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Genomic Epidemiology of ST34 Monophasic *Salmonella enterica* Serovar Typhimurium from Clinical Patients from 2008 to 2017 in Henan, China

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ABSTRACT

Salmonella enterica serovar 4,[5],12:i:- (*S.* 4,[5],12:i:-) is a monophasic variant of *Salmonella* enterica serovar Typhimurium that has emerged as a global serovar causing public health concern. To date, the epidemiology and genomic characterization of this pathogen in China have not been well described. We investigated the prevalence, antimicrobial resistance (AMR) phenotypes, and population genomics of sequence type 34 (ST34) *S.* 4,[5],12:i:- among cases of human salmonellosis in Henan Province, China. A total of 100 ST34 *S.* 4,[5],12:i:- isolates were studied from 2008 to 2017 and found mostly resistant to ampicillin (AMP), streptomycin (STR), sulfonamides (SUL), and tetracycline (TET) (ASSuT). Bayesian phylogenetic analysis demonstrated that isolates identified in China were mostly related to the European lineage and evolved into two major clades with different resistance genes and plasmid profiles. Notably, clade 1 showed a significantly higher rate of mutations in *gyrA* and plasmid-mediated quinolone resistance genes. The carrying of the resistance-containing region (encoding R-type ASSuT), including $bla_{\text{TEM-1B}}$ (conferring resistance to AMP), *strAB* (STR), *sul2* (SUL), and *tet*(B) (TET) inserted into the *fljBA* operon, was responsible for most of the monophasic variants in clade 2. IncHI2 plasmids were the dominant multi-drug resistance mobile genetic elements accounting for the transmission of acquired resistance genes in this serovar, and these were more prevalent in clade 1. Our findings highlighted the increasing prevalence of multi-drug resistant *S.* 4,[5],12:i:- in China, along with the differential characteristics of resistance gene acquisition among various lineages. Based on our data, control measures are required to address the spread of this zoonotic pathogen. Further owing to its potential origin in food-producing animals, a "One Health" approach, should be implemented to support surveillance whilst informing interventional strategies.

1. Introduction

Non-typhoidal *Salmonella enterica* (NTS) remains an important foodborne pathogen worldwide. It is responsible for the majority of deaths due to foodborne diseases, along with 4.0 million disability-adjusted life years according to the global burden of the foodborne diseases reported by World Health Organization (WHO) [1]. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a frequent serovar causing foodborne diseases. However, rapid global emergence of a new multi-drug resistant (MDR) *Salmonella* serovar, namely the monophasic variant of *S.* Typhimurium, has been recorded in the past two decades [2]. This bacterium lacks the second-phase flagellar antigen (encoded by *fljB*), giving its unique antigenic formula 4,[5],12:i:-. Since its first identification in the late 1980s, it has become one of the top five most common *Salmonella* serovars responsible for animal and

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human infections in different regions [3,4]. Furthermore, it is strongly associated with the pork food production chain suggesting a correlation with human infections. In addition, on 27 March 2022, WHO reported a multi-country outbreak of *Salmonella enterica* serovar 4,[5],12:i:- (*S.* 4,[5],12:i:-) infection linked to chocolate products, indicating novel food vehicles associated with the transmission of this bug [5].

Previously, three distinct clones (Spanish clone, European clone as well as US clone) with diverse phage types (PTs) and antimicrobial resistance (AMR) patterns have been reported. The original *S.* 4,[5],12:i:-, identified as the "Spanish clone" in 1997, is ascribed to PT U302 and sequence type 19 (ST19), and differs from *S.* Typhimurium LT2 by five major deletions [6]. It displays plasmid-mediated resistance to up to seven antimicrobial agents, including ampicillin (AMP), chloramphenicol (CHL), gentamicin (GEN), streptomycin (STR)/spectinomycin, sulfonamides (SUL), tetracycline (TET), and trimethoprim (ACGSSuTTp type). The clonal lineage referred to as the "US clone" emerged in 2004. It was characterized by a defined genomic deletion pattern surrounding the *fljBA* operon but maintaining *hin* and *iroB* genes and the STM1053-1997 region (all of which are absent from the "Spanish clone") [3]. Moreover, the MDR phenotype is rare among the "US clone" isolates (denoted in this study as clade B-I ST19 and devoid of resistance mechanisms against AMP, STR, SUL, and TET (R-type ASSuT)), a different feature from the isolates of "Spanish clone". The major European clone identified in 2005, was ascribed to PTs DT193/DT120 and ST34, and lacked the typical *S.* Typhimurium virulence plasmid pSLT. This major European clone was characterized by chromosomally-encoded resistance to ASSuT harboring the same resistance genes (bla_{TEM-1}, *strAB*, *sul2*, and *tet*(B)) with two diverse genetic contexts (denoted as resistance region (RR)1-RR2). It has now spread to several countries across the European Union, thus becoming a pandemic clone [7–9]. In addition, an extensively drug resistant ST313 *S.* 4,[5],12:i:- sublineage was emerging and caused on-going outbreak in Africa [10].

Meat production has shown a sharp growth of about 50% since 2000, thus indicating a continuous increase, especially in the low- and middle-income countries. The increasing use of antimicrobial agents in animals is both an enabler and a consequence of this scale-up in the demand for animal protein [11]. Typically, the misuse of antimicrobial compounds in animal production has contributed to the emergence of MDR bacteria. Similarly, most *S.* 4,[5],12:i:- isolates are derived from animal food and described as MDR pathogens. Furthermore, MDR *Salmonella* is recognized as a serious threat to human health [12]. With its high transmission capacity, MDR *S.* 4,[5],12:i:- is increasingly becoming a global hazard and must be controlled.

In China, several previous studies show the increasing prevalence of *S.* 4,[5],12:i:- since the recent decade, and the resistance to multiple drugs is also a cause of concern. Most of the animal-derived isolates in China were from pigs, with a high resistance rate against TET, AMP, SUL, STR, nalidixic acid (NAL), amoxicillin-clavulanic acid, and CHL [13,14]. ST34 *S.* 4,[5],12:i:- is the dominant subtype among isolates from both pigs and humans [15–17]. China is the world's biggest producer and consumer of pork. Further, global trade (of food-producing animals and/or food products) and international travel have been identified as the underlying causes of the emergence and spread of infectious diseases as exemplified by the transmission of other foodborne pathogens. While there is a good understanding of the genomic landscape of *S.* 4,[5],12:i:- in Europe, the United States, and Australia [7–9,12], such an extensive investigation has not yet been conducted for *S.* 4,[5],12:i:- originated from China. Therefore, understanding the emergence of foodborne diseases is crucial in preventing such events in future.

Herein, we present the genomic epidemiology and AMR data from 100 *S.* 4,[5],12:i:- isolates collected from 2008 to 2017 in Henan, China. Using antimicrobial susceptibility data and whole-genome sequencing (WGS) analysis, we determined the prevalence and mechanisms of resistance and conducted a comparative study using isolates from different countries in an effort to identify potential drivers of variation in AMR profiles and clonality. These isolates, collected from human cases of diarrhea provided a unique opportunity to investigate different lineages and elucidate the role of this zoonotic pathogen in the spread of AMR in China and elsewhere.

