic resistance gene screening. The parameters were set to a minimum coverage of 60% and minimum similarity of 80%. The evolutionary tree was constructed with WGS single nucleotide polymorphism (wgSNP) data. MUMmer v3.23 (University of Maryland, College Park, MD, USA) was used to find SNPs in all strains and to merge these. To improve the overall quality of data, a random strain was selected as the reference sequence (FC15971). SNPs less than 5 bp away and those carrying unspecified nucleotides ("N") were removed, and an evolutionary tree for wgSNP sequences was constructed using fasttree [19–23]. Antibiotic resistance genes (ARGs) were identified based on the best alignment with the ResFinder database, with thresholds of 90% DNA sequence identity and minimum coverage of 80%. A multilocus sequence typing (MLST) scheme was used to subtype the isolates using BLASTn and 7 housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) [24].

2.3 Antimicrobial Susceptibility Testing and AMR Correlation

Antimicrobial susceptibility testing (AST) of the *S.* Enteritidis isolates was performed with the broth microdilution method (Lot: D-012XS, Xingbai Biotechnology, Shanghai, China) and interpreted according to guidelines from the 2017 Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST; https://eucast.org/). A bacterial suspension with a precisely adjusted concentration of 0.5 McFarland units was first prepared. The recommended protocol for introducing the control sample and thoroughly amalgamating it with the bacterial solution was meticulously adhered to. Subsequently, the resultant mixture was kept in an incubation chamber at a constant temperature of 35 °C for 18 to 24 hours. This process was carefully conducted with the ultimate aim of determining the minimum inhibitory concentration (MIC). Susceptibility to the following antimicrobials was assessed: β-lactam antimicrobials, which includes ampicillin (AMP), ceftazidime (CAZ), cefoxitin (CFX), cefotaxime (CTX), cefazolin (CFZ), ampicillin-sulbactam (AMS) and imipenem (IPM); the tetracycline antibiotic, which includes tetracycline (TET); the quinolone antibiotic, which includes nalidixic acid (NAL) and ciprofloxacin (CIP); the macrolide azithromycin (AZM); the amphenicol antibiotic chloramphenicol (CHL); aminoglycosides, which included gentamicin (GEN); and the sulfonamides, which included the pediatric compound sulfamethoxazole (SXT). We defined an MDR strain as being resistant to three or more different classes of antibiotics at the same time. Strains were otherwise defined as non-MDR. *Escherichia coli* ATCC 25922 was used as the quality control strain. ResFinder software was used to detect antibiotic resistance-related genes based on the WGS data, and their association with MIC phenotypes.

2.4 Phylogenetic Analysis

Illumina reads were mapped to the reference genome using Bowtie 2 v2.2.8 (Johns Hopkins University, Baltimore, MD, USA), SNPs were identified using Samtools v1.9 (Shanghai Institute of Life Sciences, Shanghai, China), and the data was combined as described previously [25]. All high-quality SNPs (HQ snps) supported by $>$5 reads with a mapping quality of 20 were investigated further (https://github.com/generality/iSNV-calling). Phylogenetic analysis was performed based on the remaining core genome sequences. The best-fitting substitution model (K3P+ASC+R2) was identified using ModelFinder and selected to build a maximum likelihood phylogenetic tree using IQ-TREE v2.0.6 (Center for Computational Biology, Stockholm, Sweden). The phylogenetic relationships and distribution of resistance genes were displayed using iTOL v6.8.1 (Max Planck Institute for Developmental Biology, Tübingen, Germany) [26–28].

2.5 Statistical Methods

Statistical analysis was performed using SPSS software (Version 16.0, IBM, Armonk, NY, USA). Comparisons of antibiotic resistance between clinical isolates and chicken-origin isolates were performed by Chi-square/Fisher exact test for categorical variables. $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1 Salmonella Serotyping

All 83 isolates were identified as *S.* Enteritidis ST11 by MLST after WGS analysis (see **Supplementary Table 1**).

3.2 Identification of Virulence Genes

A total of 124 virulence genes were detected in the 83 *Salmonella* genomes. All 83 isolates were positive for 111 virulence genes. Seven known virulence factors showed different distributions in the VFDB matrix (*rck*, *pefB*, *pefD*, *pefC*, *spvR*, *spvC*, *spvB*), with a low carriage rate in chicken isolates (see **Supplementary Table 1**). These virulence genes are located on the virulence plasmid of *S.* Enteritidis.

3.3 Antimicrobial Susceptibility Profiles

Analysis of the antibiotic resistance phenotypes of *S.* Enteritidis isolated from different sources revealed a minor level of resistance in the clinical isolates. The multiple drug resistance rate of chicken-origin isolates was higher than that of clinical isolates ($p > 0.05$). For the penicillin antibiotics AMP, CAZ, CFX, CTX, and CFZ, the resistance rates of chicken isolates were slightly higher (except for CFZ) than those of the clinical isolates, but the difference was not statistically significant ($p > 0.05$). All strains were sensitive to AZM and resistant to the first-generation quinolone drug NAL. The clinical isolates were sensitive to CIP, but there were some resistant strains in chicken-origin isolates. In addition, clinical isolates were sensitive to the aminoglycoside drug GEN and the chloramphenicol antibiotic CHL, but chicken isolates were significantly more resistant ($p < 0.05$) (see Table 1).

