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Comparative genomics analysis of *Salmonella* Enteritidis isolated from clinical cases associated with chicken

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Abstract

Salmonella Enteritidis is a major foodborne pathogen, and the emergence of multidrug-resistant (MDR) *S.* Enteritidis poses a serious public health challenge. In this study, we report the genomic characterization of five *S.* Enteritidis isolates from clinical. These isolates exhibited resistance to seven classes of antimicrobials with four of the five characterized as MDR. Isolate 33 A exhibited resistance to colistin and polymyxin B, while no associated antimicrobial resistance genes (ARGs) were identified in its genome. Isolate 21 A and 44 A were extended-spectrum beta-lactamases-producing (ESBLs). Whole genome sequencing analysis revealed the presence of multiple mobile genetic elements (MGEs), including plasmids, prophages, and genomic islands, which may have facilitated the acquisition and dissemination of ARGs. Notably, several ARGs, including $bla_{CTX-M-55}$, $bla_{TEM-141}$, bla_{TEM-1B}, *aph(3')-IIa*, *aph(3")-Ib*, *aph(6)-Id*, *tet(A)*, *floR*, *fosA3*, and *sul2*, were identified on plasmids. In addition, chromosomal point mutations in *gyrA* (D87G and D87Y) and *acrB* (F28L and L40P) were also observed in each isolate. Multiple virulence genes associated with the type III secretion system were identified on *Salmonella* pathogenicity islands (SPIs) SPI-1 and SPI-2. Phylogenetic analysis revealed that the five isolates, along with a clinical and chicken origin isolates in the database, clustered together, suggesting a probable common source of infection. Our findings highlight the intricate genetic mechanisms behind MDR in *S.* Enteritidis, emphasizing the ongoing necessity for surveillance and appropriate antimicrobial usage. This contributes to our understanding of *S.* Enteritidis transmission within the food chain.

Keywords Antibiotic resistance, Mobile genetic elements, Multidrug-resistant, Phylogenetic analysis, Virulence factors, Whole-genome sequencing

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Background

Salmonella enterica, a major pathogen associated with foodborne illnesses in humans, represents public health concern [1]. Among the various serotypes of *Salmonella*, Enteritidis is the most frequently reported serotype, linked to invasive infections. According to a study conducted from 2001 to 2007, *S.* Enteritidis accounted for approximately 43.5% of all *Salmonella* isolates reported from humans [2]. *S.* Enteritidis commonly presents as self-limiting gastroenteritis with symptoms of diarrhea, fever, abdominal cramps, and dehydration [3]. The majority of *S.* Enteritidis infections are primarily linked to the consumption of undercooked or raw meat, vegetables, and eggs [4]. The infection of *Salmonella* may require treatment with antimicrobials. However, the widespread use of antimicrobials is believed to contribute to the emergence of multidrug-resistant (MDR) strains. MDR *Salmonella* strains have been increasingly reported worldwide, creating significant challenges for effective treatment [5, 6]. According to the Global Antimicrobial Resistance and Use Surveillance System (GLASS), ESBLs, colistin resistance and fluoroquinolone resistance are the primary targets for epidemiologic surveillance [7]. The increase in antimicrobial resistance to *Salmonella* has emerged as a significant threat, causing severe challenges in healthcare [8].

Whole genome sequencing (WGS) has become a useful method for tracking antimicrobial resistance and virulence factors in pathogens, providing comprehensive genetic information [9]. The use of short and long-read hybrid sequencing technology makes up for the shortcomings of insufficient depth of short-read sequencing and high error frequency of long-read sequencing, and allows more accurate and complete genomic data to be obtained [10]. Hybrid sequencing technologies, combining short and long reads, improve data accuracy and allow deeper insights into the genomic landscape, such as plasmids, ARGs, MGEs, and pathogenicity islands [9–11]. Previous studies have shown that plasmids carrying ARGs are horizontally transferable and often surrounded by MGEs like transposons and integrons, contributing to the spread of resistance [12].

In our study, we performed antimicrobial susceptibility testing and WGS to determine the genetic background of antimicrobial resistance and corresponding ARGs, MGEs and virulence factors of clinical *S.* Enteritidis, in addition to phylogenetic relationship analyses with other isolates from different sources. The results will provide data for understanding the antimicrobial resistance profile, virulence factors and genomic characterization of *S.* Enteritidis strains prevalent in the local population. It will allow public health agencies to develop targeted intervention and prevention strategies based on the prevalence and characterization of MDR *Salmonella*.

Materials and methods

Salmonella isolates

The five *S. enterica* in this study were isolated from fecal samples of patients with clinical diarrhea. The isolates were identified based on biochemical tests embedded in the API-20E system (bioMérieux, France). All isolates were stored in buffered peptone water with 50% glycerol at -80 °C until further study. As the focus of this study is on bacteria, it was exempted from review by the Ethics Committee of Zhangjiagang First People's Hospital.

Antimicrobial susceptibility and ESBLs assay

Antimicrobial susceptibility testing of *S. enterica* was performed by the minimal inhibitory concentration (MIC) method using the Sensititre NARMS Gram Negative Plate (CMV3AGNF, Thermo Fisher Scientific, USA). The 17 antimicrobials used were ampicillin (AMP), ampicillin/ sulbactam (AMS), ceftriaxone (AXO), ceftazidime (CAZ), cefotaxime (CTX), cefazolin (CFZ), cefepime (FEP), azithromycin (AZM), chloramphenicol (CHL), tetracycline (TET), doxycycline (DOX), nalidixic acid (NAL), sulfafurazole (SUL), colistin (CT), polymixin B (PB), kanamycin (KAN), treptomycin (STR). The MIC breakpoints of each antimicrobial were used as recommended by the current Clinical and Laboratory Standard Institute (CLSI) [13] and European Committee on Antimicrobial susceptibility Testing (ECAST) guidance [14]. *S. enterica* was defined MDR when it exhibits resistance to three or more antimicrobial classes [15].

To detect ESBLs, the disc-approximation method was employed [16]. Double-disk diffusion was performed using both cefotaxime (30 μg) and ceftazidime (30 μg) disks individually, as well as in combination with clavulanic acid (cefotaxime/clavulanic acid (30/10 μg) and ceftazidime/clavulanic acid (30/10 μg). *Escherichia coli* ATCC 25922 served as quality control strains for the ESBLs screening test. The phenotypic presence of ESBLs in the isolates was ascertained by measuring the increase in the diameter of the inhibition zone around the clavulanate disk in comparison to the corresponding β-lactam antimicrobial disk. If the enhancement value was greater than 5 mm, the isolate was presumptively considered an ESBLs producers [17].

DNA extraction and WGS

Genomic DNA was extracted and purified using the QIAamp DNA mini kit (Qiagen, USA) according to the instructions provided by the manufacturer. Whole-genome sequencing was performed by the BGI Genomics (Shenzhen, China) using a hybrid approach combining PacBio RS II (Pacific Biosciences, USA) long-read sequencing and DNBSEQ-G400 (MGI Tech, China) short-read sequencing. For the DNBSEQ-G400 platform, an approximately 400-bp library was constructed using

the MGIEasy FS DNA library prep set. Subsequently, 2×150-bp paired-end sequencing was performed using the DNBSEQ-G400 platform. For the PacBio Sequel platform, a 10-kbp DNA library was constructed and sequenced using single-molecule real-time (SMRT) sequencing technology by Template Prep Kit 1.0. The obtained raw data were filtered by Fastp [18] and then the sequence data were assembled using SPAdes [19] software for the DNBSEQ-G400 platform and Canu [20] software for the PacBio Sequel platform. Finally, the assembly was polished using Pilon [21].

Bioinformatics analysis

The bacterial genome was annotated using the Prokka prokaryotic gene prediction tool (v1.14.5, https://github.com/tseemann/prokka) [22]. To identify the serotype of the strain, the SeqSero2 software (v1.3.1, https://github.com/denglab/SeqSero2) was utilized [23]. The sequence type was determined through multilocus sequence typing using the PubMLST scheme (https://pubmlst.org/organisms/salmonella-spp) [24]. The plasmid type was identified using Plasmidfinder (v2.1, https://cge.food.dtu.dk/services/PlasmidFinder/) [25]. Acquired ARGs and chromosomal mutations associated with antimicrobial resistance were identified using the online tool ResFinder (v4.0, https://cge.food.dtu.dk/services/ResFinder/) [26]. Virulence factors were detected using the VFanalyzer in the Virulence Factor Database [27]. GIs were predicted using the IslandViewer 4 website (https://www.pathogenomics.sfu.ca/islandviewer/) [28], and *Salmonella* pathogenicity islands were identified using SPIFinder (v2.0, https://cge.food.dtu.dk/services/SPIFinder/) [29]. The detection of prophage regions was performed using the Phaster tool. CRISPR-Cas system types were identified with CRISPR-casfinder (https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index) [30], for the comparison of *Salmonella* chromosome genomes, the BLAST Ring Image Generator (BRIG) was employed [31], while plasmid genomes were compared using BLASTn and visualized using Easyfig (v2.2.5) [32].