2. Materials and methods

2.1. Bacteria isolates and serovar detection

A total of 100 *S.* 4,[5],12:i:- isolates were identified from 1565 cultured *Salmonella* isolates from diarrhea patients in six regional Center for Disease Control and Prevention diagnostic laboratories located in Henan Province, China, from 2008 to 2017 (Table S1 in Appendix A) [18]. All these laboratories were participants in the Enhanced *Salmonella* Surveillance Project of WHO Global *Salmonella* Surveillance for NTS. The protocols for patient sample collection have been described previously [19]. Isolates cultured from patients with typical *Salmonella* morphology were further confirmed by the API 20E test (bioMérieux, China) and the polymerase chain reaction (PCR) amplification of the *invA* gene [20]. The serovars of the confirmed *Salmonella* isolates were determined by slide agglutination with commercial *Salmonella* antisera (Statens Serum Institute, Denmark) following the Kauffmann–White scheme. Isolates identified as monophasic *S.* 4,[5],12:i:- were further confirmed by multiplex PCR as recommended by the European Food Safety Authority [21].

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for the 100 *S.* 4,[5],12:i:- isolates against a panel of 14 antimicrobial agents including AMP, cefotaxime (CTX), ceftazidime (CAZ), imipenem (IPM), meropenem (MEM), GEN, amikacin (AMI), STR, SUL, trimethoprim-sulfamethoxazole (SXT), CHL, TET, NAL, and ciprofloxacin (CIP), was performed following the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) standards [22]; for colistin (CT), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocol was followed [23]. *Escherichia coli* (*E. coli*) ATCC™25922 and *Klebsiella pneumoniae* ATCC™700603 were used as quality control strains. MDR phenotype was defined as resistance to three or more different classes of antimicrobial agents.

2.3. Whole-genome sequencing, de novo *assembly, and annotation*

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA), and its quality was assessed by gel electrophoresis and quantified using a Qubit Fluorometer 2.0 (Invitrogen, Life Technologies, USA). All DNA samples were sequenced on the Illumina NovaSeq 6000 platform (Illumina, USA); 150 bp paired-end reads were generated from a library with an average insert size of 500 bp and more than 80× sequencing depth. Raw reads were initially filtered to remove low-quality reads, following which, they were *de novo* assembled using SPAdes v3.13 [24]. Six representative isolates were further sequenced by long-read sequencing protocols on the MinION platform from Oxford Nanopore Technologies (UK) with a rapid barcoding library preparation kit and the R9.4.1 flowcell. Complete genome

sequences were obtained based on a hybrid *de novo* assembly strategy using Unicycler [25]. Sanger sequencing and MinION confirmed complex plasmid structures by long-read alignments [26]. The draft and complete genome sequences were initially annotated using Prokka [27]. The AMR genes, insertion sequences, virulence genes, and replicon types of plasmids were annotated by comparison with data on the relevant databases, including ResFinder database, ISFinder database, virulence factor database (VFDB), and PlasmidFinder database [28–31], using the BLASTn software with 90% identity and minimum length coverage of 80% as thresholds.

2.4. Phylogenetic analysis based on core-genome single nucleotide polymorphisms (SNPs)

To perform phylogenetic analysis, we identified the SNPs by mapping the sequencing reads back to the genome sequence of *S.* Typhimurium LT2 (GenBank accession No.: NC_003197) using the Bowtie2 software, followed by filtering using Samtools [32,33]. In addition to our data, sequencing reads of four *S.* 4,[5],12:i:- isolates from a previous study were also included (GenBank accession No.: PRJNA573539), which were cultured from fecal samples of pigs collected from pig farms in Henan Province in 2017 [34]. Available genome data of ST34 *S.* 4,[5],12:i:- isolates from Europe (n = 345) and the United States (n = 209) sequenced and submitted to GenBank up to 2019 were also enrolled in the phylogenetic analysis. High-quality SNPs supported by more than five reads and the adjacent mutations within 5 bp were filtered to avoid recombination. Finally, the concatenated sequences were used to perform phylogenetic analysis by the maximum likelihood method using FastTree v2.1.10 [35]. The phylogenetic relationship and carrying of genetic traits including resistance genes were displayed using iTOL [36].

2.5. Bayesian divergence estimates

Bayesian phylogenetic analysis was performed using BEAST v1.8.2 [37]. The isolation year of each isolate was used to establish a temporal framework for constructing phylogenetic relationships among the isolates and estimating parameters to describe the evolutionary dynamics of the population. Comparative analyses for different molecular clock models and tree priors were performed, and the best-fit evolutionary model for the dataset was selected as the Hasegawa–Kishono–Yano substitution model, with a normal distribution of among-site rate heterogeneity. Strict clock or random local clock models were implemented with Coalescent Constant Population or Coalescent Exponential Population priors.

2.6. Nucleotide sequence accession numbers

The complete genome sequences of 2008079, 2010112, 2016062, 2016089, 2017005, and 2017028 were submitted to the National Center for Biotechnology Information (NCBI) database under the accession numbers CP090529–CP090546, respectively (Table 1). WGS raw data was deposited in the NCBI Sequence Read Archive database (BioProject No.: PRJNA794010).

3. Results

3.1. Sources of Chinese S. 4,[5],12:i:- isolates and their antimicrobial susceptibility profiles

From 2008 to 2017, a total of 1565 *Salmonella* isolates were collected from patients from six cities in Henan Province, via the laboratory-based foodborne disease surveillance system in China (Table S1 in Appendix A). Among them, 100 *S.* 4,[5],12:i:- isolates (6.4%, 100/1565) were identified by serotyping and multiplex PCR; it was the third most common serovar in the collection (Table S1). The prevalence of *S.* 4,[5],12:i:- ranged from 0.6% to 15.6% with a sharp increase during the study period (Fig. 1).

Antimicrobial susceptibility testing for these 100 *S.* 4,[5],12:i:- isolates against a panel of 15 antimicrobial compounds showed resistance phenotypes to 13 of them. Among the 100 isolates, 97 expressed the defined MDR phenotype (Table S2 in Appendix A), whereby they showed resistance to three or more classes of antimicrobial agents. No resistance phenotype was detected against IPM nor MEM. Resistance to TET (98.0%) was the most common, followed by SUL (97.0%), AMP (91%), STR (77.0%), CHL (61.0%), NAL (56.0%), CIP (53.0%), and SXT (52.0%) (Table S3 in Appendix A). Resistance to cefotaxime (13.0%) was relatively infrequent but the

Table 1
Basic information and genomic characterization of six representative *S.* 4,[5],12:i:- isolates with complete genome sequences obtained from long-read Nanopore sequencing.