3.4 The Genome Features of S. Enteritidis

A maximum-likelihood tree of all 83 *S.* Enteritidis strains was constructed using SNPs (see Fig. 1). The phylogenic tree was broadly divided into four subclades. Of note, the genomes of MDR and non-MDR strains were distributed into different subclades. Subclade A strains were resistant to two groups of antibiotics, except strain FC9132 which was resistant to 6 groups of antibiotics. All strains of subclade B were resistant to two or three groups of antibiotics. Some strains of subclade C were resistant to 5 or 6 groups of antibiotics, and were resistant to $>$3 β-lactam antibiotics. Almost half of subclade D strains were resistant only to NAL, although strain FC15955 was resistant

Table 1. Analysis of the phenotypic resistance of *Salmonella enterica* in clinical isolates and chicken-origin isolates.

Antimicrobials	No. of resistant strains (%)		χ^2	*p* value
	Clinical isolates (n/48)	Chicken-origin isolates (n/35)		
Ampicillin	37 (77.1)	32 (91.4)	2.970	0.085
Ceftazidime	6 (12.5)	5 (14.3)	0.000	1.000
Cefoxitin	0 (0.0)	1 (2.9)	-	0.422
Cefotaxime	6 (12.5)	6 (17.1)	0.353	0.553
Cefazolin	27 (56.25)	13 (37.1)	2.960	0.085
Ampicillin-sulbactam*	32 (66.7)	15 (42.9)	4.672	0.031
Imipenem	0 (0.0)	0 (0.0)	-	-
Tetracycline*	9 (18.75)	14 (40.0)	4.563	0.033
Nalidixic acid	48 (100.0)	35 (100.0)	-	-
Ciprofloxacin	0 (0.0)	3 (8.6)	2.163	0.141
Azithromycin	0 (0.0)	0 (0.0)	-	-
Chloramphenicol*	0 (0.0)	6 (17.1)	-	0.004
Gentamicin*	0 (0.0)	5 (14.3)	4.991	0.025
Pediatric compound sulfamethoxazole*	2 (4.2)	8 (22.9)	5.025	0.025
Multidrug resistance	38 (79.2)	32 (91.4)	2.304	0.129

p values were calculated by chi-square analysis with SPSS version 16.0; * denotes a statistically significant difference.

to 5 groups of antibiotics. Additionally, subclade C were all chicken-origin, except for strain 15. After mapping the SNP phylogenic tree with antibiotic resistance-related genes, subclade D strains were indeed found to have a lower carry rate for some of the resistance genes (e.g., *tetA*, *tetR*, $bla_{\text{TEM-1}}$, and *sul2*) compared with other subclades.

3.5 Antibiotic Resistance-Related Genes Correlated with Phenotype (MIC) Based on Genotypic Data (WGS)

The 20 resistance genes associated with resistance tests were the β-lactamase resistance-related genes ($bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{TEM-206}}$, $bla_{\text{TEM-141}}$, $bla_{\text{TEM-135}}$, $bla_{\text{TEM-126}}$, $bla_{\text{TEM-106}}$, $bla_{\text{TEM-206}}$, $bla_{\text{TEM-141}}$, $bla_{\text{TEM-164}}$, $bla_{\text{TEM-163}}$, $bla_{\text{TEM-122}}$), aminoglycoside resistance gene (*rmtB*), amidohydrin resistance gene (*floR*), sulfonamide-resistance genes (*dfrA1*, *dfrA12*, *dfrA17*, *sul2*, *sul1*) and tetracycline resistance gene (*tet(A)*). Numerically dominant resistance genes were observed for different antibiotics, with $bla_{\text{TEM-1}}$ present in 67 strains, *rmtB* in 4 strains, and *floR* in 3 strains. *sul2* was present in 56 isolates and *tet(A)* in 17 isolates. WGS data was correlated with the phenotypic profiles to evaluate its ability to predict phenotypic resistance. Some resistance genes showed excellent correlation, for example $bla_{\text{TEM-1}}$ with AMP, $bla_{\text{CTX-M-55}}$ with CAZ and CTX, *rmtB* with GEN, *gyrA* with NA, and *tet(A)* with TET (see **Supplementary Table 1**). The low carry rate of other resistance genes meant they were difficult to analyze. It is worth noting that three ampicillin-resistant strains (strains 54, 15955, 15968) did not present known β-lactamase resistance genes, and one cefotaxime-resistant strains did not have known resistance genes (strain 9132). Two tetracycline-resistant *S*. Enteritidis did not have known tetracycline resistance genes (strains 19 and 49), and one phenotype that was resistant to cotrimoxazole had no known drug resistance genes (strain 15955). No known resistance genes were present in *S*. Enteritidis with the gentamicin resistance phenotype, and three chloramphenicol-resistant strains (FC15955, 9099, 9132) had no known drug resistance genes.

We next screened for mutations within the Quinolone resistance determination region (QRDRs). All strains carried the *gyrA* mutation, with the mutation types being D87Y (33/48 clinical isolates *vs* 28/35 chicken-origin isolates), D87N (10/48 *vs* 3/35), and D87G (5/48 *vs* 4/35). No significant differences in the mutation types were observed between the different isolates ($p > 0.05$). No mutations were found in *parC* or *parE*.

