ANTIMICROBIAL RESISTANCE IN FOODBORNE PATHOGENS: ECOLOGY, EPIDEMIOLOGY, AND MECHANISMS

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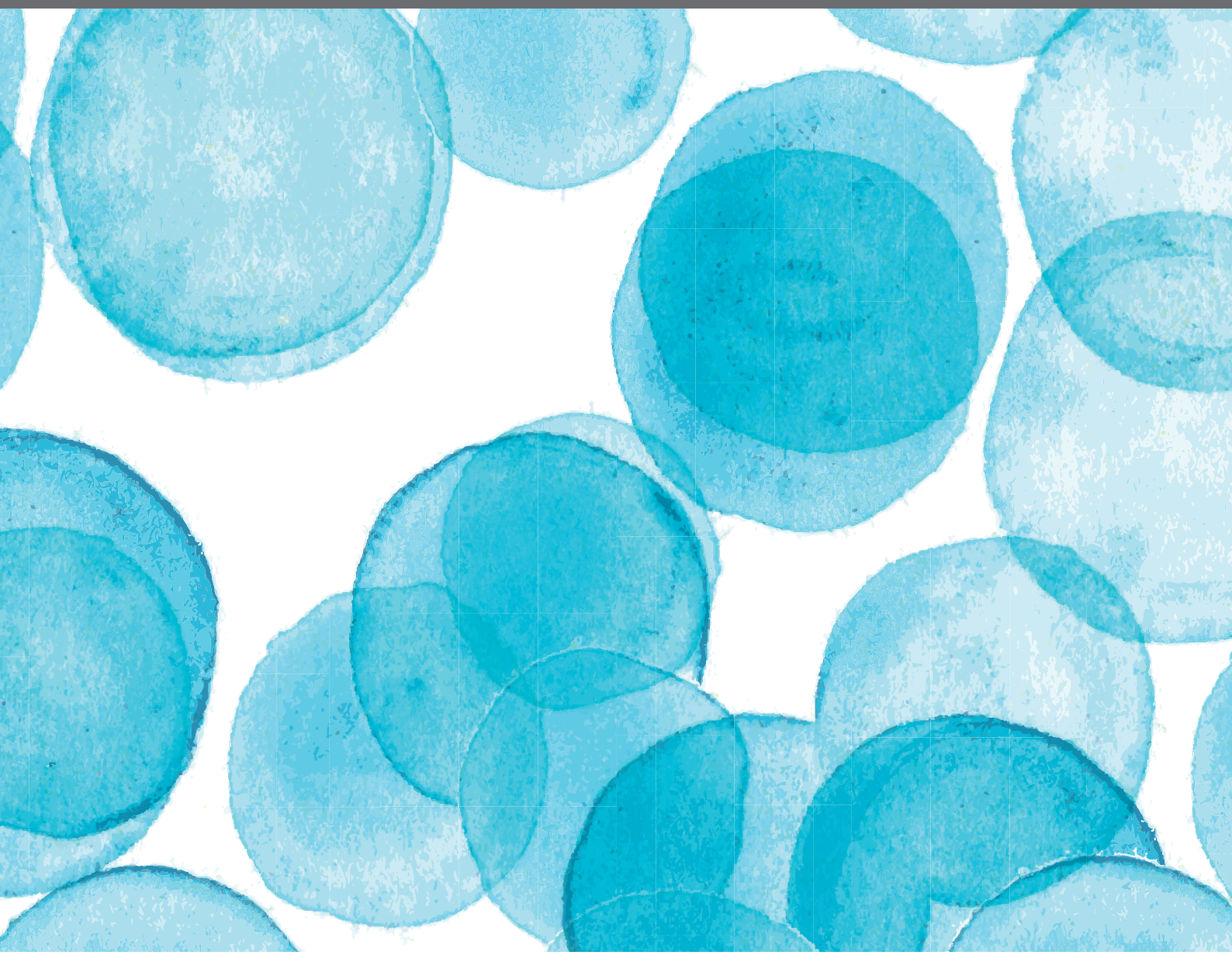

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ANTIMICROBIAL RESISTANCE IN FOODBORNE PATHOGENS: ECOLOGY, EPIDEMIOLOGY, AND MECHANISMS

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Monitoring the Microevolution of *Salmonella enterica* in Healthy Dairy Cattle Populations at the Individual Farm Level Using Whole-Genome Sequencing

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Livestock represent a possible reservoir for facilitating the transmission of the zoonotic foodborne pathogen *Salmonella enterica* to humans; there is also concern that strains can acquire resistance to antimicrobials in the farm environment. Here, whole-genome sequencing (WGS) was used to characterize *Salmonella* strains (n = 128) isolated from healthy dairy cattle and their associated environments on 13 New York State farms to assess the diversity and microevolution of this important pathogen at the level of the individual herd. Additionally, the accuracy and concordance of multiple *in silico* tools are assessed, including: (i) two *in silico* serotyping tools, (ii) combinations of five antimicrobial resistance (AMR) determinant detection tools and one to five AMR determinant databases, and (iii) one antimicrobial minimum inhibitory concentration (MIC) prediction tool. For the isolates sequenced here, *in silico* serotyping methods outperformed traditional serotyping and resolved all un-typable and/or ambiguous serotype assignments. Serotypes assigned *in silico* showed greater congruency with the *Salmonella* whole-genome phylogeny than traditional serotype assignments, and *in silico* methods showed high concordance (99% agreement). *In silico* AMR determinant detection methods additionally showed a high degree of concordance, regardless of the pipeline or database used (≥98% agreement among susceptible/resistant assignments for all pipeline/database combinations). For AMR detection methods that relied exclusively on nucleotide BLAST, accuracy could be maximized by using a range of minimum nucleotide identity and coverage thresholds, with thresholds of 75% nucleotide identity and 50–60% coverage adequate for most pipeline/database combinations. *In silico* characterization of the microevolution and AMR dynamics of each of six serotype groups (*S.* Anatum, Cerro, Kentucky, Meleagridis, Newport, Typhimurium/Typhimurium variant Copenhagen) revealed that some lineages were strongly associated with individual farms, while others were distributed across multiple farms. Numerous AMR determinant acquisition and loss events were identified, including the recent acquisition of cephalosporin resistance-conferring bla_{CMY}- and

bla_{CTX-M}-type beta-lactamases. The results presented here provide high-resolution insight into the temporal dynamics of AMR *Salmonella* at the scale of the individual farm and highlight both the strengths and limitations of WGS in tracking zoonotic pathogens and their associated AMR determinants at the livestock-human interface.

Keywords: *Salmonella*, antimicrobial resistance, serotyping, dairy cattle, whole-genome sequencing, evolution, livestock

INTRODUCTION

The foodborne pathogen *Salmonella enterica* is estimated to be responsible for 1.35 million infections, 26,500 hospitalizations, and 420 deaths each year in the United States alone (Centers for Disease Control and Prevention, 2021). Despite the fact that over 2,600 *Salmonella* serotypes have been described (Issenhuth-Jeanjean et al., 2014), fewer than 100 of these serotypes are responsible for the majority of human infections (Centers for Disease Control and Prevention, 2020). In line with this, some *Salmonella* serotypes may share strong associations with a specific host, an extreme example of which can be seen in the human-restricted nature of *Salmonella* Typhi (Uzzau et al., 2000; Boore et al., 2015). Other serotypes, while not confined exclusively to infection of a single host, may be adapted to a given reservoir; for example, *Salmonella* Choleraesuis, while largely adapted to swine, occasionally infects humans (Uzzau et al., 2000; Chiu et al., 2004).

Cattle are a potential reservoir from which humans can acquire salmonellosis, and infected animals can shed *Salmonella* at irregular intervals for varying periods of time, regardless of whether they express clinical signs of bovine salmonellosis or not (Cummings et al., 2010b; Davidson et al., 2018; Holschbach and Peek, 2018). The bovine reservoir boasts its own repertoire of serotypes that can infect humans, with bovine-associated *Salmonella* serotype Dublin, known for its rare but frequently invasive infections in humans, being arguably the most noteworthy (Taylor et al., 1982; Uzzau et al., 2000; Rodriguez-Rivera et al., 2014; Harvey et al., 2017; Mohammed et al., 2017). However, a range of *Salmonella* serotypes can persist and thrive in cattle, potentially infecting humans via either direct contact with infected animals or through food (Gutema et al., 2019). In a previous survey of 46 dairy cattle herds in New York State, *Salmonella* strains isolated from subclinically infected dairy cattle and associated farm environments spanned 26 serotypes, the most common being Cerro, Kentucky, Typhimurium, Newport, and Anatum (Rodriguez-Rivera et al., 2014). Additionally, antimicrobial resistant (AMR) isolates were observed on several farms, on numerous occasions, suggesting subclinically infected dairy cattle as a potential source of AMR *Salmonella* (Rodriguez-Rivera et al., 2014).

Numerous studies have employed whole-genome sequencing (WGS) to characterize *Salmonella* from bovine sources (Mather et al., 2013; Agren et al., 2016; Carroll et al., 2017b; Delgado-Suarez et al., 2018; Liao et al., 2019); however, little is known regarding the evolution and AMR acquisition and loss dynamics of *Salmonella* at the single herd/farm level. Furthermore, the bulk of bovine-associated *Salmonella* WGS efforts have focused on clinical veterinary samples and/or epidemic lineages (e.g., *S.* Typhimurium DT104). In this study, 128 non-typhoidal *S. enterica* strains isolated from repeated sampling on 13 New York State dairy cattle farms between 2007 and 2009 were characterized using WGS. All strains were isolated from apparently healthy, subclinically infected bovine hosts, as well as the associated farm environment (Rodriguez-Rivera et al., 2014). Using WGS, the microevolution of these persistent lineages within each herd is characterized, as well as the temporal acquisition and loss of AMR determinants among them. In addition to offering insight into the genomics of *Salmonella* isolated from healthy bovine populations at the individual herd/farm level, the accuracy and concordance of multiple *in silico* serotyping and AMR prediction tools are evaluated. Finally, an in-depth, critical analysis of the strengths and limitations of the methods used here is provided, which includes guidance to researchers who wish to employ WGS for herd-level pathogen monitoring.