Isolate	Region	Clade	Contigs (accession numbers)	Resistance genes
2008079	Luohe	C1	2008079-chromosome (CP090529) p2008079-241 (CP090530)2008079-p2 (CP090531)2008079-p3 (CP090532)	*tet*(B), *strA*, *strB*, *sul1*, *sul2*, *sul3*, bla_{TEM-1B}, bla_{OXA-1}, *dfrA12*, *catB3*, *cmlA1*, *floR*, *aac(3)-IVa*, *aac(6′)Ib-cr*, *aadA1*, *aph(3′)-Ia*, *oqxAB*, *aph(4)-Ia*, *arr-3*
2010112	Anyang	C1	2010112-chromosome (CP090541) p2010112-144 (CP090542)2010112-p2 (CP090543)2010112-p3 (CP090544)	*tet*(B), *strA*, *strB*, *sul1*, *sul2*, bla_{TEM-1B}, *oqxAB*
2016089	Zhengzhou	C1	2016089-chromosome (CP090535) p2016089-170 (CP090536)2016089-p2 (CP090537)2016089-p3 (CP090538)	*tet*(B), *sul1*, *sul2*, bla_{OXA-1}, *catB3*, *floR*, *aac(3)-IVa*, *aac(6′)Ib-cr*, *aph(4)-Ia*, *oqxAB*, $bla_{CTX-M-14}$, *arr-3*
2016062	Zhoukou	C2	2016062-chromosome (CP090539) p2016062-242 (CP090540)	*tet*(A), *tet*(B), *strA*, *strB*, *sul1*, *sul2*, bla_{TEM-1B}, *dfrA27*, *floR*, *aac(3)-IId*, *aac(6′)Ib-cr*, *aadA16*, *oqxAB*, $bla_{CTX-M-55}$, *mph*(A), *arr-3*
2017005	Zhengzhou	C2	2017005-chromosome (CP090533) p2017005-292 (CP090534)	*tet*(B), *strA*, *strB*, *sul1*, *sul2*, *sul3*, bla_{TEM-1B}, bla_{OXA-1}, *dfrA12*, *catB3*, *cmlA1*, *floR*, *aac(3)-IVa*, *aac(6′)Ib-cr*, *aadA1*, *aph(3′)-Ia*, *aph(4)-Ia*, *oqxAB*, *qnrS2*, $bla_{CTX-M-14}$, *arr-3*, *mcr-1*
2017028	Shangqiu	C2	2017028-chromosome (CP090545) p2017028-250 (CP090546)	*tet*(A), *tet*(B), *strA*, *strB*, *sul1*, *sul2*, bla_{TEM-1B}, *dfrA27*, *floR*, *aac(3)-IId*, *aac(6′)Ib-cr*, *aadA16*, *oqxAB*, *qnrB6*, $bla_{CTX-M-55}$, *mph*(A), *arr-3*

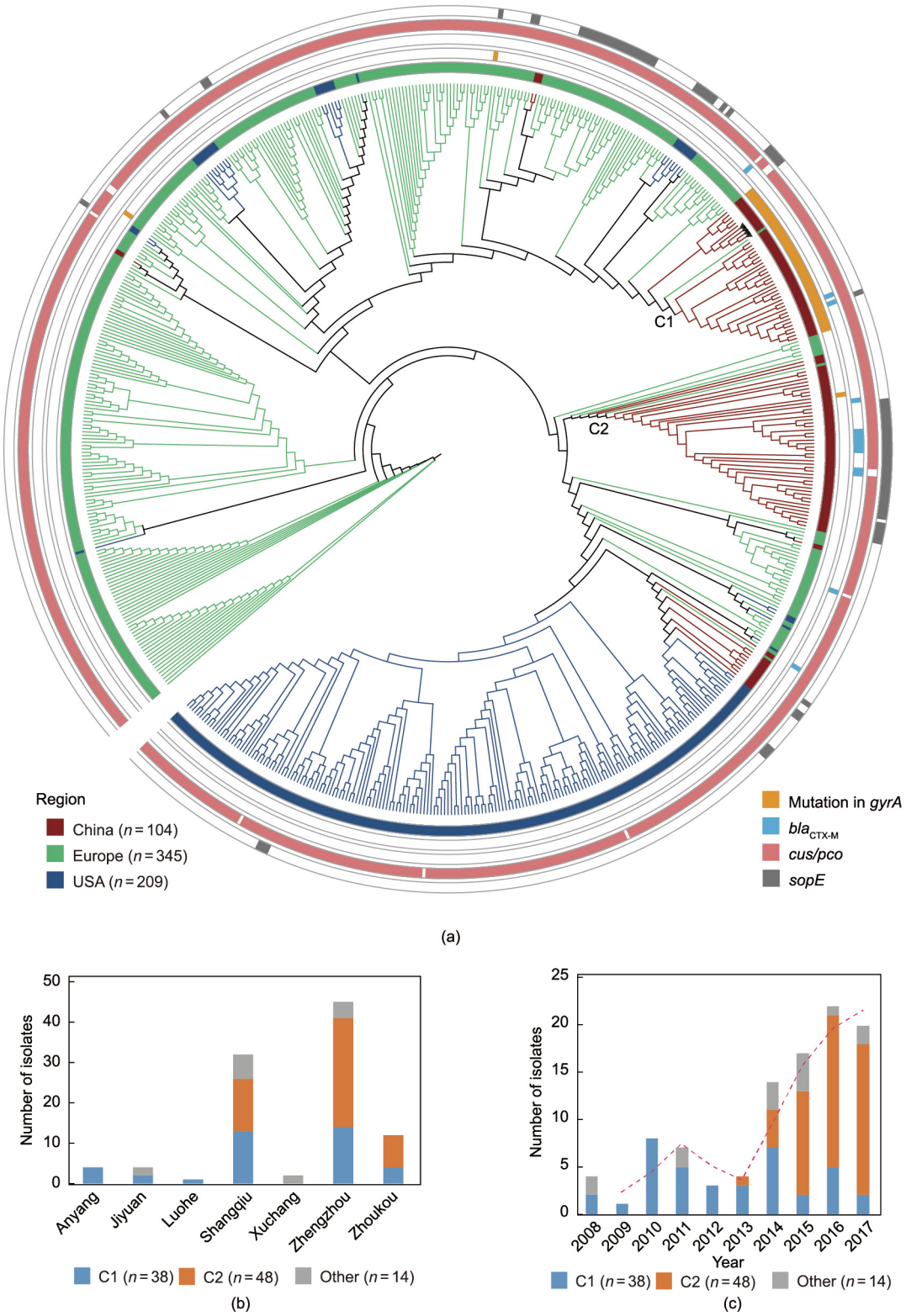

Fig. 1. (a) Phylogenetic analysis of 104 *S.* 4,[5],12:i:- isolates from China, 345 *S.* 4,[5],12:i:- from Europe, and 209 from the United States. The rings from inside to outside along the tree represent the geographical origin of the isolates, the presence of mutations in *gyrA*, the bla_{CTX-M} gene, the *cus/pco* gene cluster, and the *sopE* gene. The four Chinese isolates from pigs are marked by black triangle. (b) Distribution of *S.* 4,[5],12:i:- isolates in different regions of Henan Province, China. (c) Epidemiology trends for different *S.* 4,[5],12:i:- clades from 2008 to 2017. C1: clade 1; C2: clade 2.

12 CTX-resistant isolates were co-resistant to CIP. Resistance to CT (3.0%) and AMI (1.0%) was rare among the isolates in the study. Most (73.0%) demonstrated R-type ASSuT, wherein 42 isolates were additionally resistant to CHL. The results of antimicrobial susceptibility testing showed that the Chinese isolates were highly resistant to fluoroquinolone, aminoglycosides, and CHL (Tables S3 and S4 in Appendix A); thus, they appeared to differ from the reported profiles of European and US isolates [7,8,12].