3.6 Identification of Plasmid Replicons, Insertion Sequences, and Transposons

The WGS data was used to detect the presence and absence of transposons, plasmid replicons, and virulence genes in the 83 *Salmonella* genomes. Six types of plasmid replicons were detected: IncI1, col, IncN, IncX1, IncFIB, and IncFII. Of these, IncFIB (90.4%), IncFII (89.2%), and IncX1 (79.5%) were the most common. Only one strain (FC15968) had no plasmids, but was resistant to ampicillin and nalidixic acid. Three strains (FC15966, 17, and 78) that showed multiple drug resistance carried four types of plasmids and were resistant to 7, 7, and 3 types of antibiotics, respectively. Strains were divided into three clusters in the Inc_type matrix graph (see Fig. 2). In cluster A, the vast majority of strains were resistant only to NAL, and carried plasmids IncFIB and IncFII. In cluster C, most strains were resistant to two groups of antibiotics (with some up to 6 groups), and carried plasmids IncX1, IncFIB, and IncFII. Although most isolates in cluster B carried only the IncX1 plasmid, they still showed MDR.

We next analyzed the insertion sequence data for the 83 *S*. Enteritidis strains. A total of 562 insertion sequences

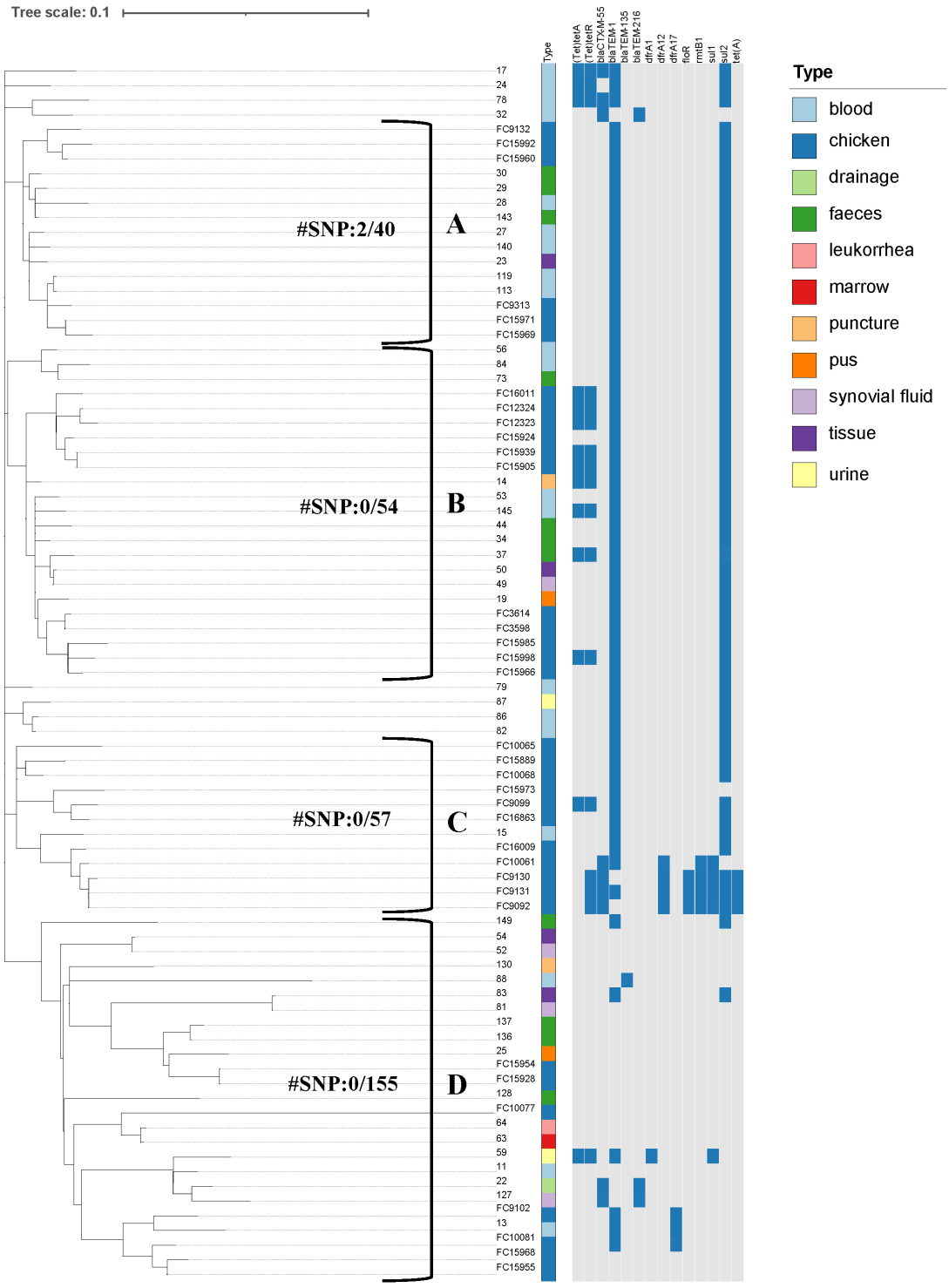

Fig. 1. Phylogenetic analysis showing antibiotic resistance patterns and the profiles of specific genetic loci in *S.* Enteritidis strains. Neighbor-joining tree based on whole-genome level single-copy genes in the 83 *S.* Enteritidis strains. The type indicates the different isolation source of strains, and the year indicates the year of isolation. The presence or absence of specific loci are denoted by blue and grey rectangles, respectively. The phylogenetic tree was divided into (A), (B), (C), and (D) subclades, with the adjacent values representing the minimum and maximum single nucleotide polymorphism (SNP) divergence between any two pairs of clades.