Phylogenetic analysis

The phylogenetic tree was constructed based on the genome single nucleotide polymorphism (SNP). The genomic data of *S. enterica* isolates from food, animal, environment, and clinical sources were collected from the NCBI database, with a total of 45 *S. enterica* strains selected for analysis (Table S1). *S. enterica* ATCC 14,028 was used as the reference genome for comparison. Sequence alignment was performed using MEGA 11 as well as the construction of a phylogenetic tree. The SNP analysis was performed by using the online tool CSI Phylogeny 1.4 [33], and then infers a phylogeny based on the concatenated alignment of the high-quality SNPs. The pipeline was run with default parameters, select 10× as the minimum depth at SNP positions, 10% as the minimum relative depth at SNP positions, 10 bp as the minimum distance between SNPs, 30 as the minimum SNP quality, 25 as the minimum read mapping quality, and 1.96 as the minimum Z-score. The maximum likelihood algorithm profile as Newick tree format was created with the FastTree (v2.1) [34]. Evolview (v3) was used to perform the display, annotation and management of the phylogenetic tree [35].

Results and discussion

Antimicrobial resistance profiles of *S.* Enteritidis

The serotype of the five *Salmonella* strains was identified as Enteritidis. Five *S.* Enteritidis isolates were subjected to an antibiotic susceptibility test using 17 antimicrobials of 7 classes. Antibiotic susceptibility testing revealed distinct resistance profiles among 5 strains of *S.* Enteritidis: strain 21 A exhibited resistance to 10 antimicrobials (AMP-AXO-CAZ-CTX-CFZ-FEP-AZM-NAL-KAN-STR), strain 33 A was resistant to 6 antimicrobials (AMP-NAL-SUL-CT-PB-STR), strain 38 A was resistant to 3 antimicrobials (AMP-NAL-KAN), strain 44 A was resistant to 14 antimicrobials (AMP-AMS-AXO-CAZ-CTX-CFZ-FEP-AZM-CHL-TET-DOX-NAL-SUL-STR), and strain 48 A was resistant to only 1 antibiotic (NAL). Notably, all isolates were resistant to nalidixic acid and 33 A was resistant to colistin and polymyxin B. Except for isolate 48 A the other four strains should be considered as MDR isolates, as they exhibit resistance to three or more classes of antimicrobials. Further, only 21 A and 44 A were identified as ESBLs-producing strains.

In our study, all isolates were resistant to nalidixic acid. This result aligns with the prevailing trend reported in literature, where resistance to nalidixic acid is frequently observed in *S. enterica* isolated from both food and clinical samples [36]. The emergence of MDR *S. enterica* strains is another additional issue for concern, with only one strain in our study proving not to be MDR. Reports from Korea and Greece have documented high rates of multiple antimicrobial resistance in *S. enterica* [37, 38], and even higher prevalence rates have been noted in earlier studies conducted in China [39]. Currently, the primary antimicrobial treatment option for salmonellosis is the use of cephalosporins [40]. However, two strains in our study were resistant to third-generation cephalosporins, including cefotaxime ceftazidime, and ceftriaxone (Table 1), and they were ESBLs-producing strains. Although the EFSA 2018/2019 reported the resistance of *Salmonella* spp.to third-generation cephalosporins at the overall low levels of 1.8% and 1.2% for cefotaxime and ceftazidime, respectively [41]. The prevalence of ESBLs phenotype was much higher in MDR strains reported

Table 1 Antibiotic resistance phenotypes of five *S.* Enteritidis strains

Strains	Antibiotics																
	Beta-lactams							Phenicols	Tetracyclines		Quinolones	Sulfonamides	Polypeptides		Aminoglycosides		
	AMP	AMS	AXO	CAZ	CTX	CFZ	FEP	AZM	CHL	TET	DOX	NAL	SUL	CT	PB	KAN	STR
21 A	R		R	R	R	R	R	R				R				R	R
33 A	R											R	R	R	R		R
38 A	R											R				R	
44 A	R	R	R	R	R	R	R	R	R	R	R	R	R				R
48 A												R					

Notes: AMP: ampicillin; AMS: ampicillin/sulbactam: AXO: ceftriaxone; CAZ: ceftazidime; CTX: cefotaxime; CFZ: cefazolin; FEP: cefepime; AZM: azithromycin; CHL: chloramphenicol; TET: tetracycline; DOX: doxycycline; NAL: nalidixic acid; SUL: sulfafurazole; CT: colistin; PB: polymixin B; KAN: kanamycin; STR: streptomycin; R: resistant

in China [42]. In particular, resistance to colistin, considered to be an antimicrobial of last resort and used to treat MDR Gram-negative bacteria, was identified in one of the strains in our study. The *mcr-1* gene, first reported in China in 2015, has been associated with colistin resistance. Several studies have reported on the mechanisms by which *mcr-1* gene variants produce new mucin resistance and their genetic characterization [33, 43]. The complex and multifaceted mechanisms of resistance to specific antimicrobials, which may not depend solely on the expression of a single gene, pose a significant threat to the effective treatment of *S. enterica* infections. This highlights the critical need for robust antimicrobial resistance surveillance and the implementation of comprehensive measures to standardize antimicrobial use in China.

Genomic features

After conducting de novo assembly using short-read and long-read data, the complete genomes of the five *S.* Enteritidis strains were obtained, revealing variations in genome sizes and G+C contents (Table 2). Strain 21A exhibited 4506 coding sequences (CDSs) and 87 tRNAs, 33A had 4452 CDSs and 86 tRNAs, 38A had 4,465 CDSs and 86 tRNAs, 44A had 4523 CDSs and 87 tRNAs, and 48A had 4,427 CDSs and 86 tRNAs. It is worth noting that each strain contained 22 rRNAs. Moreover, each strains carried 1–3 distinct plasmids, harboring multiple ARGs, including that conferring resistance to aminoglycosides (*aph(3")-Ib* and *aph(6)-Id*), β-lactam (bla_{TEM-1B}, $bla_{TEM-141}$ and $bla_{CTX-M-55}$), phenicols (*floR*), tetracyclines (*tet(A)*), fosfomycin (*fosA3*) and sulfonamide (*sul2*). In addition, all strains carried an aminoglycoside resistance gene (*aac(6')-Iaa*) on their chromosomes and predicted point mutations in gene (*gyrA*: p.D87G and p.D87Y) associated with fluoroquinolone resistance were identified. Specifically, a single point mutation in the quinolone resistance-determining region of the *gyrA* gene results in reduced sensitivity to ciprofloxacin and nalidixic acid. With *S.* Enteritidis 21 A and 48 A had a mutation in *gyrA* (D87G), while *S.* Enteritidis 33 A and 44 A and a mutation in *gyrA* (D87Y). Point mutations in the efflux pump-encoding genes *acrB* (F28L) and *acrB* (L40P) were also identified in our study (Table 2). However, the colistin resistance gene *mcr-1* was not detected in any of the *S.* Enteritidis strains. The inconsistency between colistin resistance in phenotype and genotype points to potential new resistance mechanisms, prompting further investigation. Functional annotations from the Gene Ontology (GO) database revealed that the gene products of strain 21 A were primarily associated with biological processes, including biological adhesion, regulation, cellular and metabolic processes (Fig. S1). A total of 4,295 CDSs were assigned to COG categories, comprising 23

Table 2 Summary of complete genome sequences, antibiotic resistance genotypes and plasmid profiles of five *S.* Enteritidis strains