3.2. Prevalence of resistance genes and mutations among Chinese *S.* 4,[5],12:i:- isolates

WGS data for all 100 isolates were obtained, and initial *in silico* analysis re-confirmed them as the ST34 *S.* 4,[5],12:i:- serovar [38]. These genomes were screened for known genetic determinants of AMR, including horizontally acquired genes and target gene mutations of quinolone resistance-determining regions (QRDRs) on the bacterial chromosome. Among the 100 isolates, 35 acquired AMR genes were detected, along with a single-point mutation (in *gyrA*). In comparison with European and US isolates of the same serovar, the carrying of acquired AMR genes differed significantly (Figs. 1(a), 2, and 3). Six types of acquired AMR genes were detected in more than half of the isolates. These included $bla_{\text{TEM-1B}}$ (n = 65, encoding resistance to AMP), *strA* and *strB* (n = 71, STR), *sul1* (n = 53, SUL) and *sul2* (n = 79, SUL), *tet*(B) (n = 98, TET), and *floR* (n = 51, phenicols), along with *arr-3* (n = 50, rifamycin). Alleles of dihydrofolate reductase-encoding genes (*dfr*) endowing resistance to trimethoprim were detected in 49 isolates, comprising two different variants, *dfrA12* (n = 38) and *dfrA27* (n = 11). The gene, *catB3,* encoding resistance to CHL was detected in 39 isolates. For fluoroquinolone resistance, multiple single-point mutations were detected in *gyrA* in 39 isolates (resulting in the following amino acid substitutions: D87N (n = 32), D87Y (n = 6), and S83F (n = 1)) along with four plasmid-mediated quinolone resistance (PMQR) genes, including *oqxAB* (n = 47), *qnrB6* (n = 9), *qnrS2* (n = 6), and *qepA* (n = 1). Three $bla_{\text{CTX-M}}$ subtypes were identified among the 13 extended-spectrum β-lactamase (ESBL)-positive *Salmonella* isolates, including $bla_{\text{CTX-M-55}}$ (n = 9), $bla_{\text{CTX-M-14}}$ (n = 3), and $bla_{\text{CTX-M-27}}$ (n = 1). In addition, *mph*(A) encoding for azithromycin resistance was detected in eight isolates. Among 73 R-type ASSuT, 51 isolates harbored typical resistance genes ($bla_{\text{TEM-1B}}$, *strAB*, *sul2,* and *tet* (B)) which mapped within the *fljBA* operon and disrupted it, thus giving rise to the monophasic phenotype (Table S5 in Appendix A), a common feature among European isolates [39]. Furthermore, the emerging mobile CT resistance gene, mobile colistin resistance (*mcr*)*-1*, was also detected in three isolates belonging to clade 2 (C2).

3.3. Phylogenetic relationship and resistance gene difference of *S.* 4,[5],12:i:- isolates from China, Europe, and the United States

Because all of the 100 Chinese *S.* 4,[5],12:i:- isolates we obtained were ST34, genome data representing 345 *S.* 4,[5],12:i:- isolates belonging to the ST34 type reported in Europe and 209 isolates from the United States, together with another four Chinese isolates of pig origin (Table S6 in Appendix A), were downloaded for comparative analysis. To analyze the phylogenetic relationship of these Chinese isolates with the European and the US isolates, a maximum likelihood core-genome phylogenetic tree was constructed using all the 658 genomes based on 4261 high-quality SNPs (between 0 and 138 pairwise SNPs; median = 41) (Fig. 1(a)). The Chinese isolates clustered mainly into two groups, namely clades 1 and 2 (C1 and C2), with 38 and 48 isolates, respectively. The four isolates from pigs were clustered in C1. The remaining 14 isolates were interspersed with those reported previously in Europe and the United States. The two clades belonged to distinct lineages with European ancestry, indicating their likely origin from Europe following two different evolutionary events. The two clades were widely distributed in Henan Province (Fig. 1(b)), with C2 showing emergence in 2013 and increasing to 80% (16/20) in 2017 (Fig. 1(c)). The remaining 14 isolates did not cluster closely with these two groups.

Furthermore, the carrying rates for mutations in *gyrA* along with the PMQR genes, and genes conferring resistance to aminoglycosides, CHL, trimethoprim, and rifampin among the Chinese isolates (Fig. 2), in particular, C1, were found to be higher than those in the European and US isolates (Figs. 2(a) and (d)). The distribution of resistance genes and the nature of plasmid replicon types in European and US isolates were similar but those of Chinese isolates were markedly different (Figs. 2(b) and (c)). These results indicated that the Chinese isolates may have acquired additional resistance determinants through the acquisition of new plasmid replicon types and chromosomal mutations.

3.4. Evolution of Chinese isolates with different resistance genes identified in different lineages

The phylogenetic relationships and distribution of resistance genes and plasmid types revealed that most of the Chinese isolates were originally derived from Europe but have subsequently acquired new plasmids carrying resistance genes. Therefore, we further analyzed the evolution of these Chinese isolates and the carrying of plasmids, along with the resistance genes. We calculated the divergence periods for Chinese isolates by Bayesian estimation. The results showed that the year of emergence of the most recent common ancestor of the 100 Chinese isolates was 1993 (95% highest posterior density in 1987–1998), with the isolates in C2 emerging later in 2009 (95% highest posterior density 2005–2011) (Fig. 3). By estimating the effective population size trajectories, we observed different genetic diversities between C1 and C2 over time. C1 underwent a population expansion since its original emergence in the early 2000s, which shrank after 2010. C2 showed a reverse trend, with its population expanding after 2010 (Fig. 3). These data were in line with the observed rise in the number of C2 isolates (Fig. 1(c)).

The distribution of resistance genes was clearly different between isolates in C1 and C2 (Fig. 3). The carriage rates for mutations in *gyrA* and *oqxAB* conferring resistance to fluoroquinolone, aminoglycosides (*aadA1*, *aph(3′)-Ia*, *aph(4)-Ia, aac(6′)Ib-cr*, and *aac(3)-Iva*), chloramphenicol (*catB3, cmlA1,* and *floR*), and rifampin (*arr-3*) among C1 isolates were significantly higher than those in C2 ($p < 0.05$), whereas the rates of $bla_{\text{TEM-1B}}$ conferring resistance to ampicillin and *strAB* to STR in C1 isolates were significantly lower relative to C2 ($p < 0.05$, Fig. 2(d) and Table S7 in Appendix A). On examining the resistance genes in greater detail, for penicillin, trimethoprim, and SUL, different resistance genes were more common in each clade; $bla_{\text{OXA-1}}$, *dfrA12*, *sul1*, and *sul3* were predominant in C1, while, $bla_{\text{TEM-1B}}$ and *strAB* were predominant in C2. Furthermore, *Salmonella* Genomic Island 4 (SGI-4), an about 80 kbp mobile genetic element that encodes genes expressing tolerance to metals including silver, arsenic, and copper, was detected in all of the European, US, and Chinese isolates, thus indicating its typical conservation in *S.* 4,[5],12:i:- isolates. In addition, among the 14 isolates that did not cluster in C1 or C2, similar profiles of resistance genes and plasmid types as the C2 isolates were observed (Fig. 3).

3.5. Polymorphism and complex structures of AMR-encoding plasmids in different regions

The distribution of plasmid types was different between C1 and C2. The IncQ1 replicon gene was predominant in C2, whereas IncHI2 and IncHI2A were predominant in C1 (Fig. 3). Comparative genomic analysis with *S.* Typhimurium LT2 showed that the IncQ1 replicon gene was mainly inserted in chromosomal *fljBA* operon along with resistance genes including $bla_{\text{TEM-1B}}$, *strAB*, *sul2*, and *tet*(B) encoding ASSuT. We further analyzed the presence of resistance genes in the *Salmonella* genomes carrying the two replicons, IncHI2 and IncHI2A; the results suggested different resistance gene profiles in isolates from different regions (Fig. 4(a)). Sixteen resis-