MATERIALS AND METHODS

Isolate Selection

Salmonella enterica isolates (n = 128) obtained from one of 13 dairy farms in New York State were selected to undergo WGS for this study (**Supplementary Table 1**). All strains were isolated from farms that had undergone surveillance for *Salmonella* for a period of at least 12 months as described previously (Cummings et al., 2010a; Rodriguez-Rivera et al., 2014). Strains were isolated from repeated sampling on each farm between October 2007 and August 2009, from either (i) fecal samples from healthy, subclinically infected dairy cows (referred to hereafter as "bovine" isolates), or (ii) farm environmental swabs (referred to hereafter as "farm environmental" isolates) (Cummings et al., 2010a). All isolates underwent serotyping, phenotypic antimicrobial susceptibility testing, and pulsed-field gel electrophoresis (PFGE) as described previously (Rodriguez-Rivera et al., 2014).

Whole-Genome Sequencing and Data Pre-processing

Genomic DNA extraction and sequencing library preparation were performed as described previously (Carroll et al., 2017b), and the genomes of all 128 *Salmonella* isolates were sequenced using an Illumina HiSeq platform and 2 × 250 bp paired-end reads. Illumina sequencing adapters and low-quality bases were trimmed using Trimmomatic version 0.33 (using default parameters for Nextera paired-end reads) (Bolger et al., 2014), and FastQC version 0.11.9 (Andrews, 2019) was used to confirm adapter removal and assess read quality. SPAdes version 3.8.0

(Bankevich et al., 2012) was used to assemble genomes *de novo* (using the "careful" option and *k*-mer sizes of 21, 33, 55, 77, 99, and 127), and QUAST version 4.5 (Gurevich et al., 2013) and the "lineage_wf" workflow implemented in CheckM version 1.1.3 (Parks et al., 2015) were used to assess the quality of the resulting assemblies. MultiQC version 1.8 (Ewels et al., 2016) was used to aggregate genome quality metrics. Genome quality statistics are available for all isolates (**Supplementary Table 1**).

In silico Serotyping

In addition to undergoing traditional serotyping in a laboratory setting (i.e., serological detection of expressed O and H antigens using the White-Kauffmann-Le Minor scheme) as described previously (Rodriguez-Rivera et al., 2014), all 128 assembled *Salmonella* genomes (see section "Whole-Genome Sequencing and Data Pre-processing" above) underwent *in silico* serotyping using the command line implementations of (i) the *Salmonella in silico* Typing Resource (SISTR) version 1.0.2 (Yoshida et al., 2016) and (ii) SeqSero2 version 1.1.1 (Zhang et al., 2019) (using SeqSero2's *k*-mer based workflow). Serotypes assigned using all three methods are available for all 128 isolates (**Supplementary Table 1**). In cases where a discrepancy existed among the traditional serotype designation and one or more of the *in silico* methods, the serotype assigned using two out of the three methods was selected as the final serotype to be reported (e.g., when assigning strain names to isolates in the manuscript, for phylogeny annotation). To confirm that all serotype assignments were reasonable, a phylogeny was constructed using core single nucleotide polymorphisms (SNPs) detected in all *Salmonella* genomes in this study (see section "Reference-Free Single Nucleotide Polymorphism Identification and Phylogeny Construction" below).

In silico Antimicrobial Resistance Determinant Detection

Antimicrobial resistance determinants were detected in each of the 128 *Salmonella* genomes using five separate approaches: (i) ABRicate[1] version 0.8 (Seemann, 2018), (ii) AMRFinderPlus version 3.2.3 (Feldgarden et al., 2019), (iii) ARIBA version 2.14.1 (Hunt et al., 2017), (iv) BTyper version 2.3.3 (Carroll et al., 2017a), and (v) SRST2 version 0.2.0 (Inouye et al., 2014). Assembled genomes were used as input for the ABRicate and BTyper approaches, while trimmed Illumina reads were used as input for the SRST2 and ARIBA approaches. Prokka version 1.12 (Seemann, 2014) was used to annotate each assembled genome, and the resulting GFF (.gff) and FASTA (.faa and .ffn) files were used as input for the AMRFinderPlus approach. For the ABRicate approach, the following AMR gene databases were tested (each accessed June 11, 2018 via ABRicate's abricate-get_db command): (i) the Antibiotic Resistance Gene-ANNOTation database (ARG-ANNOT) (Gupta et al., 2014), (ii) the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017), (iii) the National Center for Biotechnology Information's (NCBI's) Bacterial AMR Reference Gene Database (NCBI) (Feldgarden et al., 2019), and (iv) the ResFinder database (ResFinder) (Zankari et al., 2012). For each genome and database combination, minimum AMR gene identity and coverage thresholds ranging from 50 to 100% (5% increments) and 0–100% (10% increments) were tested, respectively. For the BTyper approach, the (i) ARG-ANNOT v3 and (ii) MEGARes version 1.0.1 (Lakin et al., 2017) databases available with BTyper version 2.3.3 were used, with the minimum AMR gene identity and coverage thresholds varied in a manner identical to the ABRicate approach. For the SRST2 approach, the (i) ARG-ANNOT and (ii) ResFinder databases available with SRST2 version 0.2.0 were tested, using default thresholds. For the ARIBA approach, the following databases were tested (each accessed June 13, 2019 using ARIBA's getref command): (i) the version of ARG-ANNOT available with SRST2, (ii) CARD, (iii) MEGARes, (iv) NCBI, and (v) ResFinder, with all default thresholds used. For the AMRFinder approach, the latest version of the AMRFinder database was used (accessed December 6, 2019), along with the organism-specific database for *Salmonella*.

[1]https://github.com/tseemann/abricate

In silico Prediction of Antimicrobial Minimum Inhibitory Concentration Values

The PATRIC3 antimicrobial minimum inhibitory concentration (MIC) prediction model for *Salmonella* (Nguyen et al., 2019) (accessed June 13, 2019) was used to predict MIC values for each of the 128 *Salmonella* isolates in this study, using the assembled genome of each as input (**Supplementary Text**).

Prediction of Phenotypic Susceptible-Intermediate-Resistant Classifications Using *in silico* Methods

All 128 *Salmonella* isolates underwent phenotypic antimicrobial susceptibility testing with a panel of 15 antimicrobials (i.e., amikacin, amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole-trimethoprim, sulfisoxazole, and tetracycline) using the Sensititre system (Trek Diagnostic Systems Ltd., Cleveland, OH, United States) available at Cornell University's Animal Health Diagnostic Center as described previously (Rodriguez-Rivera et al., 2014). A "true" (i.e., phenotypic) susceptible-intermediate-resistant (SIR) classification for each of the 15 antimicrobials was obtained for 126 *Salmonella* isolates by comparing raw MIC values to NARMS breakpoints for *Salmonella* (accessed March 23, 2020; **Supplementary Table 1**). For streptomycin, the 1996–2013 NARMS breakpoints were used, as this was compatible with the concentrations used at the time of phenotypic testing (Rodriguez-Rivera et al., 2014). For sulfisoxazole, isolates with MIC > 256 were classified as resistant, as a concentration of 512 μg/mL was not tested. While raw MIC values were unavailable for two isolates (BOV_KENT_16_04-03-08_R8-0967 and ENV_MELA_01_01-10-08_R8-0165; **Supplementary Table 1**), both isolates had previously been categorized as pan-susceptible to all 15 antimicrobials (a classification that was maintained here, as all *in silico* methods correctly classified these isolates as pan-susceptible).

Known AMR determinant/phenotype associations for AMR determinants detected by each of the AMR determinant detection pipeline/database combinations described above (see section "*In silico* Antimicrobial Resistance Determinant Detection") were obtained from (i) Supplementary Table 4 of the AMRFinder validation paper (Feldgarden et al., 2019) and (ii) CARD (**Supplementary Table 2** and **Supplementary Text**). An isolate was predicted to be resistant to a particular antimicrobial if it possessed one or more AMR determinants known to confer resistance to that antimicrobial; if it did not possess any AMR determinants known to confer resistance to that antimicrobial, the isolate was predicted to be susceptible to that antimicrobial (**Supplementary Table 2**). For each AMR determinant detection pipeline/database combination, the caret package (Kuhn, 2008) in R version 3.6.1 (R Core Team, 2019) was used to construct a confusion matrix and calculate accuracy scores, Cohen's kappa coefficients, and other statistics (**Supplementary Table 3**) by treating "true" susceptible/resistant classifications obtained using phenotypic susceptibility testing as a reference. Cases of intermediate phenotypic resistance were treated as susceptible, as it resulted in slightly higher accuracy scores for all pipeline/database combinations for this particular data set. Because *in silico* prediction of susceptibility/resistance was highly dependent on prior knowledge of AMR determinants and the antimicrobials to which they conferred resistance, the concordance of all pipeline/database combinations was assessed by comparing each pipeline/database combination to results obtained using the SRST2 pipeline/ARG-ANNOT database combination.

To assess the ability of the MIC prediction method implemented in PATRIC3 to predict *Salmonella* SIR classification (see section "*In silico* Prediction of Antimicrobial Minimum Inhibitory Concentration Values" above), predicted MIC values for 14 antimicrobials produced using PATRIC3 were used to predict the SIR status of each of the 128 *Salmonella* isolates using the same NARMS breakpoints used for phenotypic testing. Azithromycin MICs produced by PATRIC3 were excluded, as azithromycin was not among the 15 antimicrobials used here for phenotypic testing. The ability of PATRIC3 to predict amikacin resistance was also not evaluated, as amikacin is not among the antimicrobials queried by PATRIC3. A confusion matrix was constructed as described above, using predicted SIR classifications derived from predicted MIC values produced by PATRIC3 and NARMS breakpoints. Additionally, the deviation of raw MIC predictions produced by PATRIC3 ($MIC_{PATRIC3}$) from "true" raw MIC predictions produced using phenotypic testing ($MIC_{Phenotypic}$) in number of dilution factors ($N_{dilution\ factors}$) was assessed using the following equation:

$$N_{dilution\ factors} = \frac{\ln(\frac{MIC_{PATRIC3}}{MIC_{Phenotypic}})}{\ln(2)}$$

where ln corresponds to the natural logarithm. For example: if PATRIC3 predicted an MIC value of 8 and the "true" MIC value obtained with phenotypic testing was 2, then ln(8/2)/ln(2) = 2; this means that the PATRIC3 prediction of 8 is +2 dilution factors away from the "true" MIC of 2 (as dilution used for MIC are 2 fold serial dilutions, e.g., 2, 4, and 8 μg/mL).