Strain	Sequence type	Total length (bp)	GC content (%)	Acquired antibiotic resistance genes	Chromosomal point mutations	Replicons
21 A	Chromosome	4,679,872	52.18	*aac(6')-Iaa*	*gyrA*: p.D87G; *acrB*: p.F28L; *acrB*: p.L40P	
	p21A_1	67,678	52.30	$bla_{CTX-M-55}$, $bla_{TEM-141}$, *aph(3')-IIa*		IncFII(pHN7A8)
	p21A_2	60,200	51.95	-		IncFIB(S), IncFII(S)
33 A	Chromosome	4,686,594	52.18	*aac(6')-Iaa*	*gyrA*: p.D87Y; *acrB*: p.F28L; *acrB*: p.L40P	
	p33A_1	74,492	49.92	bla_{TEM-1B}		IncFIB(S), IncFII(S)
38 A	Chromosome	4,682,650	52.18	*aac(6')-Iaa*	gyrA: p.D87Y; *acrB*: p.F28L; *acrB*: p.L40P	
	p38A_1	17,635	43.56	-		IncX1
	p38A_2	74,492	49.92	bla_{TEM-1B}		IncFIB(S), IncFII(S)
44 A	Chromosome	4,679,994	52.18	*aac(6')-Iaa*	*gyrA*: p.D87Y; *acrB*: p.F28L; *acrB*: p.L40P	
	p44A_1	64,327	51.76	bla_{TEM-1B}		IncFIB(S), IncFII(S)
	p44A_2	59,138	52.72	*fosA3*, $bla_{TEM-141}$, $bla_{CTX-M-55}$		IncFII(pHN7A8)
	p44A_3	23,819	47.89	*sul2*, *aph(3")-Ib*, *aph(6)-Id*, *tet(A)*, *floR*		IncX1
48 A	Chromosome	4,679,962	52.18	*aac(6')-Iaa*	*gyrA*: p.D87G; *acrB*: p.F28L; *acrB*: p.L40P	
	p48A-1	59,372	51.94	-		IncFIB(S), IncFII(S)

functional categories. Predicted encoding genes were mainly involved in cell wall/membrane/envelope biogenesis, signal transduction mechanisms, transcription, amino acid transport and metabolism, carbohydrate transport and metabolism and inorganic ion transport and metabolism (Fig. S2). KEGG pathway analysis highlighted that the predicted encoding genes were predominantly distributed among gene entries associated with human diseases, with the highest proportion of ARGs (Fig. S3). Similar results were found regarding the functional classification of genes in the genomes of four other *S.* Enteritidis (not shown).

Whole genome sequencing coupled with antibiotic resistance gene databases allows for the identification of all resistance genes present in the strains. Li et al. [44] screened *Salmonella* isolates from the pork production chain for antibiotic resistance genes, finding high prevalence rates of 46.75% for *floR* (chloramphenicol resistance) and 87.8% for *tet(A)* (tetracycline resistance). These genes are widely distributed among *Salmonella*. Pornsukarom et al. [45] similarly identified *sul1*, *tet(R)*, and *tet(A)* as the most common resistance genes in 200 *Salmonella* strains. In this study, strain 21 A and 44 A harbored extended-spectrum β-lactamase (ESBL) genes, particularly $bla_{CTX-M-55}$, which is becoming increasingly prevalent in China and is often found both chromosomally and on plasmids [46]. ESBLs of CTX-M now outnumber TEM and SHV types as the most common in *Enterobacteriaceae*. A comparative analysis of whole genome data and antibiotic resistance gene databases revealed nine major classes of resistance genes in the studied *Salmonella* strains. *aac(6')-Iaa* was the most prevalent, followed by *sul1*, *sul2*, *floR*, and *tet(A)*, consistent with previous findings [47]. Furthermore, point mutations in the *acrB* and *gyrA* genes were identified using the Pointfinder database. Mutations in *gyrA* are associated with quinolone antibiotic resistance [48]. Hooda et al. found that *acrB* mutations confer azithromycin resistance in *S.* Typhimurium [49]. This highlights the importance of considering *acrB* mutations in addition to the *mphA* gene when studying azithromycin resistance.

To further characterize the genomes of the five *S.* Enteritidis, a comprehensive BLAST comparison was performed using the complete genomes of five *S. enterica* strains from different serotypes. These genomes included *S.* Enteritidis SE211, *S.* Typhimurium ATCC 14,028, *S.* Kentucky PU131, *S.* Typhi CT18 and *S.* Typhimurium LT2 (Fig. 1). Notably, the genome sequences of the five strains showed high similarity to the previously reported *S.* Enteritidis SE211 [3], with 99% query coverage and 100% identity, which was isolated from a chicken in 2010 in Henan, China.

Virulence factors and SPIs

Virulence factor analysis was conducted on 50 *S. enterica* genomes, which included 45 genomes obtained from the NCBI database. A total of 265 virulence genes were identified. These virulence genes encoded by the chromosome and plasmid were categorized into 15 VF classes encompassing adherence determinants, macrophage inducible genes, magnesium uptake, secretion system, stress adaptation, immune evasion, iron uptake and others. A detailed result of virulence gene mapping for 50 *S. enterica* genomes is listed in Table S2. The virulence gene pattern of five strains in this study was similar to every other strain, and a core set of virulence genes was conserved in all strains, regardless of geographic origin and isolation source. The result suggests that the five clinical

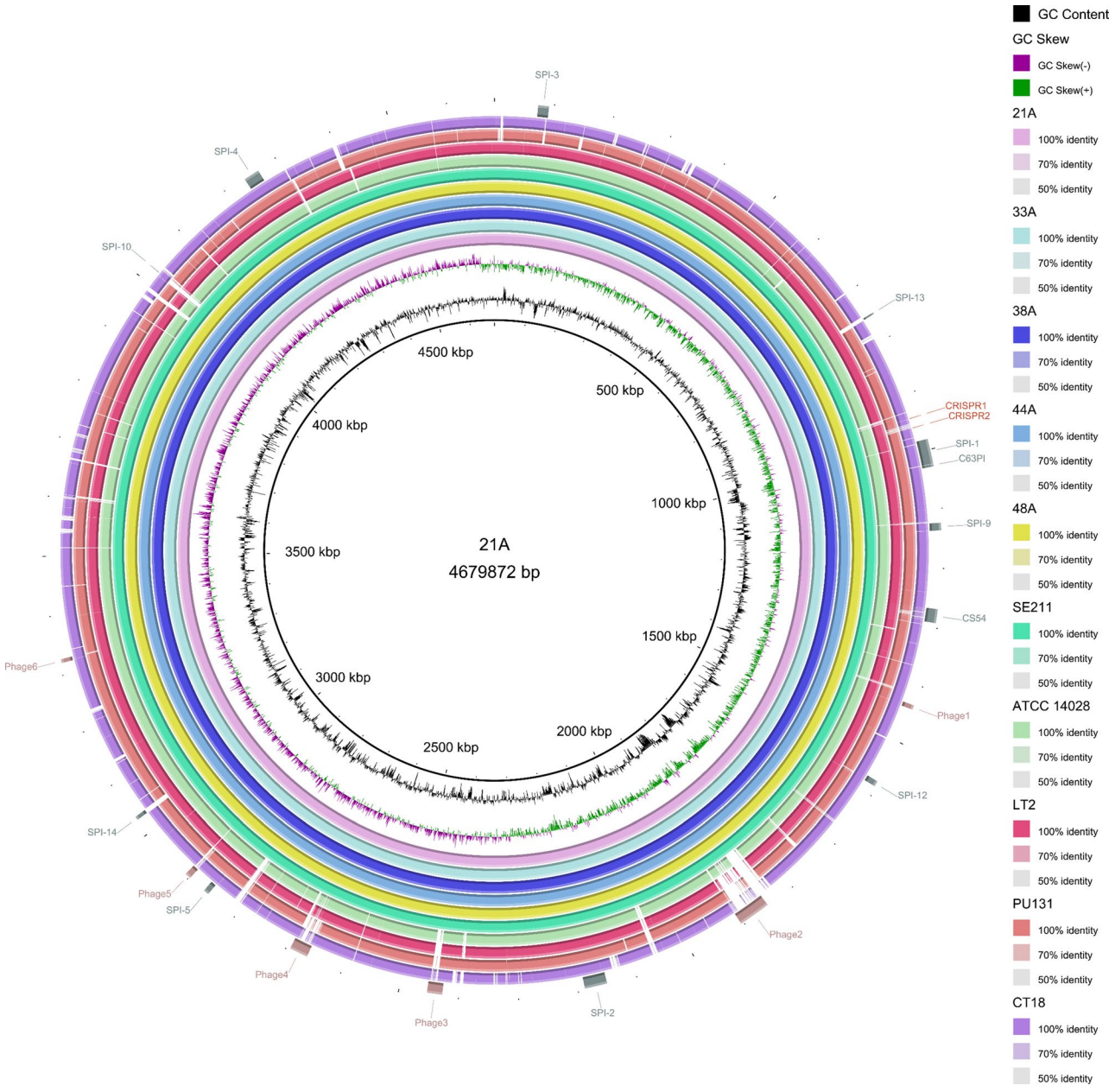

Fig. 1 Chromosome sequences comparison of five *S.* Enteritidis with reference genome *S.* Typhimurium ATCC 14,028 (CP102669.1), *S.* Typhimurium LT2 (NC_003197.2), *S.* Enteritidis SE211 (CP084532.1), *S.* Kentucky PU131 (CP026327.1) and *S.* Typhi CT18 (NC_003198.1). BLASTN match results with identity between 50–100% are shown in gradient color. The outer circles are CRISPR, prophage and *Salmonella* pathogenicity islands, respectively

isolates in this study contain the same virulence factors as the food and environmental isolates. The virulence gene profiles exhibited greater similarity within the same sequence types (STs) (Fig. 2). Moreover, most genes associated with intracellular bacterial invasion and type III secretion systems (TTSS) were found to be common to all *S. enterica*, highlighting fundamental similarities in these virulence-related mechanisms across the strains [50].