were found, including ISEcp1, ISCro1, IS1R, ISSso4, and IS186B unique to aseptic clinical sites, and IS5075, ISVsa3, and ISCR1 unique to chicken. Most strains contained ISSaen1, IS1230B, IS285, IS26, ISSwi1 and IS1351. Isolates that were resistant only to nalidixic acid showed the insertion pattern ISSaen1-IS1230B-IS285-IS1351-IS1351.

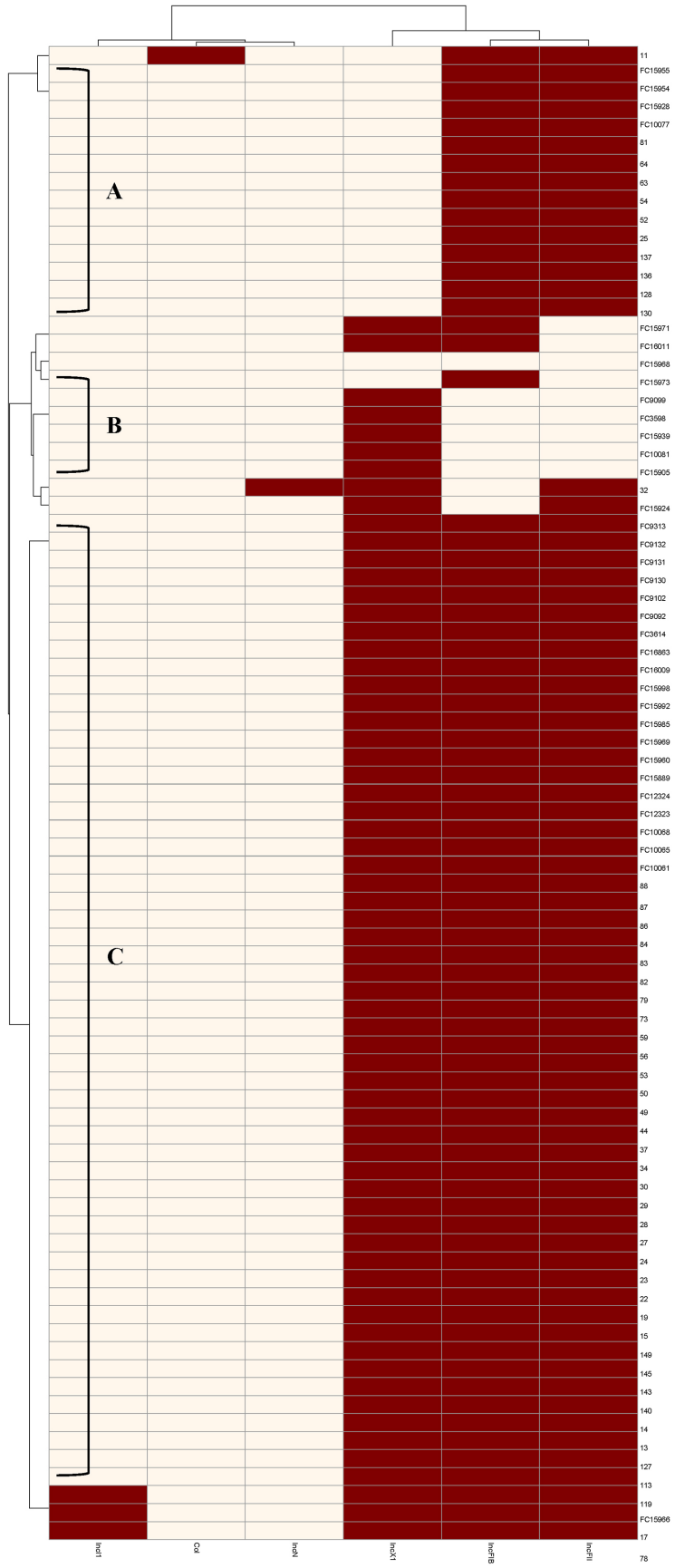

Fig. 2. Distribution of Inc types in different strains. The abscissa shows the Inc type and the ordinate shows the strain number. The presence or absence of Inc are denoted by red and beige rectangles, respectively.

Some strains that were resistant to two or three types of drugs carried IS26 and ISSwi1, while all strains resistant to four types of drugs carried IS26 and ISSwi1 (except strain 59). FC9130 and FC9131 were resistant to 6 groups of drugs and carried the additional insertion sequence of IS50R-IS1294-IS5075-ISVsa3-ISCR1.