Re-testing of Isolates With Highly Incongruent Antimicrobial Resistance Phenotypes

Several (n = 21) isolates possessed a phenotypic AMR SIR profile which was deemed to be highly incongruent with its predicted *in silico* AMR profile, regardless of the *in silico* pipeline/database used (**Supplementary Table 4**). For example, *S.* Cerro isolate BOV_CERO_35_10−02−08_R8−2685 was resistant to nine antimicrobials but did not harbor any known acquired AMR genes (**Supplementary Table 4**). Similarly, *S.* Newport isolate ENV_NEWP_62_03−05−09_R8−3442 itself was pan-susceptible, but harbored multiple acquired AMR genes (e.g., bla_{CMY-2}, *floR*, *sul2*, and *tetA*), which conferred multidrug resistance in closely related *S.* Newport isolates (**Supplementary Table 4**). To address these incongruencies, 21 selected *Salmonella* isolates underwent phenotypic antimicrobial susceptibility re-testing (conducted September 16, 2020) as described above (see section "Prediction of Phenotypic Susceptible-Intermediate-Resistant Classifications Using *in silico* Methods"), with the exception of amikacin and kanamycin, as the contemporary panel did not include these antimicrobials (**Supplementary Table 4**).

Kanamycin testing was conducted separately using a gradient diffusion assay (Jorgensen and Ferraro, 2009) according to the manufacturer's instructions (BioMérieux Kanamycin Strip KM 256, product number 412381). Briefly, bacterial isolates were streaked for single colonies onto Brain Heart Infusion [BHI, Becton Dickinson (BD), Franklin Lakes, NJ, United States] agar plates from frozen glycerol stocks. Pre-cultures were prepared by inoculating a single colony in 3 mL Mueller-Hinton (MH) broth (BD Difco), followed by incubating at 37°C with shaking at 200 rpm for 12–14 h. The pre-cultures were used to inoculate tubes with 5 mL MH broth at 1:200 dilution, and the tubes were incubated at 37°C with shaking at 200 rpm for 5 h. Four mL of melted MH soft agar medium (0.7% agar) were mixed with 100 μL of culture and poured onto Petri plates containing 15 mL of MH agar medium (0.7% agar), and the plates were dried for 5 min. Kanamycin gradient strips were laid on top of the soft agar, and the plates were incubated at 35°C for 18 h. MIC values were determined by evaluating the inhibition zone using a magnifying lens according to the manufacturer's instructions.

Minimum inhibitory concentration values obtained from re-testing these isolates were interpreted within NARMS breakpoints as described above (see section "Prediction of Phenotypic Susceptible-Intermediate-Resistant Classifications Using *in silico* Methods") and are reported in the main manuscript (with the exception of amikacin; due to its exclusion from the contemporary panel, original MIC values are reported). Original and updated MIC and SIR values for all 21 isolates are available in **Supplementary Table 4**.

In silico Plasmid Replicon Detection

Plasmid replicons were detected in all *Salmonella* genome assemblies using ABRicate and the PlasmidFinder database

(accessed June 11, 2018 via ABRicate's abricate-get_db command). For a plasmid replicon to be considered present in a genome, minimum nucleotide BLAST (BLASTN) (Camacho et al., 2009) identity and coverage values of 80 and 60%, respectively, were used (Carattoli et al., 2014).

Reference-Free Single Nucleotide Polymorphism Identification and Phylogeny Construction

A reference-free approach was used to compare the 128 *Salmonella* genomes sequenced in this study to 442 of the 445 *Salmonella* genomes described by Worley et al. (2018); three genomes were omitted because their Sequence Read Archive (SRA) data was not publicly available at the time of access (February 20, 2019). Raw reads for each of the 442 publicly available genomes were downloaded from SRA (Leinonen et al., 2011; Kodama et al., 2012) and processed and assembled as described above (see section "Whole-Genome Sequencing and Data Pre-processing" described above). kSNP3 version 3.1 (Gardner and Hall, 2013; Gardner et al., 2015) was used to identify core SNPs among all 570 assembled *Salmonella* genomes, using the optimal *k*-mer size determined by Kchooser ($k = 19$). IQ-TREE version 1.6.10 (Nguyen et al., 2015) was used to construct a maximum likelihood (ML) phylogeny using the resulting core SNPs and the optimal nucleotide substitution model identified using ModelFinder [determined using model Bayesian Information Criteria (BIC) values; **Supplementary Text**]. Bootstrapping was performed using 1,000 replicates of the Ultrafast Bootstrap method (Minh et al., 2013; Hoang et al., 2018). The resulting ML phylogeny was annotated in R using the bactaxR package (Carroll et al., 2020b; **Supplementary Text**).

Pan-Genome Characterization

GFF files produced by Prokka (see section "*In silico* Antimicrobial Resistance Determinant Detection" above) were used as input for Roary version 3.12.0 (Page et al., 2015), which was used to identify orthologous gene clusters at a 70% protein BLAST (BLASTP) identity threshold. The resulting gene presence/absence matrix produced by Roary was used as input for besPLOT[2] (Carroll et al., 2020a), which was used to perform non-metric multidimensional scaling (NMDS) (Kruskal, 1964) and construct plots in two dimensions using a Jaccard distance metric (**Supplementary Text**).

Clustering based on gene presence/absence was assessed for each of the following grouping factors: (i) serotype, (ii) farm, and (iii) isolation source (i.e., bovine or farm environmental). For each of the three grouping factors, the following three statistical tests were performed, using the gene presence/absence matrix produced by Roary, a Jaccard distance metric, and 10,000 permutations: (i) the permutest and betadisper functions in R's vegan package (Oksanen et al., 2019) were used to conduct an ANOVA-like permutation test (Anderson, 2006) to test if group dispersions were homogenous (referred to hereafter as the PERMDISP2 test); (ii) analysis of similarity (ANOSIM) (Clarke, 1993) using the ANOSIM function in the vegan package in R was used to determine if the average of the ranks of within-group distances was greater than or equal to the average of the ranks of between-group distances (Anderson and Walsh, 2013); (iii) permutational analysis of variance (PERMANOVA) (Anderson, 2001) using the adonis2 function in the vegan package in R was used to determine if group centroids were equivalent. For all tests, a Bonferroni correction was applied to correct for multiple comparisons.

Potential clustering based on AMR gene presence/absence was additionally assessed for the same three grouping factors (serotype, farm, and isolation source), using the presence and absence of AMR determinants detected by AMRFinderPlus as input (i.e., AMR and stress response determinants identified using the "plus" option in AMRFinderPlus). All steps were performed as described above, and a Bonferroni correction was used to correct for multiple comparisons.

Reference-Based Core Single Nucleotide Polymorphism Identification Within Serotypes

For each individual serotype, core SNPs were identified among genomes assigned to that serotype using a reference-based approach. For each serotype, Snippy version 4.3.6[3] (Seemann, 2019) was used to identify core SNPs among all representatives assigned to the serotype, using the trimmed Illumina paired-end reads of each genome as input (see section "Whole-Genome Sequencing and Data Pre-processing" above) and one of six high-quality assembled genomes from isolates in this study as a reference genome (**Supplementary Table 1** and **Supplementary Text**). Gubbins version 2.3.4 (Croucher et al., 2015) was used to identify and remove recombination within the full alignment that resulted, and the filtered alignment produced by Gubbins was queried using snp-sites version 2.4.0 (Page et al., 2016) to produce an alignment of core SNPs for each serotype.

Construction of Within-Serotype Phylogenies

For each serotype, IQ-TREE version 1.6.10 was used to construct a ML phylogeny, using core SNPs detected among all isolates assigned to the serotype as input (see "Reference-Based Core Single Nucleotide Polymorphism Identification Within Serotypes" section above), the optimal ascertainment bias-aware nucleotide substitution model selected using ModelFinder, and 1,000 replicates of the UltraFast bootstrap approximation. The temporal structure of each resulting ML phylogeny was assessed using the R^2 value produced by the best-fitting root in TempEst version 1.5.1 (**Supplementary Table 5**; Rambaut et al., 2016).

A tip-dated phylogeny was then constructed for each serotype using BEAST version 2.5.0 (Bouckaert et al., 2014, 2019), using the serotype's corresponding core SNP alignment as input (**Supplementary Text**, **Supplementary Table 5**, and **Supplementary Figure 1**). For a detailed

[2]https://github.com/lmc297/besPLOT

[3]https://github.com/tseemann/snippy

description of all temporal phylogeny construction steps, see the **Supplementary Text**.

Data Availability

Illumina reads are available for all isolates sequenced in this study under NCBI Bioproject Accession PRJNA756552. NCBI BioSample accession numbers for each individual isolate, as well as all associated metadata and genome quality statistics, are available in **Supplementary Table 1**. All BEAST 2 XML files used for temporal phylogeny construction are available at https://github.com/lmc297/zru_farms.