In our study, we identified 164 virulence genes in the genomes of *S.* Enteritidis 21 A, 33 A, and 48 A, while 163 and 162 virulence genes were found in the genomes of *S.* Enteritidis 38 A and 44 A, respectively. The virulence genes of the strains were distributed in 8 of the 15 VF classes. In the genome of *S.* Enteritidis 21 A, a total of 30 TTSS genes (*hilACD*, *iacP*, *iagB*, *invABCEFGHIJ*, *orgABC*, *prgHIJK*, *sicAP*, *sipD*, *spaOPQRS* and *sprB*) were located on SPI-1, 26 TTSS genes (*ssaCDEGHIJKLMNOPQRTUV*, *sscAB*, *sseBCDE* and *ssrAB*) were located on SPI-2, 10 TTSS-1 translocated effectors (*avrA*, *sipABC*, *sopAB/sigD*, *sopDEE2* and *sptP*), 14 TTSS-2 translocated effectors (*pipBB2*, *sifAB*, *spvCD*, *sseFGJK1K2L*, *sseI/srfH*

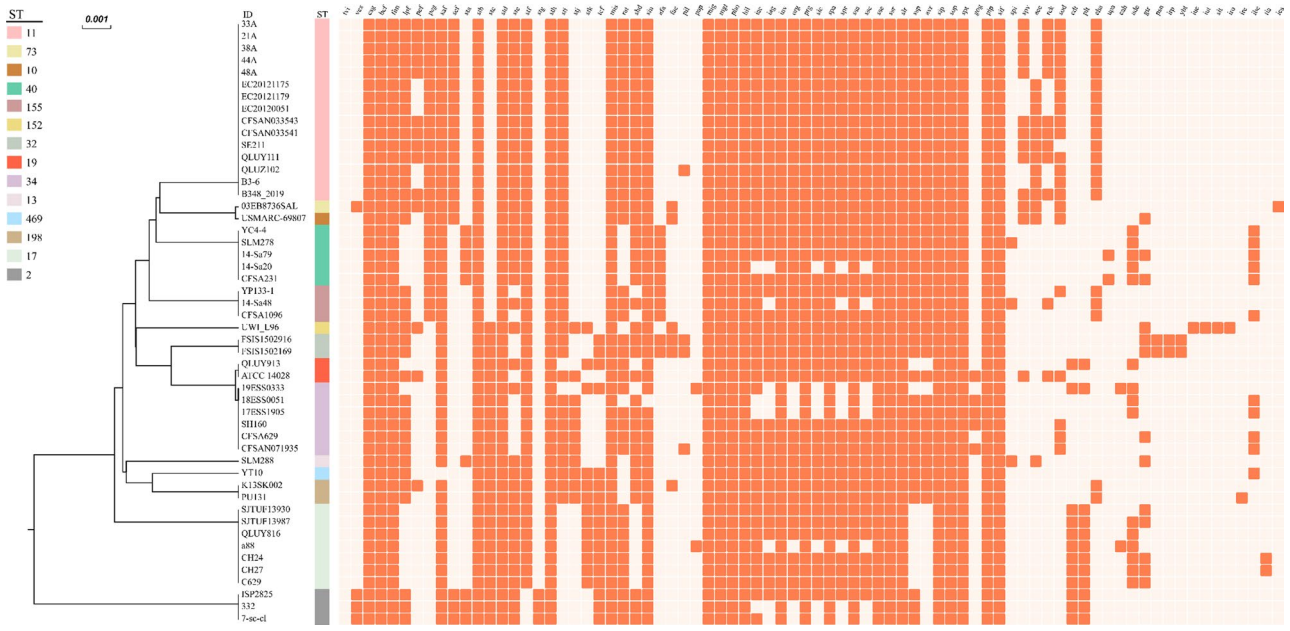

Fig. 2 Binary heatmap analysis of virulence genes carried by five *S.* Enteritidis and other 45 *S. enterica*. The tree shown on the left was constructed using MEGA by linking the sequences of the 7 housekeeping genes. The tree shown on the left was built using 7 Housekeeping Genes by MEGA. Light color means missing results for virulence gene

and *sspH2*) and 2 TTSS effectors (*slrP* and *sspH1*) were identified through two systematic translocations. *S.* Enteritidis 33 A and 48 A possessed the same virulence genes as 21 A. 44 A had fewer the adherence determinant genes *stbA* than 21 A, and in addition to this, 44 A and 38 A had less of the TTSS1 translocation effector *sopE* than 21 A. Notably, each strain carries virulence plasmid genes, including plasmid-encoded fimbriae (*pefABCD*), a macrophage inducible gene *mig-5*, virulence plasmid genes (*spvBCD*), and resistance to complement killing *rck*. Some virulence factors can directly produce toxins, such as the cytolethal distending toxin cdtB and the pertussis-like toxin pltA, which may contribute to pathogenicity in humans and animals, and which were detected with a 24% probability in 50 genomes. However, none of these toxin-producing factors were identified in the five *S.* Enteritidis strains studied here.

Salmonella pathogenicity islands (SPIs) are clusters of genes responsible for encoding the various virulence factors [51]. A total of 12 SPIs were identified using SPI-Finder in five *S.* Enteritidis strains, including SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-9, SPI-10, SPI-12, SPI-13, SPI-14, C63PI and CS54 (Table S3). A common feature of many SPIs of enteric pathogens is their insertion at tRNA loci with different G+C content compared to the rest of the genome [52]. For example, the *valV* tRNA locus is the site of SPI-2 integration, the *selC* tRNA locus is the site of SPI-3 integration in *S. enterica* and the site of the LEE pathogenicity island integration in enteropathogenic *E. coli* strains [53]. The subset of GIs which mediate the horizontal transfer of genes encode numerous resistance and virulence factors such as TTSS. C63PI, reported in a variety of *S. enterica*, known to encode protein constituents of the manganese (II) and iron (II) uptake systems [54], suggesting its significance. CS54, another pathogenicity island found in some *S. enterica* strains, a contains genes associated with virulence, antimicrobial resistance, and fitness, including genes encoding efflux pumps, iron acquisition systems, and proteins involved in biofilm formation [55]. The SPIs such as SPI-1, SPI-2, SPI-3, SPI-4, and SPI-5 play an important role in adhesion, invasion, and the intracellular lifestyle of the pathogen [56]. Among them, SPI-1 and SPI-2 are particular important, frequently found in *S. enterica*, functioning to deliver effector proteins to the host cell cytoplasm. These two islands lead to pathogenic processes from invasion to survival and intracellular replication.

Genomic islands

Horizontal gene transfer (HGT) was a core event in genome evolution and microbial adaptation to the ecological niche, and GIs are usually clusters of genes in a bacterial or archaeal genome acquired through HGT. A total of 29 GIs were predicted 21 A and 33 A, while 30 GIs were predicted in 38 A, 44 A, and 48 A, designated as GI-1 to GI-30 (Table S4). Among the gene islands contained in 21 A, GI-29, GI-6, GI-14, GI-15, GI-18, and GI-26 contained T1SS, T3SS, and T4SS protein-coding genes, respectively. In addition, the GI-23 and GI-27 regions contain genes encoding predicted proteins

associated with virulence, including putative positive transcriptional regulators, virulence regulon transcriptional activators, fimbrial protein, and the bacterial toxin CadB targeting DNA gyrase [57]. The analysis of three other GIs, GI-7 GI-16 and GI-20, also revealed the presence of genes thought to be associated with virulence. For example, the *Salmonella* type III effector PipB2 was identified in GI-7 with a sequence similar to the previously identified effector PipB of the same system [58]. The *Salmonella* type III effectors SseJ and SseI were found in GI-16 and GI-20, respectively. Notably the phage tail fiber assembly protein TfaE was also found in GI-20, suggesting that this GI is likely to be a phage remnant obtained from this strain. GI-19 contained the putative metabolite transporter protein YjhB along with two IS3 family transposases, suggesting that this strain has the capacity to actively acquire genomic elements, which may increase its pathogenicity. Furthermore, some *Salmonella* GIs that are known as unstable pathogenicity islands, possess the ability to cut and transfer between bacteria [59].