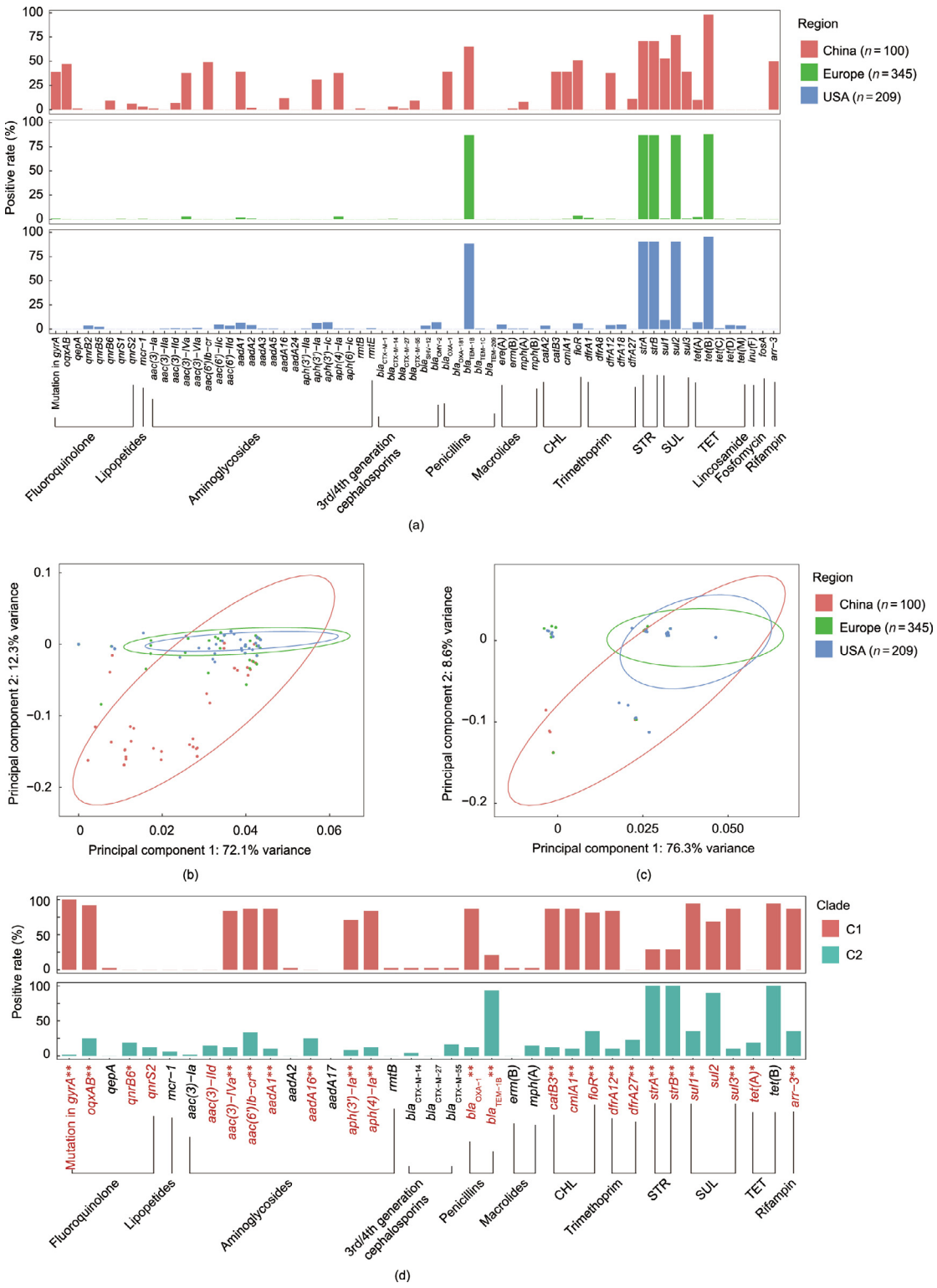

Fig. 2. (a) Positive rates of diverse resistance genes among different *S.* 4,[5],12:i:- isolates in China, Europe, and the United States. (b, c) Principal component analysis for resistance genes and plasmid types in the three lineages. (d) Different positive rates of resistance genes in two major Chinese clades. The carriage rates of genes in red are significantly different between the two clades ($p < 0.05$), a single asterisk (*) after the gene name indicates $p < 0.01$, and two (**) indicates $p < 0.001$.

tance genes, including *tet*(D), *dfrA18*, bla_{SHV-12}, and *catA2* were commonly identified in US isolates, while seventeen other genes, including *qnrB6*, *dfrA27*, $bla_{CTX-M-55}$, and *mph*(A) were often found in isolates of the Chinese clade C2. Furthermore, the distribution of 14 resistance genes was highly correlated with the two IncHI2 and IncHI2A types, whereby the distributions of bla_{TEM-1B}, *strAB*, *sul2*, and *tet*(B) were highly correlated to IncQ1, and those of *aph(3′)-Ic*, *tet*(A), *tet*(M), *aadA2*, and bla_{CMY-2} were highly correlated to IncA/C2 (Fig. 4(b)). To further examine the genetic context of resistance genes and their associated plasmids, we determined

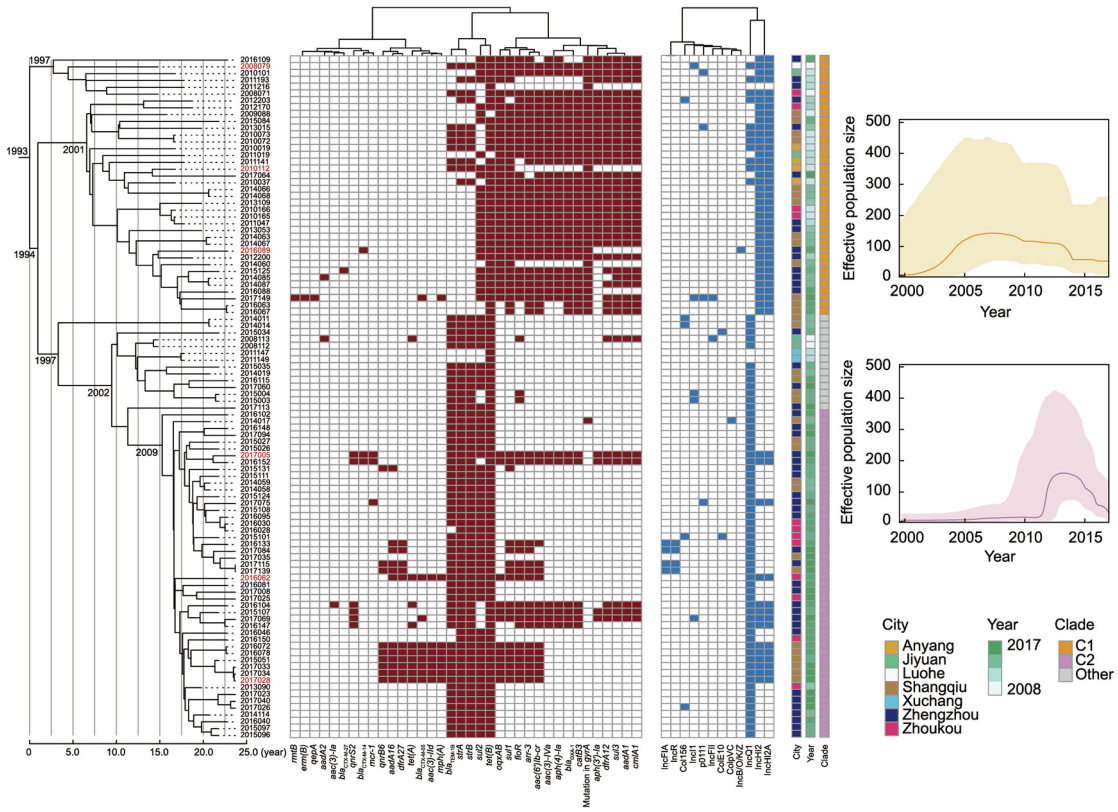

Fig. 3. Bayesian phylogenetic analysis of Chinese *S.* 4,[5],12:i:- isolates (left) correlate with the distribution of resistance genes and plasmid types (middle). The estimated emerging time is labeled on the nodes of the tree. The six isolates subjected to Nanopore long-read sequencing are in red, and the estimation of the effective population size trajectories of C1 and C2 are represented on the right.

the complete genomes of six representative Chinese isolates from the two clades (Figs. 3 and 4(c)) by long-read sequencing and obtained complete and circular genome sequences (Table 1).