RESULTS

In silico Serotyping of Bovine-Associated *Salmonella* Resolves Incongruencies Between Traditional Serotyping and Whole-Genome Phylogeny

A total of 128 *Salmonella* strains isolated from healthy (i.e., subclinically infected) dairy cattle (n = 39) and their associated farm environments (n = 89) on 13 different New York State farms underwent WGS (**Supplementary Table 1**). In addition to undergoing traditional serotyping in a laboratory setting, all isolates were assigned serotypes *in silico* using both (i) SISTR and (ii) SeqSero2 (**Supplementary Table 1**). Importantly, serotypes assigned *in silico* using SISTR and/or SeqSero2 were able to resolve all un-typable and/or ambiguous serotypes assigned using traditional serotyping (**Supplementary Table 1**). Furthermore, *in silico* serotypes assigned using (i) SISTR's core-genome multi-locus sequence typing (cgMLST) approach and (ii) SeqSero2 were both highly congruent with the *Salmonella* whole-genome phylogeny (**Figure 1**) and highly concordant with each other: 127 of 128 (99.2%) *Salmonella* isolates sequenced in this study were assigned to identical *in silico* serotypes using both SISTR cgMLST and SeqSero2 (**Supplementary Table 1**), with 100% concordance observed for six of seven observed *in silico* serotype groups (i.e., *S.* Anatum, *S.* Cerro, *S.* Meleagridis, *S.* Minnesota, *S.* Newport, and *S.* Typhimurium and its variants, assigned to n = 15, 13, 20, 1, 16, and 27 isolates, respectively). Among *S.* Kentucky (n = 36), a single incongruent isolate was observed (ENV_KENT_16_12-04-07_R8-0061), as SeqSero2 could not detect an O-antigen within the genome and was thus unable to assign this isolate to any serotype. This isolate was assigned a serotype of 8,20:-:z6 using traditional serotyping (*S.* Kentucky has antigenic formula 8,20:i:z6); SISTR classified the isolate as *S.* Kentucky, and the isolate clustered among the *S.* Kentucky isolates sequenced in this study within the *Salmonella* whole-genome phylogeny (**Figure 1** and **Supplementary Table 1**).

When variants of the *S.* Typhimurium serotype (n = 27) were considered, discrepancies were observed among traditional serotype assignments and both *in silico* methods (**Supplementary Table 1**). While SeqSero2 could differentiate between *S.* Typhimurium and the O5- variant of *S.* Typhimurium (also known as *S.* Typhimurium variant Copenhagen; "*S.* Typhimurium Copenhagen" is used hereafter), SISTR was unable to differentiate the two (**Supplementary Table 1**), as noted previously (Ibrahim and Morin, 2018; Zhang et al., 2019). However, *S.* Typhimurium and *S.* Typhimurium Copenhagen serotype assignments obtained using SeqSero2 and traditional serotyping did not always agree, as five of 27 *S.* Typhimurium/*S.* Typhimurium Copenhagen assignments (18.5%) differed between the two methods (**Supplementary Table 1**). For four of the five incongruent isolates, SeqSero2 assigned an isolate to *S.* Typhimurium Copenhagen, while traditional serotyping assigned a serotype of *S.* Typhimurium; for one isolate, the opposite scenario applied (**Supplementary Table 1**). Furthermore, the lineages formed by isolates classified here as *S.* Typhimurium Copenhagen using either traditional serotyping or SeqSero2, as well as two *S.* Typhimurium Copenhagen genomes from a previous study (Worley et al., 2018), were polyphyletic (**Figure 1**); consequently, the whole-genome phylogeny could not be used to reliably differentiate these two variants.

For the remainder of this study, a serotype assigned consistently with at least two out of the three methods (i.e., traditional serotyping, SeqSero2, and SISTR cgMLST) was selected as the final serotype to be reported for each isolate. Nine of the 13 farms surveyed here harbored *Salmonella* isolates that belonged to a single serotype, while two farms harbored two serotypes or serotype variants (Farms 25 and 35 harbored Typhimurium/Typhimurium Copenhagen and Cerro/Newport, respectively; **Supplementary Table 1**). The remaining two farms harbored three *Salmonella* serotypes (Farms 17 and 62 harbored Kentucky/Newport/Typhimurium and Cerro/Minnesota/Newport, respectively; **Supplementary Table 1**).

In silico Methods Predict Antimicrobial Susceptibility and Resistance Among Bovine-Associated *Salmonella* With High Accuracy and Concordance

Using a 15-antimicrobial panel and NARMS breakpoints for *Salmonella*, more than half of all isolates in this study (81 of 128; 63.3%) were classified as susceptible to all 15 antimicrobials tested, while 38 isolates (29.7%) were classified as resistant to two or more antimicrobials (obtained after the 15-antimicrobial panel was re-run for 22 isolates to resolve discrepancies between *in silico* predictions and phenotypic AMR data; **Supplementary Tables 1, 4**).

Regardless of choice of AMR determinant detection pipeline and AMR determinant database, all pipeline/database combinations performed nearly identically when given the task of predicting phenotypic AMR susceptibility/resistance to 15 antimicrobials using known AMR determinant-phenotype associations (**Figure 2**, **Table 1**, and **Supplementary Tables 2, 3**). Furthermore, all pipeline/database combinations showed an extremely high degree of concordance (98.0% or greater for all pipeline/database combinations; **Supplementary Figure 2**). The overall accuracy of all *in silico* AMR determinant detection pipeline/database combinations ranged from 95.8 to 97.4%, with the SRST2 AMR detection tool/ARG-ANNOT AMR

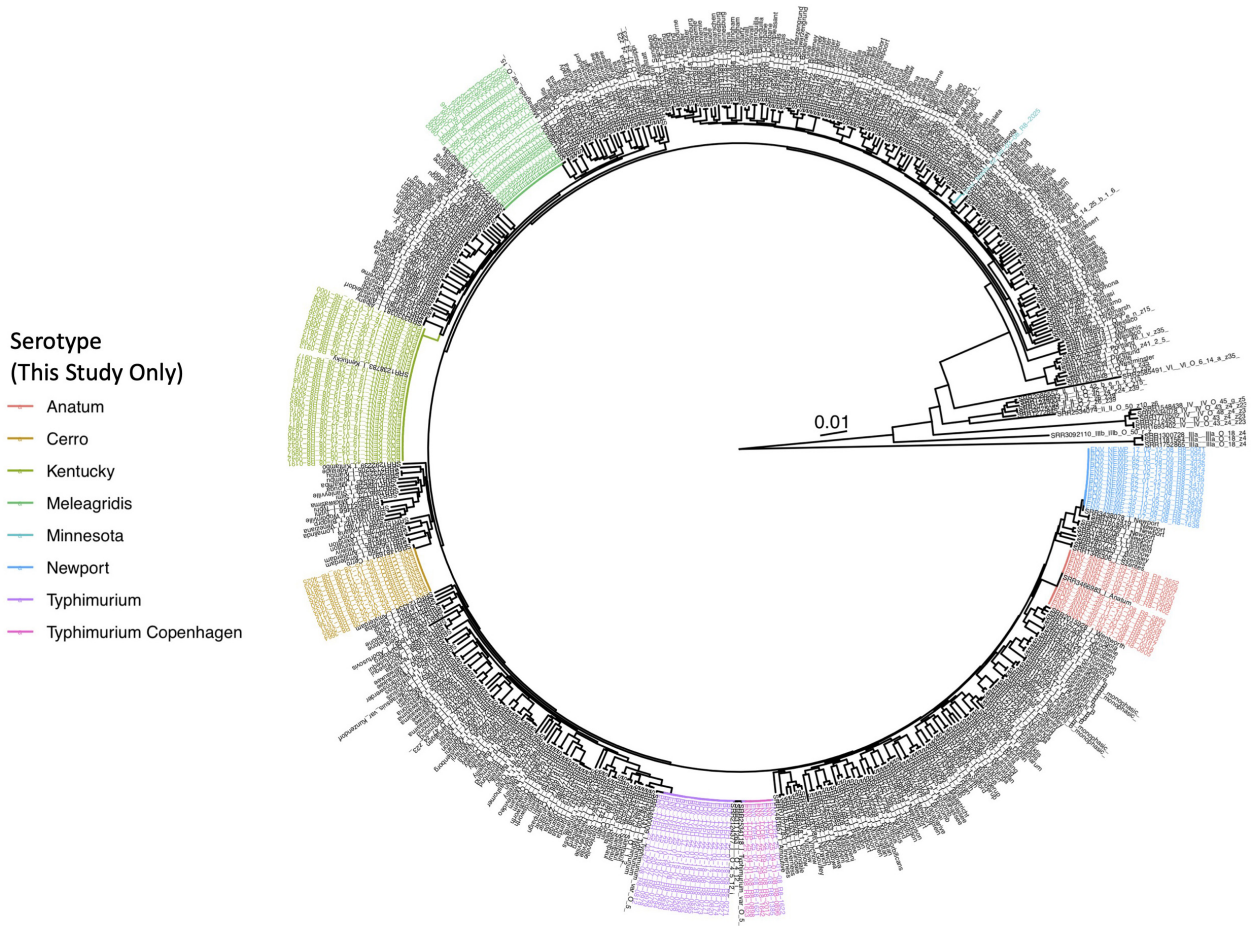

FIGURE 1 | Maximum likelihood phylogeny constructed using core SNPs identified among 570 *Salmonella* isolate genomes. Publicly available genomes are denoted by black tip labels (n = 442), while genomes of bovine- and bovine farm-associated strains isolated in conjunction with this study are denoted by colored tip labels (n = 128). In cases where a discrepancy existed between the traditional serotype designation of an isolate and one or more *in silico* methods (i.e., SISTR and SeqSero2), the serotype assigned using two out of the three methods was selected as the final serotype to be used for phylogeny annotation. The phylogeny is rooted at the midpoint with branch lengths reported in substitutions per site. Core SNPs were identified among all genomes using kSNP3, while the phylogeny was constructed and annotated using IQ-TREE and bactaxR/ggtree, respectively.

determinant database combination achieving the highest accuracy for this data set (**Figure 2**, **Table 1**, and **Supplementary Table 3**). The ARIBA/CARD pipeline/database combination achieved the highest specificity, although all pipeline/database combinations were able to predict phenotypic AMR with high specificity (>99.0%; **Figure 2**, **Table 1**, and **Supplementary Table 3**). Sensitivity ranged from 71.8 to 84.4%, with SRST2 achieving the highest sensitivities (84.4 and 84.0% for the ARG-ANNOT and ResFinder databases, respectively; **Table 1** and **Supplementary Table 3**).