GI-1 and GI-10 contain genes of cytochrome c biogenesis.GI-12 is the largest GI detected in this strain, with a size of 74,696 bp, and contained several genes in this GI, including DNA-binding protein Hns, phage integrase IntA, and crossover junction endodeoxyribonuclease RusA, in addition to a large number of hypothetical proteins. These GIs provide a major drive for genome evolution, which can enhance pathogens survival through parenteral transmission [59, 60]. This may be one of the reasons for the high prevalence of *Salmonella* in foods or animals. Similar results were observed in three other strains. Notably *S.* Enteritidis 38 A, 44 A and 48 A one additional GI than 21 A, carrying genes including the HTH-type transcriptional regulator HmrR, copper-transporting P-type ATPase ActP, and efflux pump periplasmic junction BepF. However, there have been numerous previous reports indicating that some GIs encode ARGs that are common in *Salmonella* and other food-borne pathogens [61, 62]. While no GIs associated with antimicrobial resistance were found in any of the five strains in this study, the many GIs in these strains provide a potential opportunity for the acquisition of such ARGs.

Phage and CRISPR

Six prophage regions, named Phages 1 to 6, were predicted on the chromosomal genome of *S.* Enteritidis 21 A, including two complete (Phage2 and 4) and four incomplete regions (Phage1, 3, 5, and 6) (Fig. 1). The two complete prophages were Salmon_118970_sal3 and Gifsy_2. In addition, non-*Salmonella* prophage sequences were contained in 4 other incomplete prophages. For example, Phage1 was a phage of *Escherichia* spp. (Escher_500465_2), and Phage3 and Phage6 were prophages of *Shigella* spp. (Shigel_Stx and Shigel_SflV), which may indicate horizontal gene transfer or polyvalent prophages (Table S5). Understanding the roles of bacteriophages in *Salmonella* pathogenesis has been challenging due to the diversity of prophages and *Salmonella* strains. However, it is increasingly evident that prophages play significant roles by delivering functional effector molecules and contributing to the *Salmonella* virulence regulatory networks. For example, we identified multiple virulence factors present in Phage2 (*seek2*), Phage4 (*sopE* and *sodC1*) and Phage5 (*sseI*). The distribution of these phage-encoded effector molecules is often serovar and even strain specific, suggesting their incorporation via transducing prophage [63]. Gifsy-2 phages, known to regulate *Salmonella* virulence, were represented by Phage 4 and Phage 5, carrying corresponding virulence genes crucial for *Salmonella* overall virulence [64]. Similar results were observed in the chromosomes of four other *S.* Enteritidis strains, with the difference that they carried plasmids containing different incomplete prophages. Furthermore, the prophage region contains a large number of putative proteins whose exact role in the biology of the five *S.* Enteritidis remain to be explored.

The CRISPR-Cas system in bacteria and archaea hosts is an adaptive prokaryotic immune system that can be resistant to foreign genetic material such as MGEs. All strains carried CRISPR loci with encoded Cas proteins, and such systems belonged to Type I-E. All strains were found to have 2 CRISPR and 1 Cas type I-E (Table S6). CRISPR was classified using levels of evidence on a scale of 1 to 4. CRISPRs with an evidence level of 4 were designated as highly predictive candidate motifs. The level of evidence for CRISPR 1 and CRISPR 2 was 4 in all strains. Eight *cas* genes including *cas1*, *cas2*, *cas3*, *cas5*, *cas6*, *cas7*, *cse1* and *cse2* were identified. Similarly, two CRISPR regions were also found in the chromosome of SE211, as well as eight *cas* genes belonging to the type I-E CRISPR-Cas system, as previously reported [3]. Furthermore, in addition to its involvement in viral defense, it is possible that the CRISPR-Cas system could be involved in the regulation of virulence gene expression. Bacterial strains with the CRISPR-Cas system tended to have a greater ability to form biofilms and were more likely to colonize mouse organs than bacteria lacking the CRISPR-Cas system or the core Cas proteins [65]. This suggests a potential link between the CRISPR-Cas system and bacterial virulence, highlighting the need for careful consideration of its impact.

Phylogenetic tree

Multilocus sequence typing (MLST) showed that all strains in this study belonged to ST11. MLST cluster analysis yielded a total of 14 well-defined *Salmonella* groups, which closely matched the detected ST types.

Strains of the same ST type clustered together (Fig. 2). ST11 is reported as the most prevalent *Salmonella* STs in China [66, 67]. A phylogenetic tree based on high-quality SNPs was constructed in order to gain a deeper understanding of the phylogenetic relationships among the 50 *S. enterica* and between *S.* Enteritidis and other *Salmonella* serotypes of isolates. The phylogenetic tree in Fig. 3 illustrates that *S.* Enteritidis isolates have a relatively closer genetic relationship with Dublin serotypes, which is consistent with previous findings [68, 69]. *S.* Dublin is a bovine host-adapted serotype but can still cause human infections with high pathogenicity and mortality [70]. Large numbers of MDR *S.* Dublin have been reported globally, showing resistance patterns and harboring numerous ARGs [71]. The close genetic relationship observed between Enteritidis and Dublin serotypes raises concerns gives us a warning that about the potential transfer of MDR *S.* Enteritidis from food to humans through the food supply chain. This scenario could lead to outbreaks, emphasizing the urgent need to enhance epidemiological surveillance and management of MDR *S.* Enteritidis to minimize its threat to food safety and public health.

Furthermore, the genetic relationships of intestinal *Salmonella* strains isolated from various sources such as clinical, food, environmental, and animal origins were investigated through phylogenetic analysis. Clustering appeared to be independent of the year, source, or geographic location of isolation. Notably, in this study, five strains of enteric *Salmonella* were clustered together, each associated with environmental strain B3-6, clinical strain B348_2019, and animal strain SE211, respectively. The ranges of SNPs between them ranged from 21 to 76, with 44 A and B348_2019 differing by 27 SNPs; B348_2019 was isolated from the feces of diarrhea patients, and both belonged to clinical sources. Similarly, 48 A and SE211 differed by only 21 SNPs, with SE211 isolated from chickens. The detection of *S.* Enteritidis in