Among these six isolates, one to three plasmids each were identified along with IncHI2–IncHI2A plasmids encoding multiple resistance genes. IncHI2 plasmids are broad-host-range plasmids that contribute to the spread of antibiotic resistance genes. The plasmids of the IncHI2 type frequently recombine with those of other replicon types [40,41]. Four IncHI2–IncHI2A plasmids (denoted as plasmids p2010112-144, p2016089-170, p2008079-241, and p2017005-292) and two heterozygous plasmids of IncHI2–IncHI2A–IncQ1 (p2016062-242 and p2017028-250) were characterized. The lengths of the four IncHI2–IncHI2A plasmids ranged from 144 to 292 kbp (Table 1). As compared to the other three IncHI2–IncHI2A plasmids in this study, plasmid p2017005-292, contained *mcr-1*, as well as a copper resistance gene cluster, *cusCFBA*. Although these four plasmids are of the IncHI2–IncHI2A type, their molecular structures and resistance genes are different due to the plasticity of the MDR region. This is consistent with the fact that IncHI2 plasmids are highly flexible and genetically plastic, a feature that facilitates bacteria to acquire diverse antibiotic resistance markers [42]. BLASTn comparison of the IncHI2–IncHI2A plasmid, p2017005-292, with those in the NCBI database showed high similarity to two other IncHI2–IncHI2A plasmids of *S.* Typhimurium, pSH16G4498 (MH522423) and pS585_1 (CP061116) (Fig. 5(a)). Interestingly, the IncQ1 replicon gene was found on chromosomes of isolates 2008079, 2010112, 2016062, 2017005, and 2017028. The IncQ1-bearing MDR chromosomal regions contained multiple IS*26* inserted into the *fljBA* operon, thus resulting in the disruption of the phase 2 flagellin-encoding gene [8]. The IncQ1 replicon gene was shared by plasmids and chromosomes in isolates 2016062 and 2017028, implying that the MDR region linked to this replicon could recombine between plasmids and chromosomes mediated by insertion sequences such as IS*26*; however, direct evidence is needed to support this hypothesis.

The two IncHI2–IncHI2A–IncQ1 plasmids were 242 and 250 kbp in length, respectively, and the circular sequence comparison indicated similar plasmid backbone structures. In the NCBI database, two plasmids found in *E. coli* denoted as plasmids, pE105-4 (CP072315) and pEC22-CTX-M-15 (CP084901), were of the IncHI2–IncHI2A–IncQ1 type and possessed similar backbone structures (Fig. 5(b)). These IncHI2–IncHI2A–IncQ1 plasmids harbor 14–18 resistance genes, including *floR*, $bla_{\text{CTX-M-55}}$, *tet*(A), and *sul1*. Online BLASTn indicated that coexistence of IncHI2–IncHI2A and IncQ1 was common in bacteria, and with the IncQ1-harboring mobile elements enabling insertion into the chromosomal *fljBA* operon, monophasic phenotypes were produced and the *S.* 4,[5],12:i:- antigenic formula was generated.

3.6. Sequence variations in chromosomal regions related to Salmonella *monophasic variants*

We also analyzed the chromosomal sequence changes that affected the *fljBA* operon and accounted for the monophasic variations observed among the six Chinese isolates. Except for 2016089, the insertion fragments were almost always the same, comprising resistance genes, including $bla_{\text{TEM-1B}}$, *strAB,* and *tet*(B), thus accounting for the ASSuT resistance profile and bilaterally flanked by IS*26*. A minor sequence difference was noted which included a fragment comprised of *hin* and *fliC* adjacent to $bla_{\text{TEM-1B}}$ in isolate 2008079. In addition, the loci that the latter fragment was inserted into were different. In 2008079 and 2010112, these fragments were inserted into *fljB* and caused deletion of *hin* and a part of *fljC* (Fig. 6(a)); in 2016062, 2017005, and 2017028, the fragments were