For the AMR determinant detection pipelines that relied on nucleotide BLAST (i.e., ABRicate and BTyper), a range of minimum percent nucleotide identity and coverage thresholds were additionally tested (i.e., all combinations of 50–100% nucleotide identity in increments of 5% and 0–100% coverage in increments of 10%; **Supplementary Figure 3**) so that the optimal combination(s) could be established for the isolate genomes sequenced here. For ABRicate/ARG-ANNOT, ABRicate/NCBI, and ABRicate/ResFinder, maximum accuracy was achieved using minimum coverage thresholds of 60, 50, and 50–60%, respectively, and 75–95% nucleotide identity thresholds (**Supplementary Figure 3**). For ABRicate/CARD, minimum thresholds of 60% coverage and 75% nucleotide identity were optimal (**Supplementary Figure 3**). For BTyper/ARG-ANNOT, maximum accuracy was achieved using 60% coverage and 50–95% nucleotide identity; for BTyper/MEGARes, 50–60% coverage and 95% nucleotide identity were the optimal thresholds (**Supplementary Figure 3**).

The performance of the PATRIC3 *in silico* MIC prediction method was additionally evaluated (**Figure 3** and **Supplementary Figure 4**). PATRIC3 was able to correctly classify *Salmonella* isolates as SIR based on NARMS breakpoints with an overall accuracy of 92.9% [95% confidence interval 91.6–94.1%, accuracy P-value (accuracy > no information rate) < 1.25E-26; **Figure 3**]. At the individual antimicrobial level, PATRIC3 achieved >90% SIR prediction accuracy for 12 of 14 antimicrobials; only

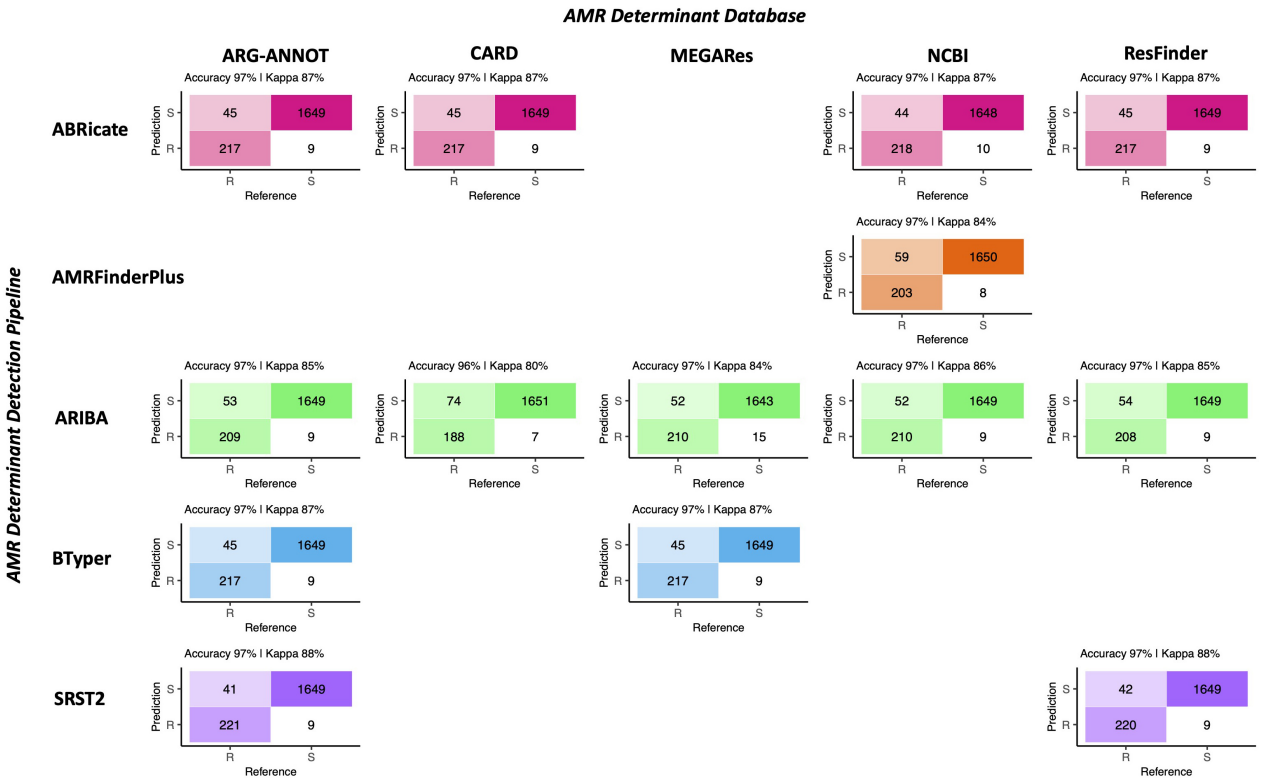

FIGURE 2 | Confusion matrices showcasing agreement between susceptible-intermediate-resistant (SIR) classification of 128 *Salmonella* isolates obtained using phenotypic resistance testing (denoted as the matrix "Reference") and five *in silico* methods (denoted as the matrix "Prediction") for 15 antimicrobials. Accuracy values above each matrix denote the percentage of correctly classified instances out of all instances. Kappa values above each matrix denote Cohen's kappa coefficient for the matrix, reported as a percent. For the phenotypic method, SIR classification was determined using NARMS breakpoints for *Salmonella* (accessed March 23, 2020). For the *in silico* antimicrobial resistance (AMR) determinant detection approaches, combinations of five pipelines and one to five AMR determinant databases were tested; isolate genomes that harbored one or more AMR determinants previously known to confer resistance to a particular antimicrobial were categorized as resistant to that antimicrobial ("R"), while those which did not were categorized as susceptible ("S"; see **Supplementary Table 2** for all detected AMR determinants and their associated resistance classifications). For all AMR determinant detection methods, isolates that showed intermediate phenotypic resistance to an antimicrobial were categorized as susceptible ("S") rather than resistant, as this classification produced slightly better accuracy scores for all pipeline/database combinations. For AMR determinant detection methods that relied on nucleotide BLAST (i.e., ABRicate and BTyper), the confusion matrix obtained using the nucleotide identity and coverage threshold combination that produced the highest accuracy are shown (**Supplementary Figure 3**); for all other methods, confusion matrices obtained using default detection parameters are shown.

sulfisoxazole and tetracycline resistance prediction accuracies were <90% (83.6 and 68.0%, respectively; **Figure 3** and **Supplementary Figure 4**).

Genomic Antimicrobial Resistance Determinants of Bovine-Associated *Salmonella* Are Serotype-Associated

Based on the presence and absence of pan-genome elements among all 128 *Salmonella* isolates sequenced here, the *Salmonella* pan-genome was more similar within serotype and within farm than between serotype and between farm, respectively (PERMANOVA and ANOSIM $P < 0.05$ after a Bonferroni correction; **Figure 4** and **Table 2**), with serotypes showing a higher degree of pan-genome dissimilarity (ANOSIM $R = 0.99$) and accounting for a larger proportion of the variance (PERMANOVA $R^2 = 0.93$) than farms (**Figure 4** and **Table 2**); however, dispersion among both serotypes and farms differed (PERMDISP2 $P < 0.05$ after a Bonferroni correction; **Table 2**), indicating that the ANOSIM and/or PERMANOVA tests could potentially be confounding dispersion with serotype/farm. Additionally, subclinical bovine *Salmonella* isolates did not significantly differ from strains isolated from the associated farm environment based on pan-genome element presence/absence (PERMANOVA, ANOSIM, and PERMDISP2 $P > 0.05$ after a Bonferroni correction; **Figure 4** and **Table 2**).

Based on the presence and absence of AMR and stress response determinants detected among all 128 *Salmonella* genomes, isolates were more similar within serotype than between serotypes (PERMANOVA and ANOSIM $P < 0.05$ and PERMDISP2 $P > 0.05$ after a Bonferroni correction; **Figure 4** and **Table 2**). Additionally, isolates were more similar within farm than between farm based on their AMR and stress response gene presence/absence profiles (PERMANOVA and ANOSIM $P < 0.05$; **Figure 4** and **Table 2**), although significant, potentially confounding dispersion differences among farms were present

TABLE 1 | Statistics for the 15-antimicrobial phenotypic susceptibility/resistance prediction task for all antimicrobial resistance (AMR) determinant pipeline/database combinations[a].

AMR Pipeline	AMR Database	% Accuracy (95% Confidence Interval)	Cohen's Kappa (%)	Corrected Accuracy *P*-Value[b]	Corrected McNemar's Test *P*-Value[b]	Sensitivity (%)	Specificity (%)
ABRicate	ARG-ANNOT	97.2 (96.3–97.9)	87.3	1.72E-59	2.67E-05	82.8	99.5
ABRicate	CARD	97.2 (96.3–97.9)	87.3	1.72E-59	2.67E-05	82.8	99.5
ABRicate	NCBI	97.2 (96.3–97.9)	87.4	1.72E-59	9.94E-05	83.2	99.4
ABRicate	ResFinder	97.2 (96.3–97.9)	87.3	1.72E-59	2.67E-05	82.8	99.5
AMRFinderPlus	NCBI	96.5 (95.6–97.3)	83.9	1.41E-50	1.41E-08	77.5	99.5
ARIBA	ARG-ANNOT	96.8 (95.9–97.5)	85.3	7.37E-54	6.63E-07	79.8	99.5
ARIBA	CARD	95.8 (94.8–96.6)	79.9	3.08E-42	3.14E-12	71.8	99.6
ARIBA	MEGARes	96.5 (95.6–97.3)	84.3	1.41E-50	1.53E-04	80.2	99.1
ARIBA	NCBI	96.8 (95.9–97.6)	85.5	1.55E-54	1.06E-06	80.2	99.5
ARIBA	ResFinder	96.7 (95.8–97.5)	85.0	3.45E-53	4.15E-07	79.4	99.5
BTyper	ARG-ANNOT	97.2 (96.3–97.9)	87.3	1.72E-59	2.67E-05	82.8	99.5
BTyper	MEGARes	97.2 (96.3–97.9)	87.3	1.72E-59	2.67E-05	82.8	99.5
SRST2	ARG-ANNOT	97.4 (96.6–98.1)	88.4	1.69E-62	1.63E-04	84.4	99.5
SRST2	ResFinder	97.3 (96.5–98.0)	88.1	9.85E-62	1.04E-04	84.0	99.5