3.7 Genetic Context of Resistance Genes

The only subtype of the $bla_{\text{CTX-M}}$ resistance gene found in this study was CTX-M-55. A total of 9 *S.* Enteritidis strains carried $bla_{\text{CTX-M-55}}$, of which 5 were clinical isolates and 4 were chicken-origin isolates. These accounted for 10.42% (5/48) and 11.43% (4/35) of the respective groups, with no significant difference in the carry rate between them ($p > 0.05$). Further analysis showed the $bla_{\text{CTX-M-55}}$ resistance gene was located on the plasmid in all clinical isolates carrying this gene, whereas in half of the chicken isolates it was located on the chromosome (see Fig. 3). After alignment, two clinical strains with $bla_{\text{CTX-M-55}}$ had the same genetic background and were located on the insertion sequence ISEcp1. The two strains (strains FC10061 and FC9131) with the $bla_{\text{CTX-M-55}}$ gene located on the chromosome had the same ISSwi1-CTX-M-55 transposable element.

The most common β-lactam antibiotic-related gene, $bla_{\text{TEM-1}}$, was carried by 37 strains of clinical isolates and 30 strains of chicken-origin isolates. The TEM-1 resistance gene was located on the plasmid in 55 strains, with no significant difference in frequency between clinical and chicken-origin isolates (33/37 *vs* 22/30 respectively, $p > 0.05$). ISSwi1 was the most frequent accompanying insertion sequence in these strains.

A total of 8 isolates carried the *drfA* resistance gene, of which two strains were clinical isolates (all located on plasmids) and 6 strains were chicken-origin isolates (four strains located on plasmids and two strains located on chromosomes, all belonging to the *dfrA17* subtype) (see Fig. 4). The two strains (FC10081 and FC9102) with *dfrA17* located on the chromosome had similar genetic contexts to the one strain (strain 13) located on the plasmid. A total of 56 *S.* Enteritidis isolates had the *sul2* gene (29 clinical isolates and 27 chicken-origin isolates). The *sul1* gene was found in one clinical isolate and in four chicken-origin isolates. All *sul1* and *sul2* genes were located on plasmids, with no significant difference in frequency between clinical and chicken-origin isolates ($p > 0.05$), and no accompanying insertion sequence.

Three *S.* Enteritidis isolates, all of chicken-origin, carried the *floR* gene on the plasmid, thereby conferring a higher resistance rate to chloramphenicol in the chicken-origin isolates compared to clinical isolates.

Seventeen strains carried *tet(A)* resistance genes, including 7 clinical isolates and 10 chicken-origin isolates. In all cases the genes were located on plasmids, with no significant difference in the frequency between the clinical and chicken isolates ($p > 0.05$).

Several highly resistant chicken isolates were examined (strains FC15955, FC9092, FC9130, FC9131, FC9132, and FC10061). Most were found to carry special resistance genes such as *floR*, *tet(A)*, *sul1*, and *rmtB*, and to carry more insertion sequences (e.g., IS1230B, IS285, ISSaen1) than other isolates.

4. Discussion

Salmonellosis caused by *S.* Enteritidis is an important foodborne infectious disease [29]. The current study investigated 83 *S.* Enteritidis strains isolated from different sources. All *S.* Enteritidis strains were identified as ST11, consistent with the epidemiological characteristics of ST typing for *S.* Enteritidis [30,31]. In addition, the results showed high genomic similarity between *S.* Enteritidis isolates from different sources and different regions, similar to the results of Davis *et al.* [32].

Differences in the virulence of pathogens can often be attributed to virulence factors [33]. To identify virulent *S.* Enteritidis isolates and to find genetic markers that distinguish invasive *S.* Enteritidis, we conducted a genome-wide association analysis of virulence factors using different sources of isolates as phenotypes. The three sources of isolates were aseptic sites in patients with extra-intestinal infections, feces from patients with diarrhea, and foodborne chicken-origin isolates. Screening of the VFDB database revealed a lower carriage rate for virulence genes from the *Salmonella* Virulence Plasmid in chicken-origin isolates compared to clinical isolates. This indicates the virulence plasmid plays an important role in the pathogenic process of *S.* Enteritidis.

A SNP-based phylogenetic tree describing the evolution of *S.* Enteritidis identified four distinct subclades. The differential resistance observed between subclade C and subclade D phenotypes implies the SNP differences may be because of differences in the resistance genes. We analyzed the resistance phenotypes of different isolates and found the MDR rate to be up to 83.1%, which was higher than a previous study [34]. Chicken isolates showed greater resistance to almost all antibiotics than clinical isolates, with a higher frequency of MDR. This indicates a broader drug resistance profile in the chicken-origin isolates. We are unable to speculate on possible differences in antibiotic use between the provinces from which the isolates were collected, and no clusters were visible in the phylogenetic tree according to the different provinces.

Analysis of the resistance genes indicated differences in the evolutionary characteristics between chicken-origin and clinical isolates. Several highly resistant chicken isolates (FC15955, FC9092, FC9130, FC9131, FC9132, and FC10061) were found to carry some resistance genes, such as *floR*, *tet(A)*, *sul1*, and *rmtB1*. This may explain their greater resistance compared with other strains.