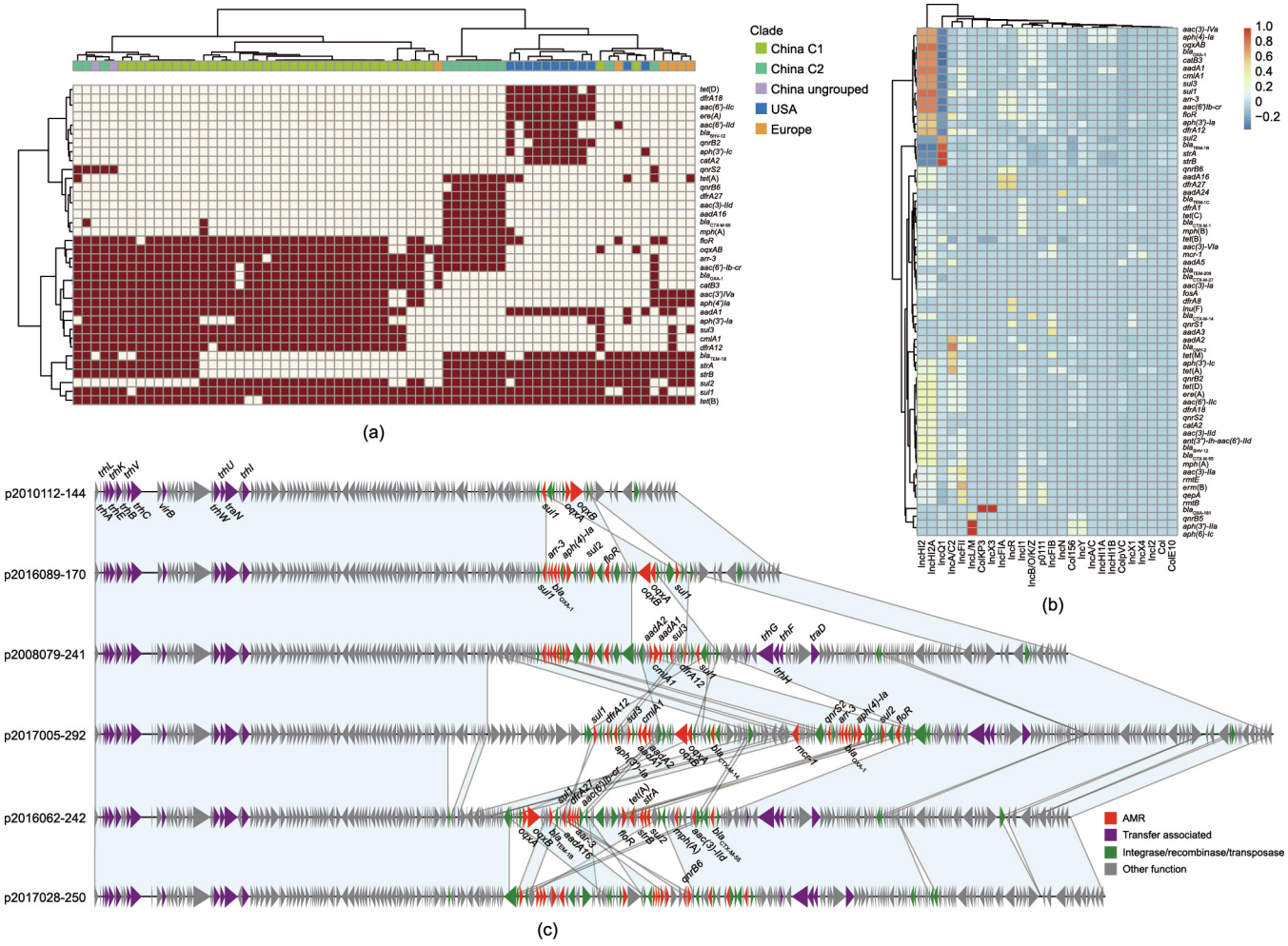

Fig. 4. (a) Distribution of resistance genes among *S.* 4,[5],12:i:- isolates with IncHI2–IncHI2A plasmids belonging to different clades. The red block in the heatmap represents the presence of resistance genes. The resistance genes found in less than five isolates are excluded. (b) Correlation analysis for resistance genes and plasmid types among the 100 isolates we obtained in this study and those with available genome data from Europe and the United States. The heatmap represents the correlation coefficients of resistance genes with plasmid types. (c) Linear plasmid sequence comparison among six MDR IncHI2 plasmids from different Chinese *S.* 4,[5],12:i:- isolates. The matched regions between two sequences are displayed by light blue blocks. The arrows represent the genes related to resistance and transfer.

inserted in reverse orientation, resulting in large deletions from STM2745 to *hin* as compared to *S.* Typhimurium LT2 (Fig. 6(b)). The insertion fragments were highly similar to those found in *S.* 4,[5],12:i:- strain 105/7/03; RR1 and RR2 in this isolate were combined in the five Chinese isolates. In isolate 2016089, we observed a different sequence variation resulting in *fljBA* operon variation. A large inversion (about 407 kbp) occurred between 2 916 036–3 323 378 bp relative to the genome of *S.* Typhimurium LT2, whereby *fljA* and a part of *fljB* were deleted (Fig. S1 in Appendix A).

3.7. Distribution of virulence-associated genes

The analysis of *Salmonella* Pathogenicity Islands (SPIs) showed that the types of pathogenic islands in *S.* 4,[5],12:i:- in China were consistent. SPIs including SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-6, SPI-9, SPI-11, SPI-12, SPI-13, and SPI-14 were distributed in all isolates (Table S8 in Appendix A). Notably, the types of virulence genes contained in *S.* 4,[5],12:i:- were similar in comparing the Chinese isolates with those reported outside the country. Each *Salmonella* isolate contains multiple virulence genes including those for adherence (*lpf, misL, ratB, shdA,* and *sinH*), invasion (*inv*), and stress survival (*sodCI*); the number of virulence factors ranges between 104 and 108 (Table S9 in Appendix A). Moreover, all the analyzed genomes lacked the typical *S.* Typhimurium virulence plasmid, pSLT.

4. Discussion

Prevalence of *S.* 4,[5],12:i:- is increasing worldwide, thus giving rise to a global public health concern, especially in Europe, the United States, Australia, and Thailand [3,9,43,44]. As an MDR monophasic variant of *S.* 4,[5],12:i:-, ST34 is thought to be originated from Europe and spread to other parts of the world; it is associated with both animal (especially pig) and human infections. As a country with large pork consumption with a high incidence of *S.* 4,[5],12:i:- in pork, the genomic epidemiology of *S.* 4,[5],12:i:-, along with the local and global transmission landscape are important to understand in China. The findings of this study demonstrated the nature of the variant that has overtaken *S.* Typhimurium to become the second most common serovar recorded in cases of salmonellosis in Henan. Phylogenetic analysis identified two major clades, C1 and C2, both belonging to European lineages. By Bayesian analysis, the Chinese lineage was shown to have originated from the European lineage; however, in 2000 it further evolved into unique Chinese lineages. Notably, the US lineage has also originated from the European lineage, whereby in

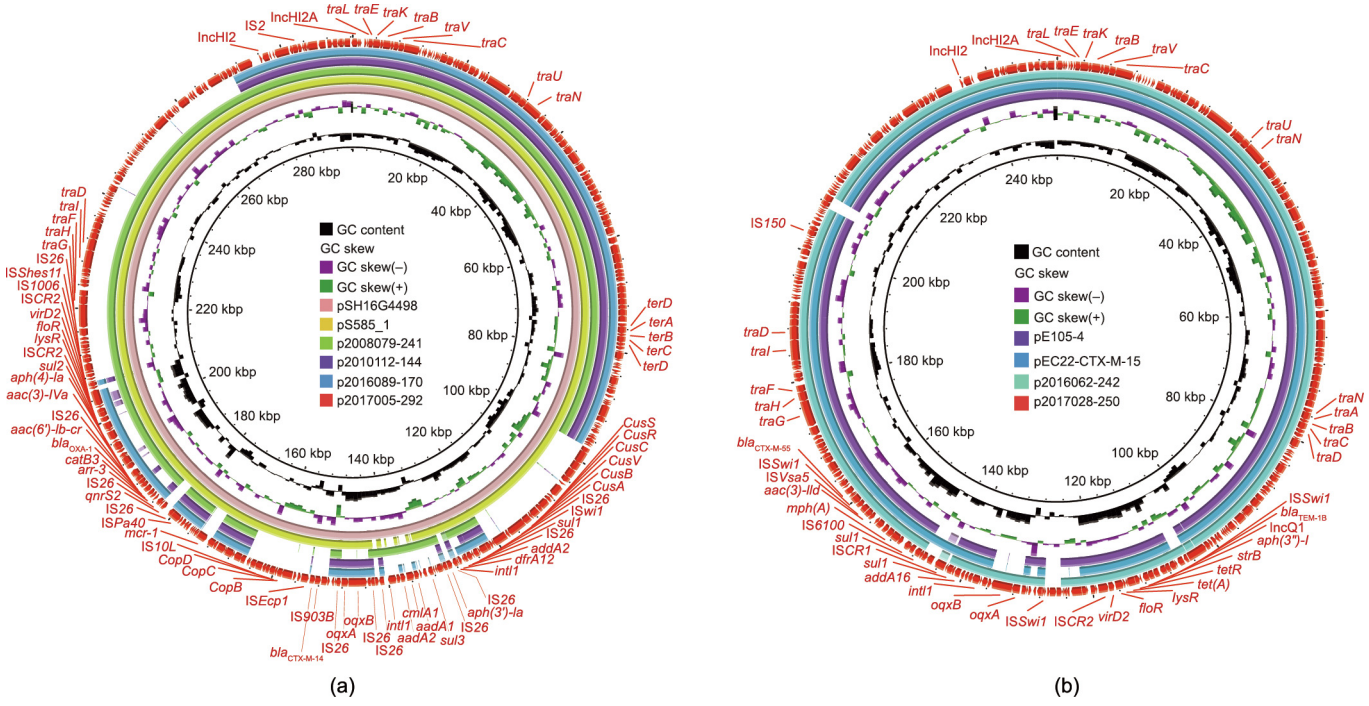

Fig. 5. (a) Comparisons between IncHI2–IncHI2A plasmids found in this study and those in the online NCBI database. (b) Comparison between IncHI2–IncHI2A–IncQ1 plasmids found in this study and those in the online NCBI database. The outmost red circles indicate the reference plasmid used for comparison.