[a] *Statistics were calculated using the confusionMatrix function in the caret package in R, with resistant ("R") phenotypes/genotypes treated as the "positive" result and susceptible ("S") phenotypes/genotypes treated as the "negative" result; See* ***Supplementary Table 3*** *for an extended version of this table.*
[b] *Adjusted using a Bonferroni correction.*

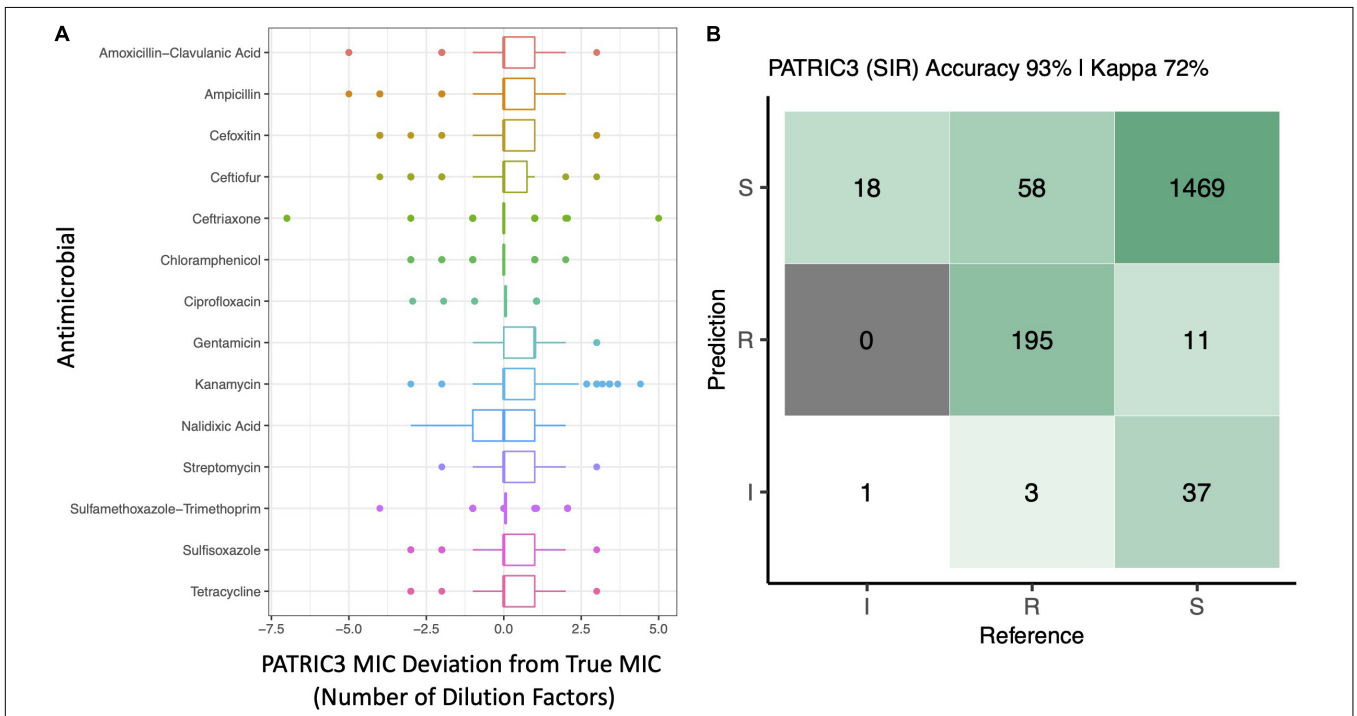

FIGURE 3 | (A) Deviation of PATRIC3 predicted minimum inhibitory concentration (MIC) values from "true" MIC values obtained using phenotypic resistance testing for 14 antimicrobials (*Y*-axis), reported in number of dilution factors (*X*-axis). For each box plot, lower and upper box hinges correspond to the first and third quartiles, respectively. Lower and upper whiskers extend from the hinge to the smallest and largest values no more distant than 1.5 times the interquartile range from the hinge, respectively. Points represent pairwise distances that fall beyond the ends of the whiskers. Only isolates with raw MIC values available are included (126 of 128 isolates). **(B)** Confusion matrix showcasing agreement between susceptible-intermediate-resistant (SIR) classification of all 128 *Salmonella* isolates obtained using phenotypic resistance testing (denoted as the matrix "Reference") and PATRIC3 (denoted as the matrix "Prediction") for 14 antimicrobials. The accuracy value above the matrix denotes the percentage of correctly classified instances out of all instances. The Kappa value above the matrix denotes Cohen's kappa coefficient for the matrix, reported as a percent. For both the phenotypic and PATRIC3 methods, SIR classification was determined using NARMS breakpoints for *Salmonella* (accessed March 23, 2020).

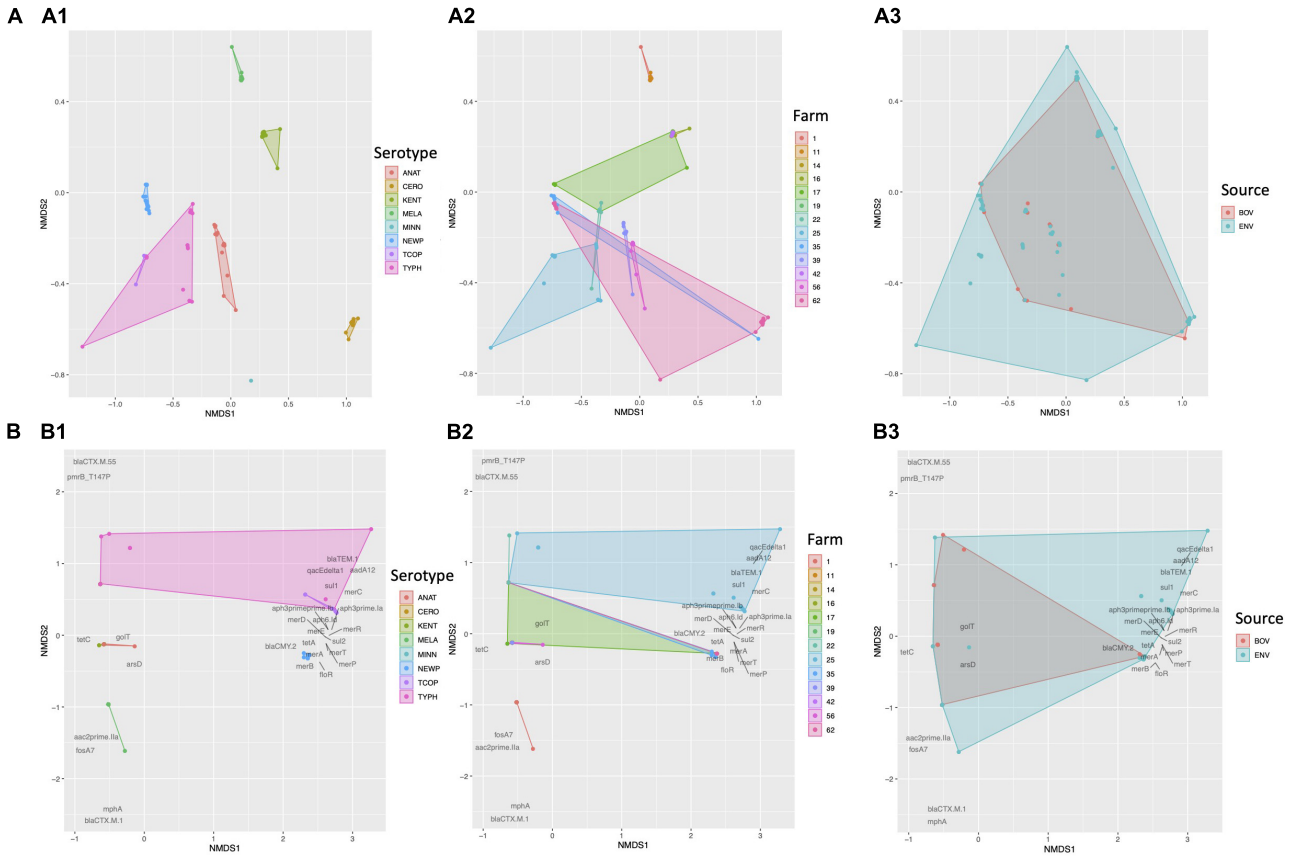

FIGURE 4 | Results of non-metric multidimensional scaling (NMDS) performed using the presence and absence of **(A)** pan-genome elements (*n* = 4,102; identified using Roary), and **(B)** antimicrobial resistance (AMR) and stress response genes (*n* = 28; detected using AMRFinderPlus) among 128 bovine-associated *Salmonella* isolates, plotted in two dimensions. Points represent isolates, while shaded regions and convex hulls correspond to isolate (1) serotypes (ANAT, Anatum; CERO, Cerro; KENT, Kentucky; MELA, Meleagridis; NEWP, Newport; TCOP, Typhimurium Copenhagen; TYPH, Typhimurium), (2) farm, and (3) source (BOV, bovine; ENV, bovine farm environment). For all plots, a Jaccard distance metric was used. For AMR/stress response genes **(B)**, gene names/scores are plotted in dark gray text.

TABLE 2 | Results of PERMDISP2, ANOSIM, and PERMANOVA tests[a].