Resistance to quinolones is related to point mutations in QRDR, with the pattern of point mutations in differ-

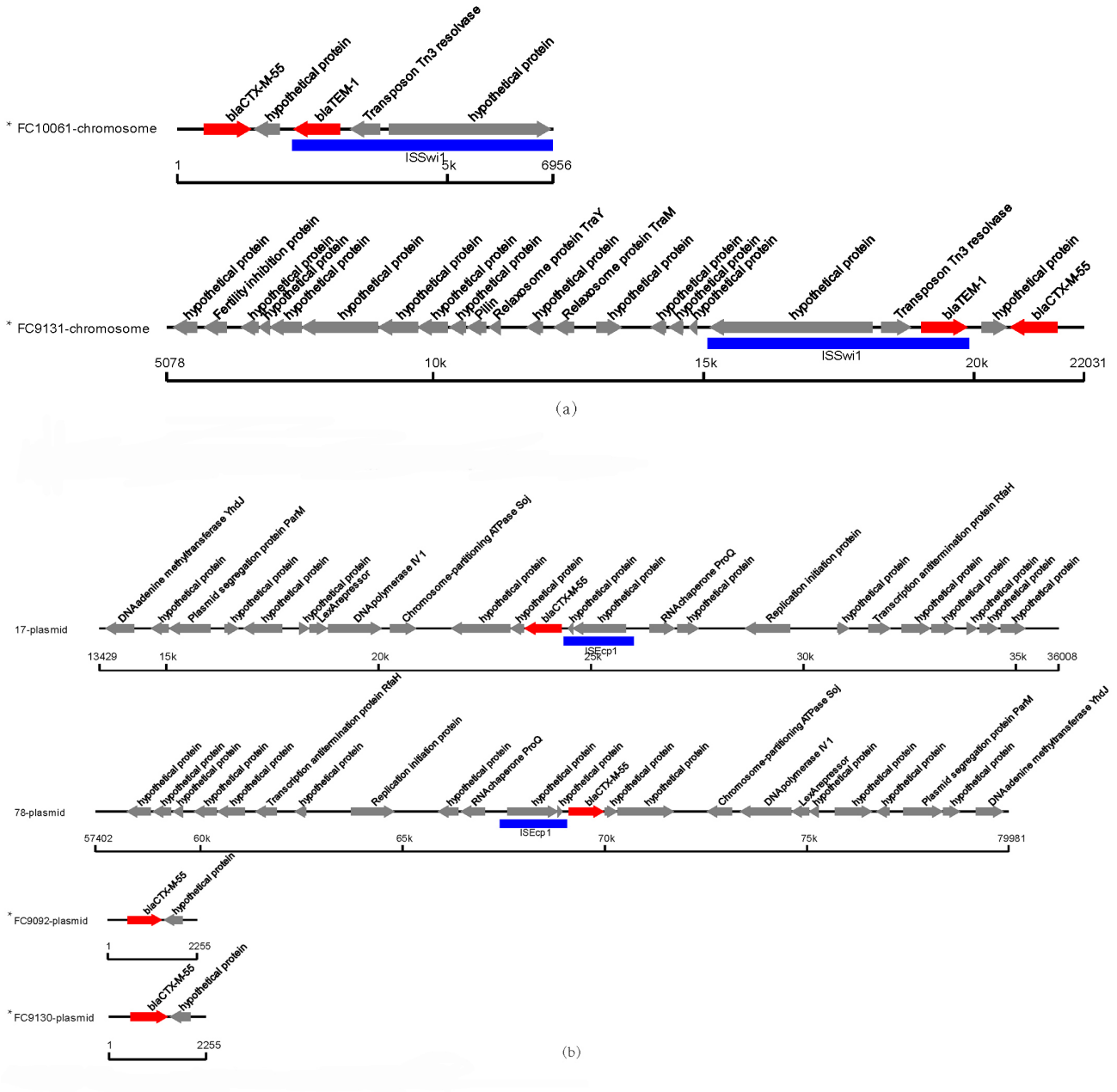

Fig. 3. Genetic environment of *bla*$_{\text{CTX-M-55}}$ in five S. Enteritidis isolates (FC10061, FC9131, 17, 78, FC9092, and FC9130). (a) *bla*$_{\text{CTX-M-55}}$ of strains located on the chromosome. (b) *bla*$_{\text{CTX-M-55}}$ of strains located on the plasmid.

ent genes being associated with different resistance phenotypes and levels. Three mutations (D87Y, D87N, and D87G) were found in *gyrA*, but none in *parC* and *parE*. Three strains (FC15955, FC9131, and FC9132) found to be resistant to ciprofloxacin had the D87G, D87Y, and D87Y mutations, respectively. The ciprofloxacin MIC values for all other strains were $<$0.5 mg/mL. In addition, the MIC values for nalidixic acid were all $>$64 mg/mL. The three *gyrA* mutations did not affect the resistance level of *S.* Enteritidis to quinolones, and no significant differences in the distribution of quinolone resistance genes and phenotypes were observed between clinical isolates and chicken-origin isolates ($p > 0.05$). The high carry rate of quinolone resistance genes and the high rate of resistance to nalidixic acid may be related to the use of quinolones in poultry and in clinical treatment [35,36].