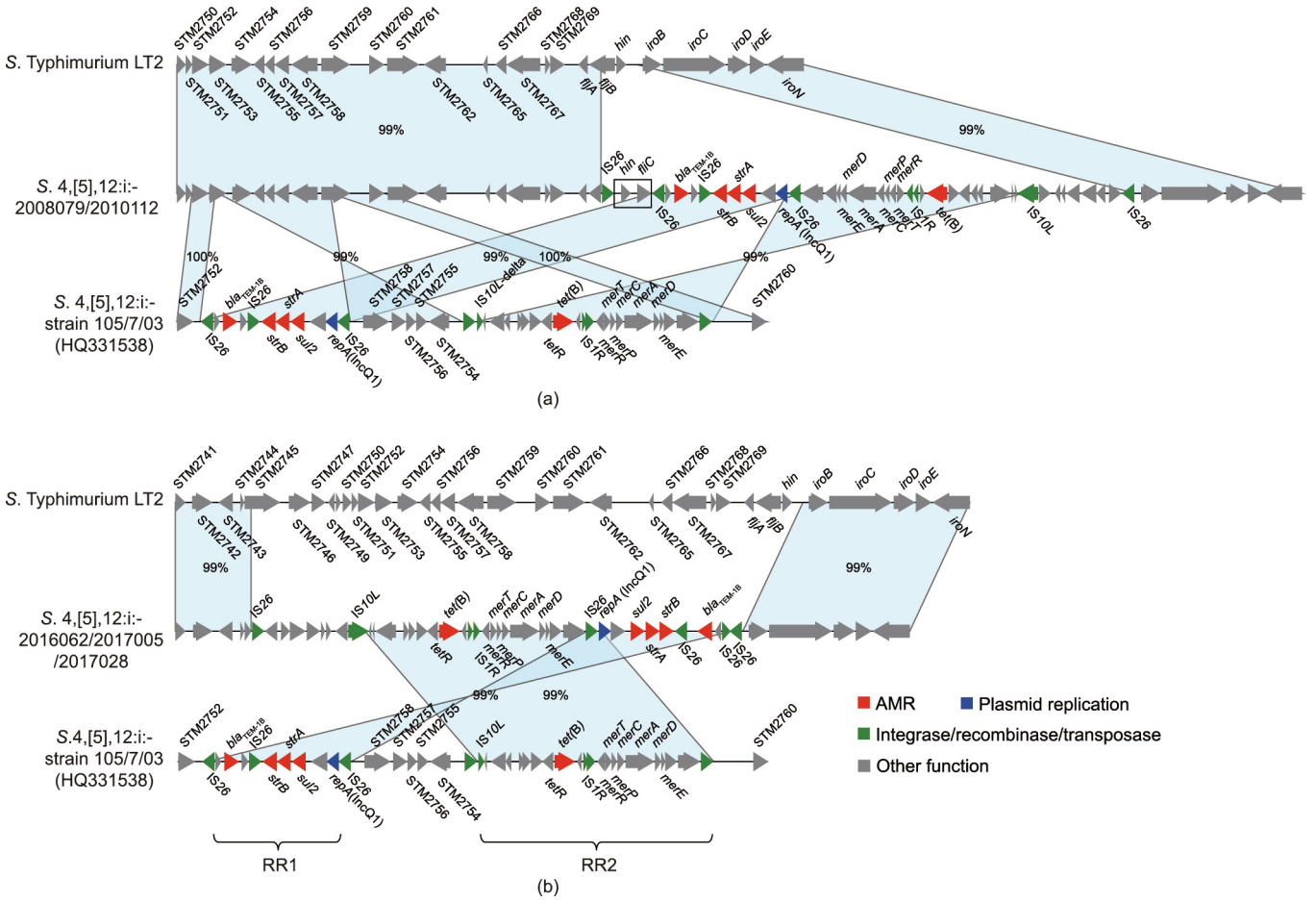

Fig. 6. Insertions cause *fljBA* operon variations in five Chinese isolates. The matched regions between two sequences are shown in light blue blocks and the identities are marked. The arrows represent genes related to resistance and transfer.

the early 2000s, it evolved independently to form the current US lineage [45]. Our data indicated that the European lineage may be the ancestor of *S.* 4,[5],12:i:-. isolates, worldwide.

The disruption of *fljBA* operon by an IS*26* associated MDR mobile element (bla_{TEM-1}, *strAB*, *sul2*, and *tet*(B), conferring R-type ASSuT) abolishes the expression of the second phase flagellar antigen, resulting in the serovar 4,[5],12:i:- [7,39,46]. The resistance genes bla_{TEM-1B} and *strAB* were found in most of the Chinese isolates except for some C1 isolates. The presence of the IncQ1 replicon gene was related to the observations for bla_{TEM-1B} and *strAB*, which form RR1 and are inserted in the chromosome, resulting in the disruption of the *fljBA* operon [7]. IncQ1 replicon gene located on both chromosomal RR1 and IncHI2 plasmids in many isolates including 2016062 and 2017028, thus indicating that RR1 may be derived from IncHI2 plasmids through IS*26*-mediated recombinational events.

Genomic analysis showed that the resistance genes mostly coexisted with plasmid replicon type IncHI2 in the Chinese isolates, indicating that IncHI2 plasmid is the main vector for resistance gene spread. Linear alignment of six IncHI2 plasmids with complete sequences demonstrated a mosaic MDR region harboring various resistance genes dispersed among diverse mobile elements. IncHI2 plasmids are important conjugative vectors for crucial resistance genes such as bla_{CTX-M}, *oqxAB*, *qnrB*, and *mcr*; these are widespread among the members of the Enterobacteriaceae family [46–51]. Cointegration of IncHI2 with other types of plasmids has been reported recently [41]. The prevalence of typical MDR IncHI2 plasmids in *S.* 4,[5],12:i:- was markedly related to the categories of resistance genes and accounted for the major role of acquiring resistance genes in this serovar [47].

All the Chinese isolates have similar virulence genes, indicating conservation in the chromosomal regions. Distributions of the SPIs were also consistent among Chinese, US, and European isolates. Besides, SGI-4 is responsible for increased resistance to copper and is a typical integrative and conjugative element [52]. Selection pressure imposed by metal supplements in pig feed is important for the retention of SGI-4, and this genomic island may soon lose during transmission [3,52,53]. The high prevalence of SGI-4 in Chinese isolates matched those in the European isolates, indicating a close relationship with pork or pig isolates. Moreover AMR *S.* 4,[5],12:i- lineages efficiently infected and survived in host phagocytes and epithelial cells without eliciting significant cellular cytotoxicity [9]. The underlying molecular mechanisms are unclear and warrant further investigations.

Although we investigated the long-term genomic epidemiology of *S.* 4,[5],12:i:- in China, some limitations to this study remain to be addressed. It is reported that contaminated pork and pig are important sources of *S.* 4,[5],12:i:- in different regions [43,44,54]. Importantly, comparison among human, animal, and food isolates would provide a better understanding of national and global transmission. Furthermore, additional regions or provinces should be included to better represent the status at the national level.

5. Conclusions

In conclusion, genomic analysis of ST34 *S.* 4,[5],12:i:- represents a first snapshot of the importance of *Salmonella* serovar prevalence in China. Different Chinese clades were likely to have been derived from European lineages; these subsequently evolved along multiple pathways resulting in different AMR profiles and distributions of mobile elements consisting of plasmids and SGIs. More comprehensive investigations including isolates with a "One Health" approach will provide a better holistic understanding of the transmission. Importantly, appropriate measures should be taken to curb the ST34 epidemic at the national and global levels.

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Compliance with ethics guidelines

Yujiao Mu, Ruichao Li, Pengcheng Du, Pei Zhang, Yan Li, Shenghui Cui, Séamus Fanning, and Li Bai declare that they have no conflict of interest or financial conflicts to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2022.05.006.

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