Group	PERMDISP2 Raw *P*-Value (*F*)[b]	ANOSIM Raw *P*-Value (*R*)[c]	PERMANOVA Raw *P*-Value (R^2)[d]
Pan-genome element presence/absence (*n* = 4,102)[e]			
Serotype	2.0E-4 (14.3)*	<1.0E-4 (0.99)*	<1.0E-4 (0.93)*
Farm	<1.0E-4 (4.43)*	<1.0E-4 (0.54)*	<1.0E-4 (0.73)*
Source	0.071 (3.40)	0.99 (−0.07)	0.32 (0.01)
Antimicrobial resistance and stress response gene presence/absence (*n* = 28)[f]			
Serotype	0.013 (4.46)	<1.0E-4 (0.79)*	<1.0E-4 (0.85)*
Farm	<1.0E-4 (5.52)*	<1.0E-4 (0.30)*	<1.0E-4 (0.54)*
Source	0.74 (0.01)	0.79 (−0.03)	0.31 (0.01)

[a] *All tests were performed using a Jaccard dissimilarity metric and 10,000 permutations; raw P-values are reported for all tests, with significant P-values (P < 0.05 after a Bonferroni correction was applied to all values) denoted with an asterisk (*).*
[b] *ANOVA-like permutation test applied to results obtained using the PERMDISP2 procedure for the analysis of multivariate homogeneity of group dispersions (i.e., variances), obtained using the betadisper and permutest functions in the vegan package in R; betadisper is a multivariate analog of Levene's test for homogeneity of variances.*
[c] *Analysis of similarities (ANOSIM) test results obtained using the ANOSIM function in the vegan package in R.*
[d] *Permutational analysis of variance (PERMANOVA) test results obtained using the adonis2 function in the vegan package in R.*
[e] *Identified using Roary and a 70% protein BLAST (BLASTP) identity threshold.*
[f] *Detected using AMRFinderPlus.*

(PERMDISP2 $P < 0.05$; **Table 2**). As was the case with the pan-genome in its entirety, subclinical bovine *Salmonella* isolates did not significantly differ from farm environmental isolates based on AMR and stress response gene presence/absence (PERMANOVA, ANOSIM, and PERMDISP2 $P > 0.05$ after a Bonferroni correction; **Figure 4** and **Table 2**).

Each of Two New York State Dairy Farms Harbors a Unique, Bovine-Associated *Salmonella* Anatum Lineage

Fifteen *S.* Anatum strains encompassing four PFGE types (**Supplementary Table 1**) were isolated from subclinical bovine sources and their associated farm environments on two different New York State dairy farms (i.e., Farms 39 and 56; **Figure 5** and **Table 3**). Notably, the *S.* Anatum lineages circulating on each farm were distinct at a genomic level, with isolates from each farm forming a separate clade [posterior probability (PP) = 1 for each; **Figure 5**]. The two farm-associated lineages were predicted to share a common ancestor circa 1836 (node age 1836.28 using median node heights; **Figure 5**), although the age of the common ancestor could not be dated reliably [node height 95% highest posterior density (HPD) interval 540.85–1978.42; **Supplementary Figure 5**].

Salmonella Anatum isolates from Farm 39 shared a common ancestor circa 2005 (node age 2004.69, node height 95% HPD

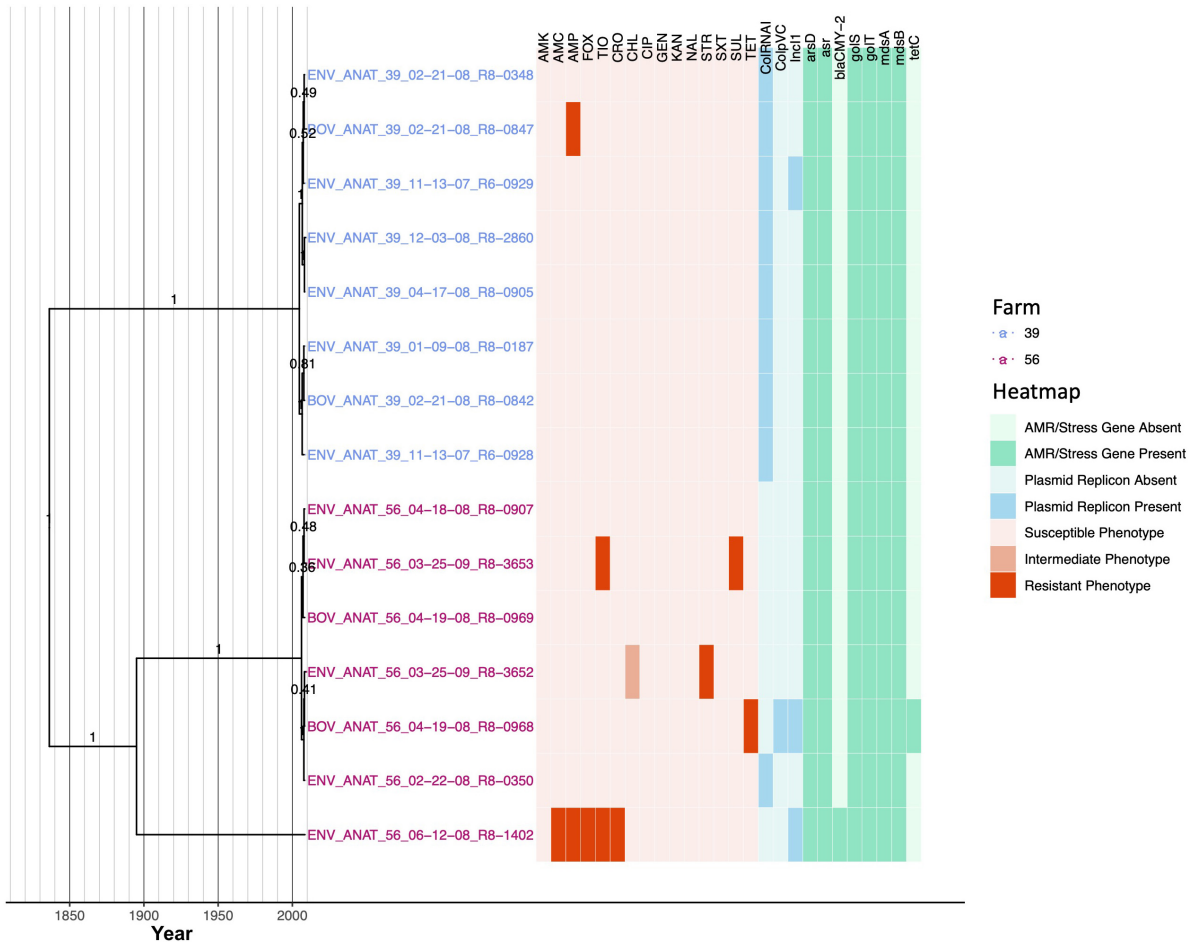

FIGURE 5 | Rooted, time-scaled maximum clade credibility (MCC) phylogeny constructed using core SNPs identified among 15 *Salmonella* Anatum genomes isolated from subclinical bovine sources and the surrounding bovine farm environment. Tip label colors denote the ID of the farm from which each strain was isolated. Branch labels denote posterior probabilities of branch support. Time in years is plotted along the *X*-axis, and branch lengths are reported in years. The heatmap to the right of the phylogeny denotes (i) the susceptible-intermediate-resistant (SIR) classification of each isolate for each of 15 antimicrobials (obtained using phenotypic testing and NARMS breakpoints; orange); (ii) presence and absence of plasmid replicons (detected using ABRicate/PlasmidFinder and minimum nucleotide identity and coverage thresholds of 80 and 60%, respectively; blue); (iii) presence and absence of antimicrobial resistance (AMR) and stress response genes (identified using AMRFinderPlus and default parameters; green). Core SNPs were identified using Snippy. The phylogeny was constructed using the results of ten independent runs using a strict clock model, the Standard_TVMef nucleotide substitution model, and the Coalescent Bayesian Skyline population model implemented in BEAST version 2.5.1, with 10% burn-in applied to each run. LogCombiner-2 was used to combine BEAST 2 log files, and TreeAnnotator-2 was used to construct the phylogeny using median node heights. Abbreviations for the 15 antimicrobials are: AMK, amikacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; FOX, cefoxitin; TIO, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; SUL, sulfisoxazole; TET, tetracycline.

1978.46–2007.69; **Figure 5** and **Supplementary Figure 5**). All Farm 39 *S.* Anatum isolates possessed identical AMR/stress response gene profiles, and all isolates were pan-susceptible except for a single isolate that was resistant to ampicillin (**Figure 5**). All Farm 39 *S.* Anatum isolates additionally harbored ColRNAI plasmids; a single isolate additionally harbored an IncI1 plasmid that appeared to harbor no AMR genes (**Figure 5**).

Salmonella Anatum isolates from Farm 56, however, were considerably more diverse than their Farm 39 counterparts; while a clade containing six of seven strains shared a very recent common ancestor (node age 2006.16, node height 95% HPD 1985.37–2008.11; **Figure 5** and **Supplementary Figure 5**), a unique lineage represented by a single environmental isolate (ENV_ANAT_56_06-12-08_R8-1402) was present among *S.* Anatum from Farm 56 (**Figure 5**). All *S.* Anatum isolates from Farm 56 were predicted to have evolved from a common ancestor that existed circa 1895 (node age 1895.03), although this node could not be reliably dated (node height 95% HPD 1036.89–1989.944; **Supplementary Figure 5**). Additionally, *S.* Anatum isolated from Farm 56 showcased a greater degree of AMR heterogeneity than those from Farm 39 (**Figure 5**). Notably, the isolate comprising the unique Farm 56 *S.* Anatum lineage possessed an IncI1 plasmid and $bla_{\mathrm{CMY-2}}$ and was multidrug resistant (MDR) (resistant to amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, and ceftriaxone; **Figure 5**). Three of six *S.* Anatum strains comprising the major Farm 56 *S.* Anatum lineage were pan-susceptible. The remaining three isolates were resistant to one of (i) tetracycline, (ii) streptomycin, or (iii) ceftiofur and sulfisoxazole; the streptomycin-resistant isolate additionally exhibited reduced susceptibility to chloramphenicol (**Figure 5**). The tetracycline-resistant isolate additionally possessed both ColpVC and IncI1 plasmids and harbored tetracycline resistance gene *tetC* (**Figure 5**). While these data suggest some *S.* Anatum lineages queried here have recently acquired AMR, the limited number of isolates and the large degree of uncertainty for some phylogeny node ages preclude reliable estimation of AMR acquisition timeframes.