The CTX-M gene is associated with anti-cefotaxime levels. Only one CTX-M gene, CTX-M-55, was detected in 9 isolates (strains FC9131, 127, 32, FC10061, FC9092, 78, FC9130, 22, and 17). No difference was observed in the prevalence of CTX-M-55 between clinical isolates and chicken-origin isolates. However, *bla*$_{\text{CTX-M-55}}$ was present on both the plasmid and chromosome in the chicken-origin isolates, whereas in clinical isolates it was only found on the plasmid. This finding suggests the *bla*$_{\text{CTX-M-55}}$ resistance gene may be able to travel between the plasmid and

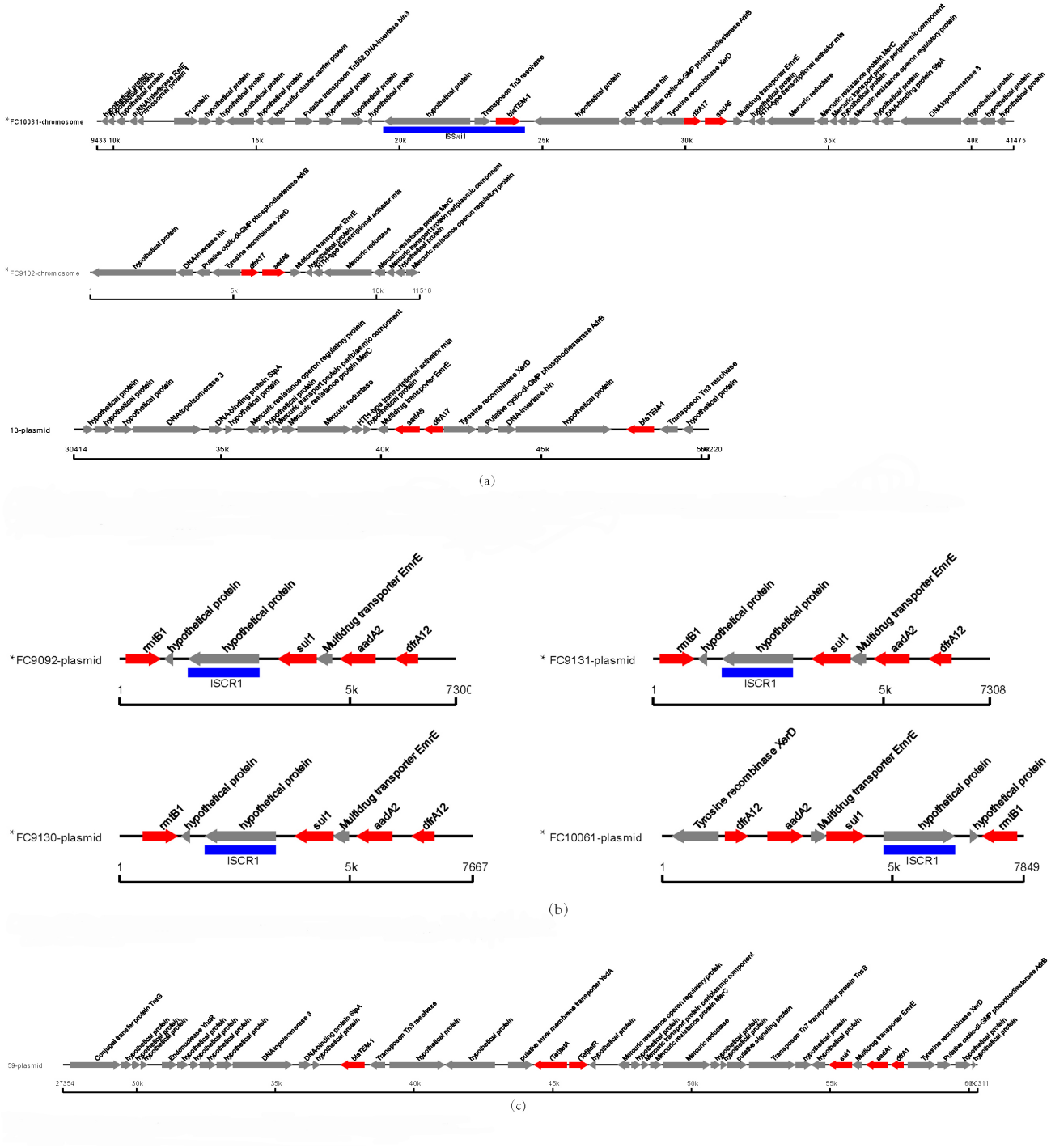

Fig. 4. Genetic environment of *drfA* in eight *S.* Enteritidis isolates (FC10081, FC9102, 13, FC9092, FC9130, FC9131, FC10061, and 59). (a) Comparison between *dfrA17*-positive strains. The *dfrA17* of strains FC10081 and FC9102 were located on the chromosome. The *dfrA17* of strain 13 was located on the plasmid. (b) The *dfrA12* of strains were located on the plasmid. (c) The *dfrA1* of strain was located on the plasmid.