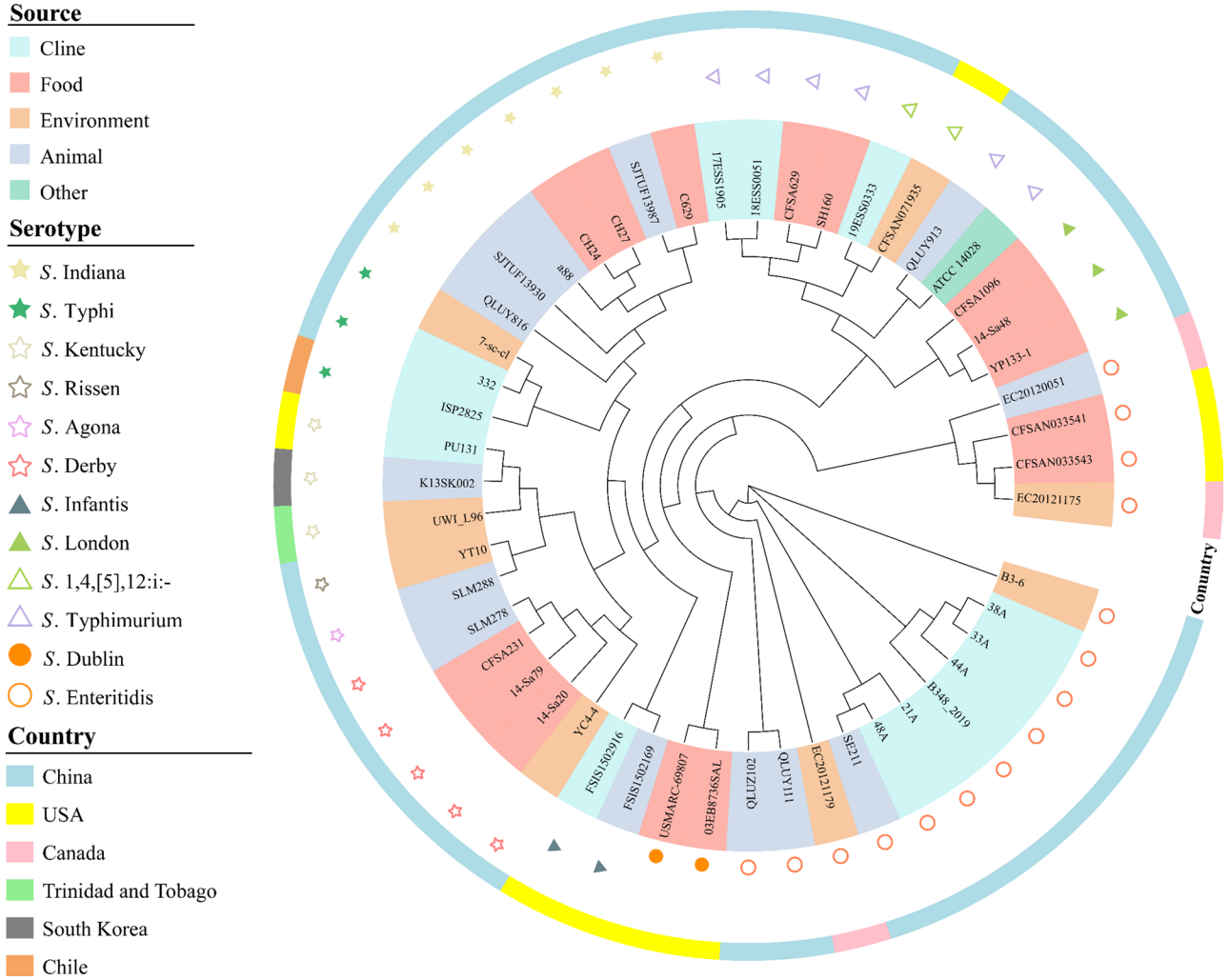

Fig. 3 Phylogenetic position of five *S.* Enteritidis, in the context of *S. enterica* isolates from different sources, countries and serotypes. Serotypes, sources and countries are annotated by colors and shapes

poultry has been reported in China on several occasions, covering different regions and multiple periods, revealing the widespread presence of this bacterium in poultry flocks [67, 72]. Overall, our findings serve as a reminder of the importance of taking action against and monitoring any *Salmonella* contamination in the food chain to avoid human *Salmonella* infections from contaminated food. The adoption of a harmonized surveillance policy at the human-food-animal-environment interface will contribute to a better understanding of the public health implications of specific serotypes and clones in China.

Plasmid characterization of *S.* Enteritidis

In this study, a total of 9 plasmids were identified, with all strains carrying at least one plasmid. The plasmids sequences were identified as belonging to the IncFII(phn7A8), IncFIB(S)-IncFII(S), and IncX1 plasmid replicon types using the PlasmidFinder tool (Table 2). The most frequently observed plasmid was IncFIB(S)-IncFII(S). Therefore, plasmid p14523B, belonging to the IncFIB(S)-IncFII(S) group, was selected as the reference plasmid for BLAST comparisons against the 9 identified plasmids. The results indicated that 5 IncFIB(S)-IncFII(S) plasmids show high similarity to the reference plasmid. Among these, p33A_1 and p38A_2 exhibit 86% query coverage and 100% identity to p14523B, while p21A_2 and p14523B display 98% query coverage and 100% identity. Notably, p44A_1 and p48A_1 have 100% query coverage and 100% identity to p14523B, which are essentially the same, but these plasmids do not share the same host, suggesting that these plasmids likely evolved from a common ancestor (Fig. 4). Some of these plasmids carry ARGs (Table 2). There are also a large number of MGEs in these plasmids, such as insertion sequences and transposons. The Tn2 transposon (bla_{TEM-1B}-*tnp*R-*tnp*A) was found in p33A_1, p38A_2, and p44A_1, and in addition to the reference plasmid p124523B was also found in pR17.1476_64K, pSE74-2, and p1.15-2E5 (Fig. 5). Tn2 carrying bla_{TEM} genes are not only present in *E. coli* [73], but have also been reported previously in *S. enterica*. However, unlike the findings in this study *IS26* will frequently insert into different sites of Tn2 and truncate *tnp*A or *tnp*R [74].

Moveable elements may also form multidrug resistance region (MRR) regions by recombining and integrating ARGs from the local environment. Plasmids p21A_1 and p44A_2 each have a MRR that carries three ARGs including $bla_{TEM-141}$, $bla_{CTX-M-55}$, *fosA3* and *aph(3')-IIa*, respectively. It was found that both of them identified the *IS26*-$bla_{TEM-141}$-*orf*-$bla_{CTX-M-55}$-*IS15* with the same transposable structure as in pAH01-3, pSE109-1 and pKP32558-4. However, pCREC-591_2 possesses a putative protein between $bla_{CTX-M-55}$ and *IS15* (Fig. 6). Furthermore, a *fosA3*-containing transposon structure (IS*26*-*orf*-*orf*-*orf*-*fosA3*-*IS26*) upstream of $bla_{TEM-141}$ in p44A_2 was identified, identical to pAH01-3. However, a second type of *fosA3* transposable unit (*IS26*-*fosA3*-*IS26*) was found in pCREC-591_2 and flanked by the opposite *IS26* sequence (Fig. 6). This observation, along with previous studies, suggest that plasmid-borne *fosA3* may be mediated by *IS26* [75]. The transposable structure of *aph(3')-IIa* was also found in p21A_1 (*IS50R*-*aph(3')-IIa*-*IS26*) similar to that in pSE109-1 and pkp32558-4, with the difference that a hypothetical protein flanks *aph(3')-IIa* (Fig. 6).

We also identified a 9,988 bp MRR in p44A_3 consisting of four ARGs, which are linked upstream to *IS1294* and downstream to *IS26* (Fig. 7). Similar transposon structures were found in the *E. coli* plasmids pP19_598a and pNDM33-4, as well as the *K. peneumoniae* plasmid Psxc4-2_tmex_350k. The difference lines in the upstream insertion sequence, which is *ISVsa3* in these plasmids instead of *IS1294*, and that the downstream insertion sequence *IS26* is flanked by another *IS5075*, with the opposite *IS26* sequence. The difference with p.E166-P2 and pAR13438_1 is also in the difference between the upstream and downstream insertion sequences, where the upstream sequence of p.E166-P2 is an incomplete *ISVsa3*, while the downstream sequence *IS26* of pAR13438_1 flanks a *TnAs3* (Fig. 7). In *Salmonella*, *IS26* is a common MGEs associated with antimicrobial resistance determinant cluster and often mediates homologous recombination of ARGs [76, 77]. Our study similarly found *IS26* sequences in several MRRs, suggesting that these MRRs were likely *IS26*-mediated, acquired through homologous recombination, and progressively integrated.

Conclusion

In this study, we analyzed the antimicrobial resistance of 5 clinical *S.* Enteritidis isolates, revealing resistance to a total of 7 classes of antibiotics, with 4 strains classified as MDR. The systematic and complete analysis of the genomes of chromosomes and plasmids revealed 11 ARGs genes, 12 SPIs and 2 complete CRISPR-Cas systems. The presence of multiple MGEs in the vicinity of multiple ARGs may lead to the formation of MRR. Phylogenetic analysis based on SNPs demonstrated significant genetic diversity among the isolates, suggesting distinct evolutionary trajectories possibly influenced by environmental factors. This divergence may influence pathogenicity, antimicrobial resistance, and epidemiological behavior, providing insights into the evolutionary history and transmission patterns of *S.* Enteritidis. By understanding the genomic and genetic characteristics of *S.* Enteritidis, we can gain a deeper understanding of the mechanisms and phylogeny of antimicrobial resistance and pathogenicity, leading to improved prevention and