chromosome via mobile elements, and then to exist stably on the chromosome [37]. Two strains with the $bla_{\text{CTX-M-55}}$ gene located on the chromosome had the same ISSwi1-CTX-M-55 transposable element, indicating that it promotes the transfer of $bla_{\text{CTX-M-55}}$ to the chromosome. Furthermore, strains FC10081 and FC9102 with *dfrA17* located on the chromosome had similar genetic contexts to strain 13 with *dfrA17* located on the plasmid, indicating that *dfrA17* can also switch between the plasmid and chromosome [38]. The plasmid incompatibility groups identified in this study were IncI1, col, IncN, IncX1, IncFIB, and IncFII. We found that *S.* Enteritidis with IncX1 exhibited MDR, in

agreement with previous results showing this plasmid carries numerous important resistance genes [39]. Subclade C (strains FC10065, FC15889, FC10068, FC15973, FC9099, FC16863, 15, FC16009, FC10061, FC9130, FC9131, and FC9092) contained more inserted sequences, suggesting more frequent gene exchange. ISEcp1 was previously confirmed as the most common insertion sequence associated with $bla_{\text{CTX-M-55}}$, consistent with the present results. SEcp1 and $bla_{\text{CTX-M-5}}$ had not been confirmed as vectors of resistance genes in our study, but this insertion may induce more gene exchange in pathogenic bacteria [40].

Lei *et al*. [41] analyzed the genetic relationship and antimicrobial resistance of *S*. Enteritidis strains isolated from Hebei province by WGS. Their isolates had a high resistance rate to NAL and AMP, and demonstrated the vertical transmission of *S*. Enteritidis from breeding chickens to commercial chickens. Li *et al*. [42] conducted a genomic analysis of dead poultry in Shandong province from 2008 to 2019. The authors found that isolated *Salmonella* strains showed greater resistance to ampicillin than to nalidixic acid. These findings are consistent with our results showing that chicken-origin isolates have high resistance to ampicillin and nalidixic acid, with even higher rates than those reported in earlier studies. Another study of resistance in samples from chicken meat products reported resistance to tetracycline only. The proportion of drug-resistant strains in the clinical isolates analyzed here was not particularly high compared to clinical isolates from other regions. A study conducted in 2013–2014 at the Hangzhou pediatric hospital reported a similar incidence of resistance to ampicillin as the present study [43]. Several studies have suggested that foodborne *Salmonella* enteritis may be the cause of clinical disease [36,44]. Other studies have proposed that co-selection and transmission of ESBL and fluoroquinolone resistance in *Salmonella* occurs through the food chain, and that transmission of resistance genes is mediated by plasmids [45,46]. A genomic surveillance study found that a pork isolate differed from a human isolate by only 10 SNPs, indicating that pork food was the likely source of human infection. Phylogenetic analysis suggests that foodborne *Salmonella* could be the source of clinical disease [47]. This Chinese study was based on enteritis caused by foodborne *Salmonella* and clinical pathogenic isolates analyzed in genome evolution, different sources isolates biggest differences in SNP number only about 155, and the drug-resistant genes may spread through the plasmid and insert sequences. We propose that foodborne MDR *Salmonella* may be transmitted to humans, and that antimicrobial resistance originating in chickens is concerning and its genomic evolution should be monitored.

5. Conclusions

Based on WGS data, this study compared virulence factors and drug resistance between *S.* Enteritidis isolates of chicken-origin and clinical origin. In contrast to clinical isolates, some chicken-origin *S.* Enteritidis isolates did not carry the virulence plasmid that plays a role in pathogenesis. Chicken-origin isolates were more resistant to drugs compared with clinical isolates, with several strains being resistant to >7 groups of antibiotics. By studying the distribution of drug-resistance genes, chromosomes, plasmids, and insertion sequences isolated from different sources, it appears that chicken isolates have more gene exchanges. The maximum number of SNP differences between isolates from different sources was only 155, leading us to speculate that foodborne MDR *Salmonella* could spread to humans. The possibility that poultry food is a source of this resistance is worthy of further investigation. Because of the limited number of strains collected, the clinical and chicken-origin isolates analyzed here did not originate from the same geographical region. This may have biased the results, and continued surveillance of these strains is warranted.

Availability of Data and Materials

The genome sequences in this study were deposited into the National Center for Biotechnology Information database under BioProject accession no. PRJNA918954.

Author Contributions

JL: Conceptualization, Methodology, Data curation, Funding Acquisition, Writing - Review & Editing. YH: Conceptualization, Methodology, Formal Analysis, Visualization, Writing - Original draft preparation. LZ: Methodology, Data curation, Writing - Review & Editing. GC: Software, Validation, Writing - Review & Editing. JC: Methodology, Writing - Original draft preparation. QZ: Software, Validation, Writing - Review & Editing. LY: Methodology, Data curation, Writing - Review & Editing. SC: Conceptualization, Methodology, Data Curation, Writing - Reviewing and Editing. CW: Conceptualization, Methodology, Data Curation, Writing - Reviewing and Editing. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Oral informed consent was obtained for all strains collected from the People's Liberation Army General Hospital between 2010 and 2017. The project had passed ethical review at the time of application, and when the experiment was carried out, it was approved by Beijing Chaoyang District Center for Disease Control and Prevention Ethics Committee (IRB Approval Number: CYCDPCIRB-20240312-1).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2903112.

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