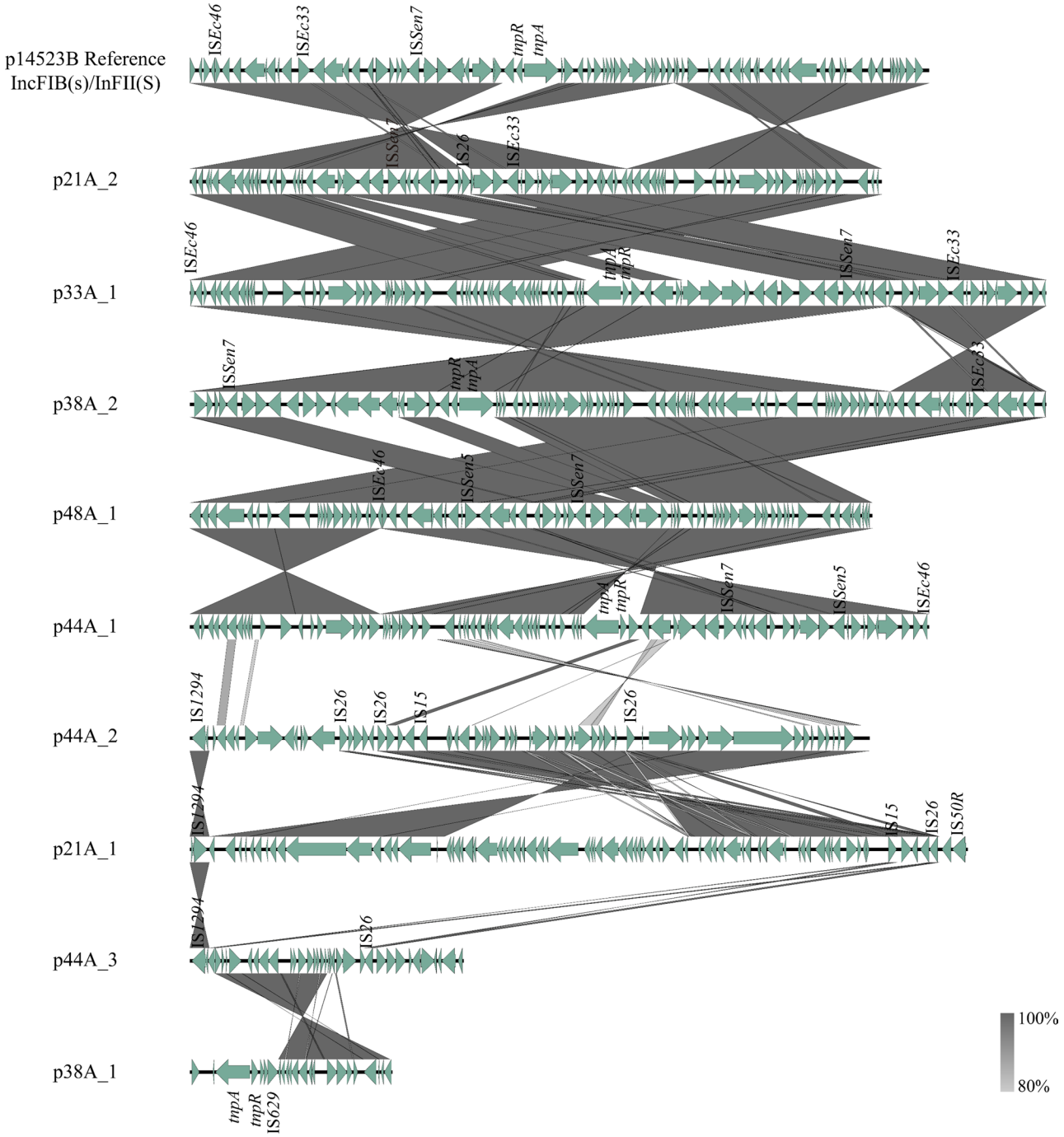

Fig. 4 Sequence comparison of plasmid of this study and reference plasmid p14523B (CP074430). The gray shaded areas indicate the homology between the corresponding gene loci on each plasmid, Arrows represent ORFs

treatment strategies. Future research could explore the transmission and antimicrobial resistance of *S.* Enteritidis across human, animal, environmental, and food sources, enhancing our understanding of its evolution and spread within the One Health framework, which emphasizes the interconnectedness of human, animal, and environmental health.

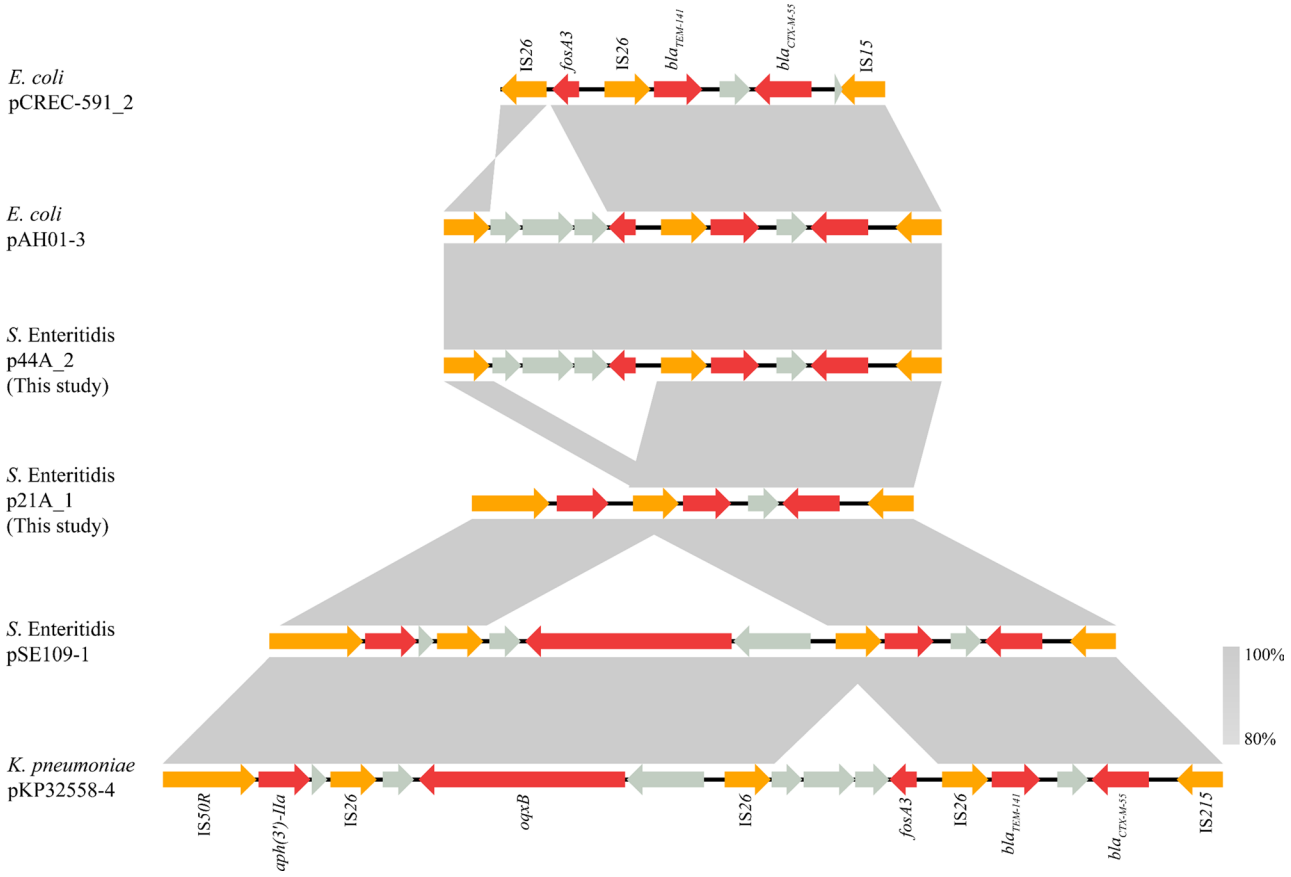

Fig. 5 Genetic comparison of bla_{TEM-1B} in plasmid p33A_1, p38A_2, p44A_1, p1.15-2E5 (MN125609), pSE74-2 (CP050725), p14523B (CP074430) and pR17.1476_64k (CP100725). The light gray shaded areas indicate the homology between the corresponding gene loci on each plasmid, Arrows represent ORFs. Red color represents antibiotic resistance genes; yellow color represents IS/transposase, and light green color represents putative proteins or other genes

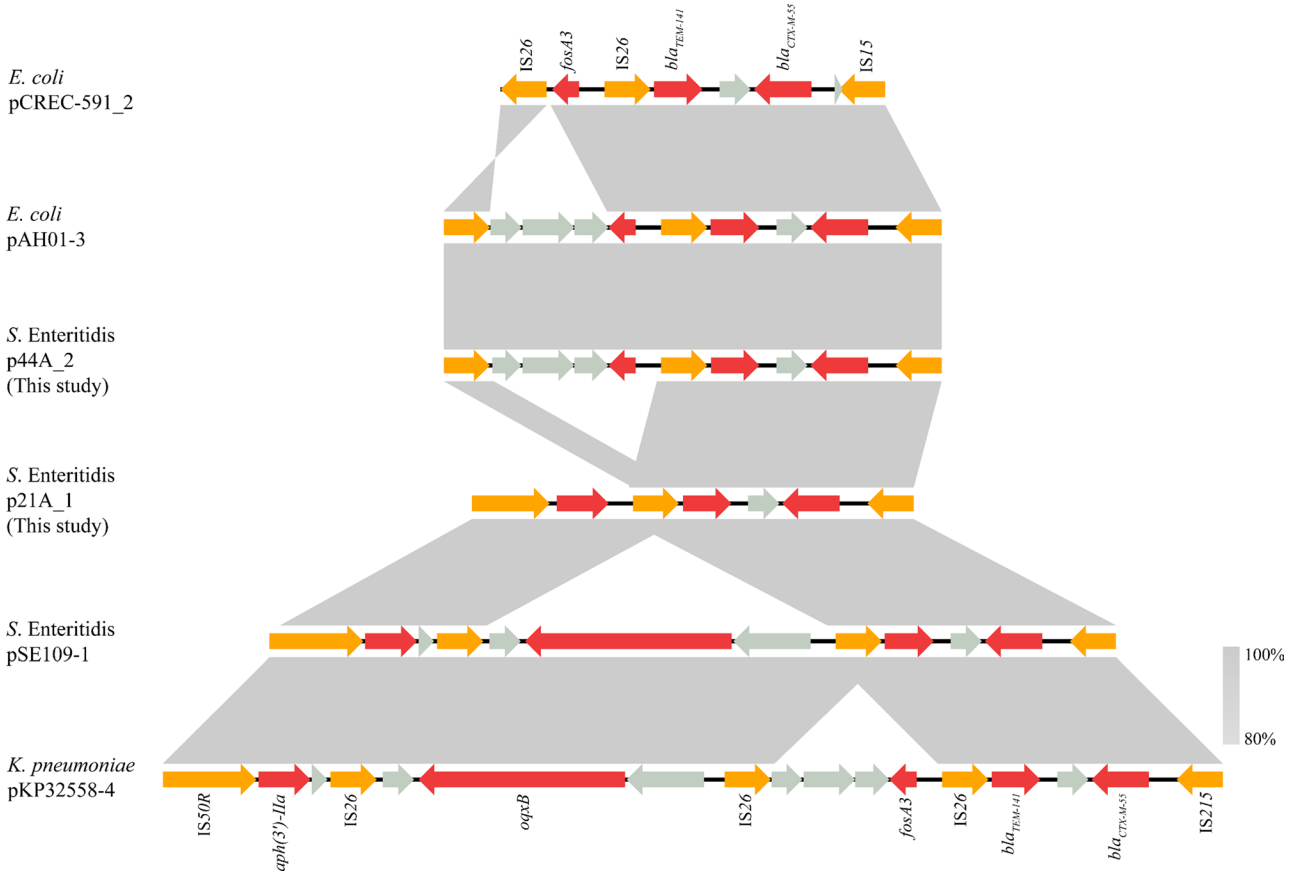

Fig. 6 Genetic comparison of fosA3, $bla_{TEM-141}$ and $bla_{CTX-M-55}$ in plasmids p21A_1, p44A_2, pCREC-591_2 (CP024823), pAH01-3 (CP055254), pSE109-1 (CP050710) and pKP32558-4 (CP076034)

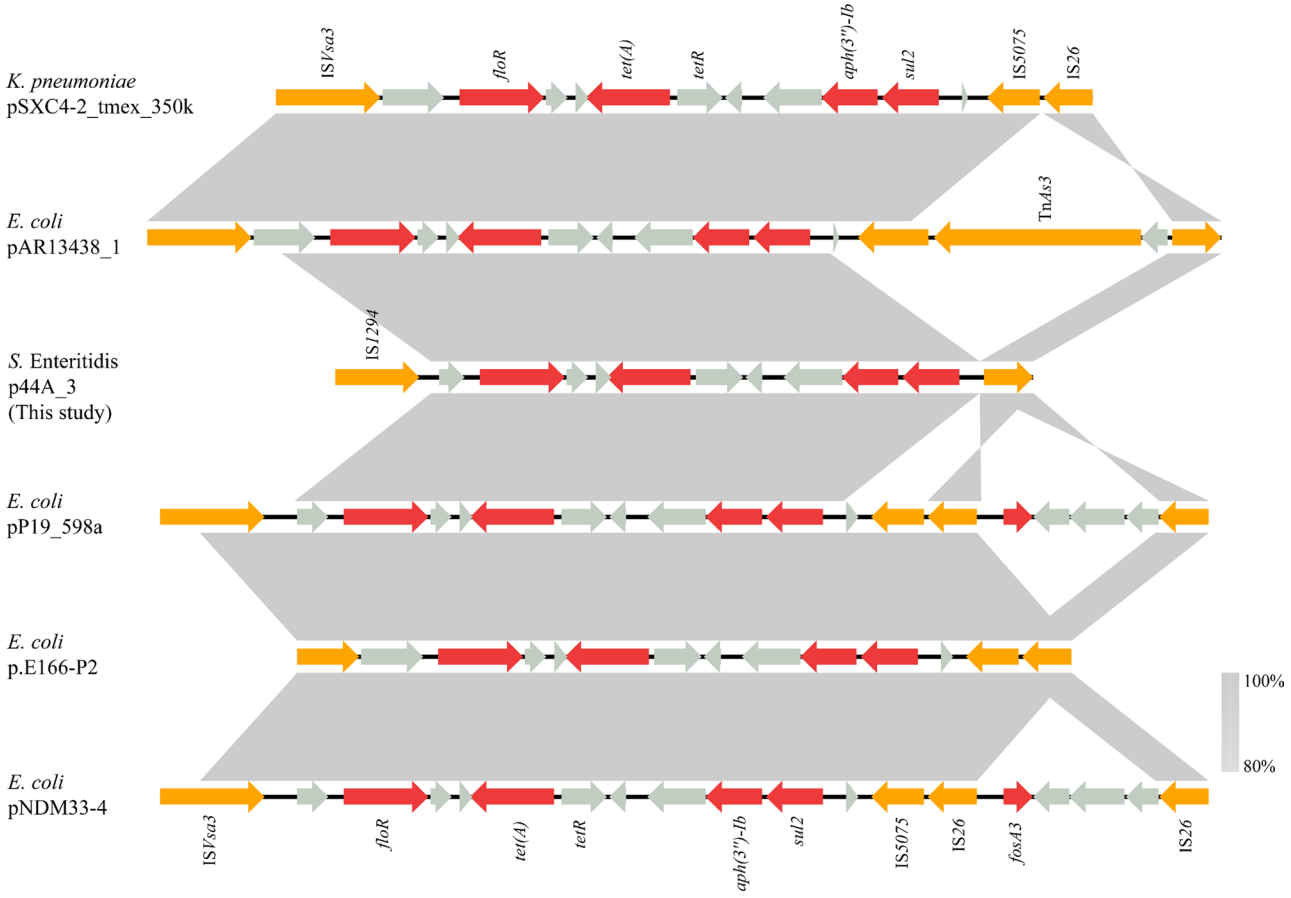

Fig. 7 Genetic comparison of *floR*, *tet(A)*, *aph(3'')-Ib* and *sul2* in plasmids p44A_3, pSXC4-2_tmex_350k (CP113193), pAR13438_1 (CP097171), pP19_598a (CP067242), p.E166-P2 (CP032068) and pNDM33-4 (CP076649). genes

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03651-4.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7
Supplementary Material 8

Author contributions

Xiangfeng Bu: Methodology, Investigation, Writing – original draft. Yufan Wu: Conceptualization, Methodology, Resources, Writing – review & editing. Yi Hong: Methodology, Writing – review & editing. Kai Jia: Methodology. Jingdong Shao: Methodology, Resources. Juping Shi: Methodology, Funding acquisition. Qingli Dong: Resources. Xiang Wang: Conceptualization, Writing – original draft, Supervision, Funding acquisition.

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Data availability

The complete sequences (including chromosome sequences and plasmid sequences) of the five S. Enteritidis strains have been deposited in the NCBI database under the Bioproject Accession Number PRJNA1051507 and the Biosample Accession Numbers SAMN38777018, SAMN38777019, SAMN38777020, SAMN38777021 and SAMN38777022.

Declarations

Ethics approval and consent to participate

All *S.* Enteritidis strains used in this study are routinely encountered during laboratory procedures at the hospital, all isolates were obtained after informed consent of the patients. The Ethics Committee of Zhangjiagang First People's Hospital waived the requirement for ethics approval.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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