A Closely Related *Salmonella* Cerro Lineage Spans Two New York State Dairy Farms

Thirteen *S.* Cerro strains encompassing two PFGE types (**Supplementary Table 1**) isolated from two dairy farms (12 from Farm 62 and one from Farm 35) were found to share a high degree of genomic similarity; isolates differed by, at most, 12 core SNPs and evolved from a common ancestor that existed circa March 2008 [node age 2008.21, common ancestor (CA) node height 95% HPD interval 2007.6–2008.6; **Figure 6**, **Table 3**, **Supplementary Figure 6**, and **Supplementary Table 5**]. While IncI1 and ColRNAI plasmid replicons were detected in all 13 *S.* Cerro isolates, only one isolate was not pan-susceptible (**Figure 6**). Notably, the isolate from Farm 35 (BOV_CERO_35_10-02-08_R8-2685) was classified as resistant to nine antimicrobials using phenotypic methods (i.e., amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline); based on the most parsimonious explanation for AMR acquisition, this lineage acquired AMR after July 2008 (node age 2008.51, CA node height 95% HPD interval 2008.14–2008.75; **Figure 6** and **Supplementary Figure 6**). However, no genomic determinants known to confer resistance to these antimicrobials were detected in the genome of the MDR isolate (**Figure 6**), and the MDR phenotype was confirmed in a second, independent phenotypic AMR test (**Supplementary Table 4**).

Salmonella Kentucky Strains Isolated Across Five Different New York State Dairy Farms Evolved From a Common Ancestor That Existed Circa 2004

Thirty-six *S.* Kentucky isolates encompassing two PFGE types (**Supplementary Table 1**) isolated across five New York State dairy farms (i.e., five, seven, nine, seven, and eight isolates from each of Farm 14, 16, 17, 19, and 42, respectively) were similar at a genomic level; isolates differed by between 0 and 30 core SNPs and shared a common ancestor that was predicted to have existed circa January/February 2004 (node age 2004.07, CA node height 95% HPD interval 2000.73–2006.8; **Figure 7**, **Table 3**, **Supplementary Figure 7**, and **Supplementary Table 5**). Two farms harbored a total of three *S.* Kentucky isolates, which were not pan-susceptible (two isolates from Farm 16 and one from Farm 17; **Figure 7**). Farm 17 harbored a tetracycline-resistant isolate (ENV_KENT_17_03-11-08_R8-0815), which possessed an IncI1 plasmid and *tetC* (**Figure 7**). The lineage represented by this isolate was predicted to have acquired tetracycline resistance after March 2007 (node height 2007.19, CA node height 95% HPD interval 2006.43–2007.84; **Figure 7** and **Supplementary Figure 7**). The two *S.* Kentucky isolates from Farm 16 additionally showed reduced susceptibility to chloramphenicol, a trait predicted to have been acquired by these lineages after December 2006/January 2007 (for the lineage represented by isolate ENV_KENT_16_12-04-07_R8-0061; node height 2006.98, CA node height 95% HPD interval 2005.95–2007.85) and May 2007 (for the lineage represented by isolate BOV_KENT_16_02-13-08_R8-0838; node height 2007.38, CA node height 95% HPD interval 2006.59–2008.10; **Figure 7** and **Supplementary Figure 7**). No corresponding genes that may encode for reduced chloramphenicol susceptibility were identified in these two isolates.

A Clonal *Salmonella* Meleagridis Lineage Is Distributed Across Two New York State Dairy Farms and Encompasses Isolates Carrying $bla_{CTX-M-1}$

Nineteen *S.* Meleagridis isolates encompassing two PFGE types (**Supplementary Table 1**) were isolated from two dairy farms (13 and six isolates from Farms 01 and 11, respectively) and were highly clonal: isolates differed by fewer than ten core SNPs and evolved from a common ancestor that existed circa May/June 2007 (node age 2007.42, CA node height 95% HPD interval 2006.91–2007.75; **Figure 8**, **Table 3**, **Supplementary Figure 8**, and **Supplementary Table 5**). All but three branches within the *S.* Meleagridis phylogeny had low

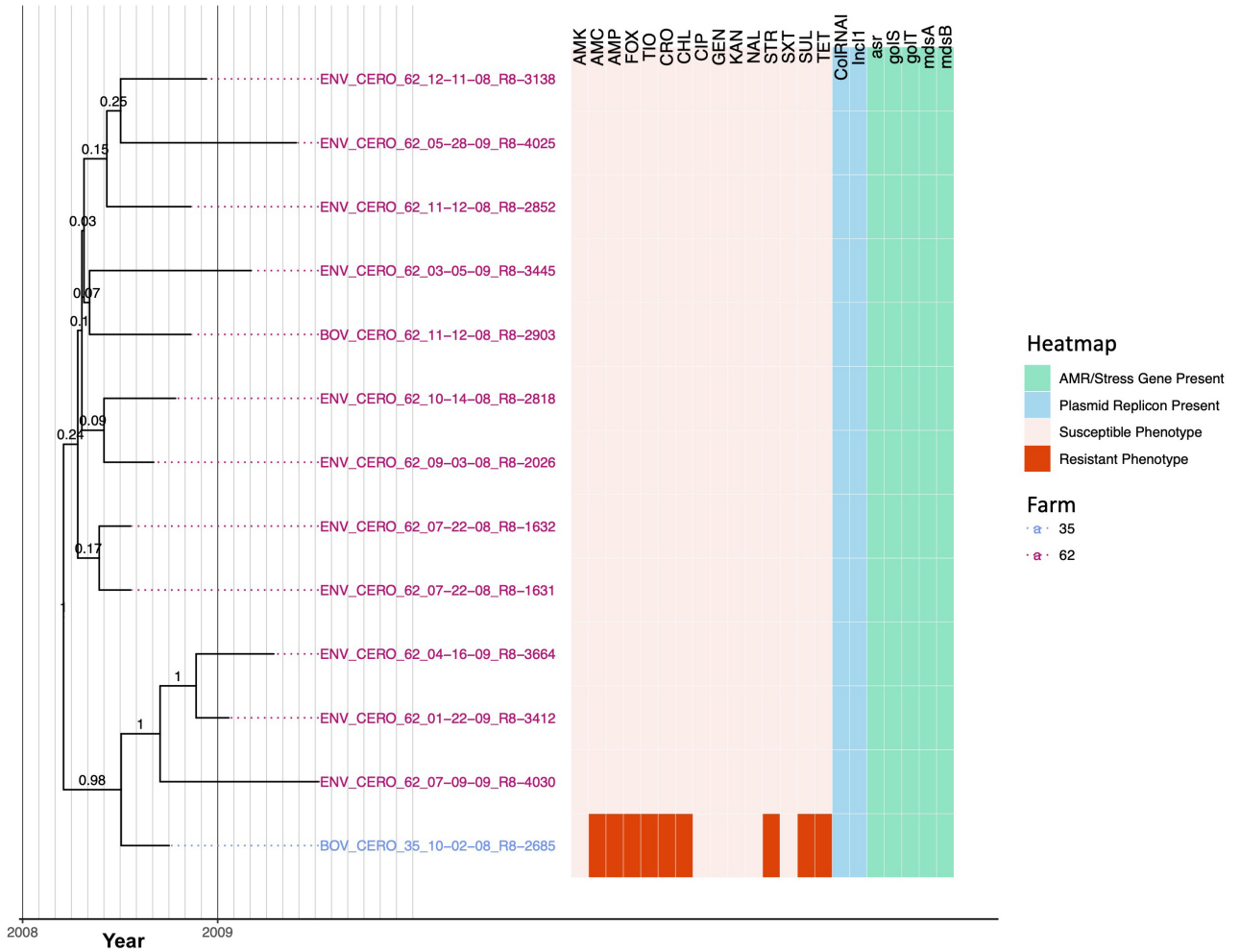

FIGURE 6 | Rooted, time-scaled maximum clade credibility (MCC) phylogeny constructed using core SNPs identified among 13 *Salmonella* Cerro genomes isolated from subclinical bovine sources and the surrounding bovine farm environment. Tip label colors denote the ID of the farm from which each strain was isolated. Branch labels denote posterior probabilities of branch support. Time in years is plotted along the *X*-axis, and branch lengths are reported in years. The heatmap to the right of the phylogeny denotes (i) the susceptible-intermediate-resistant (SIR) classification of each isolate for each of 15 antimicrobials (obtained using phenotypic testing and NARMS breakpoints; orange); (ii) presence of plasmid replicons (detected using ABRicate/PlasmidFinder and minimum nucleotide identity and coverage thresholds of 80 and 60%, respectively; blue); (iii) presence of antimicrobial resistance (AMR) and stress response genes (identified using AMRFinderPlus and default parameters; green). Core SNPs were identified using Snippy. The phylogeny was constructed using the results of ten independent runs using a strict clock model, the Standard_TPM1 nucleotide substitution model, and the Coalescent Bayesian Skyline population model implemented in BEAST version 2.5.1, with 10% burn-in applied to each run. LogCombiner-2 was used to combine BEAST 2 log files, and TreeAnnotator-2 was used to construct the phylogeny using common ancestor node heights. Abbreviations for the 15 antimicrobials are: AMK, amikacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; FOX, cefoxitin; TIO, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; SUL, sulfisoxazole; TET, tetracycline.

support (PP $\leq$ 0.41; **Figure 8** and **Supplementary Figure 8**), indicating that most nodes were unreliable, likely due to the isolates being highly clonal. All *S.* Meleagridis isolates from Farm 11 were pan-susceptible, possessed no plasmid replicons, and did not possess any acquired AMR genes (**Figure 8**). Among the *S.* Meleagridis isolates from Farm 01, one isolate (ENV_MELA_01_10-02-07_R6-0938) was resistant to ampicillin, ceftiofur, and ceftriaxone, and possessed an IncN plasmid, macrolide resistance gene *mph(A)*, and beta-lactamase $bla_{\mathrm{CTX-M-1}}$ (**Figure 8**). Two additional *S.* Meleagridis isolates from Farm 01 each exhibited reduced susceptibility to either (i) cefoxitin, sulfisoxazole, and tetracycline, or (ii) ceftiofur (**Figure 8**).

Kanamycin Resistance Among Each of Three New York State Dairy Farms Harboring a Distinct, Multidrug-Resistant *Salmonella* Newport Lineage Is Farm-Associated

Sixteen *S.* Newport isolates encompassing three PFGE types (**Supplementary Table 1**) were isolated from one

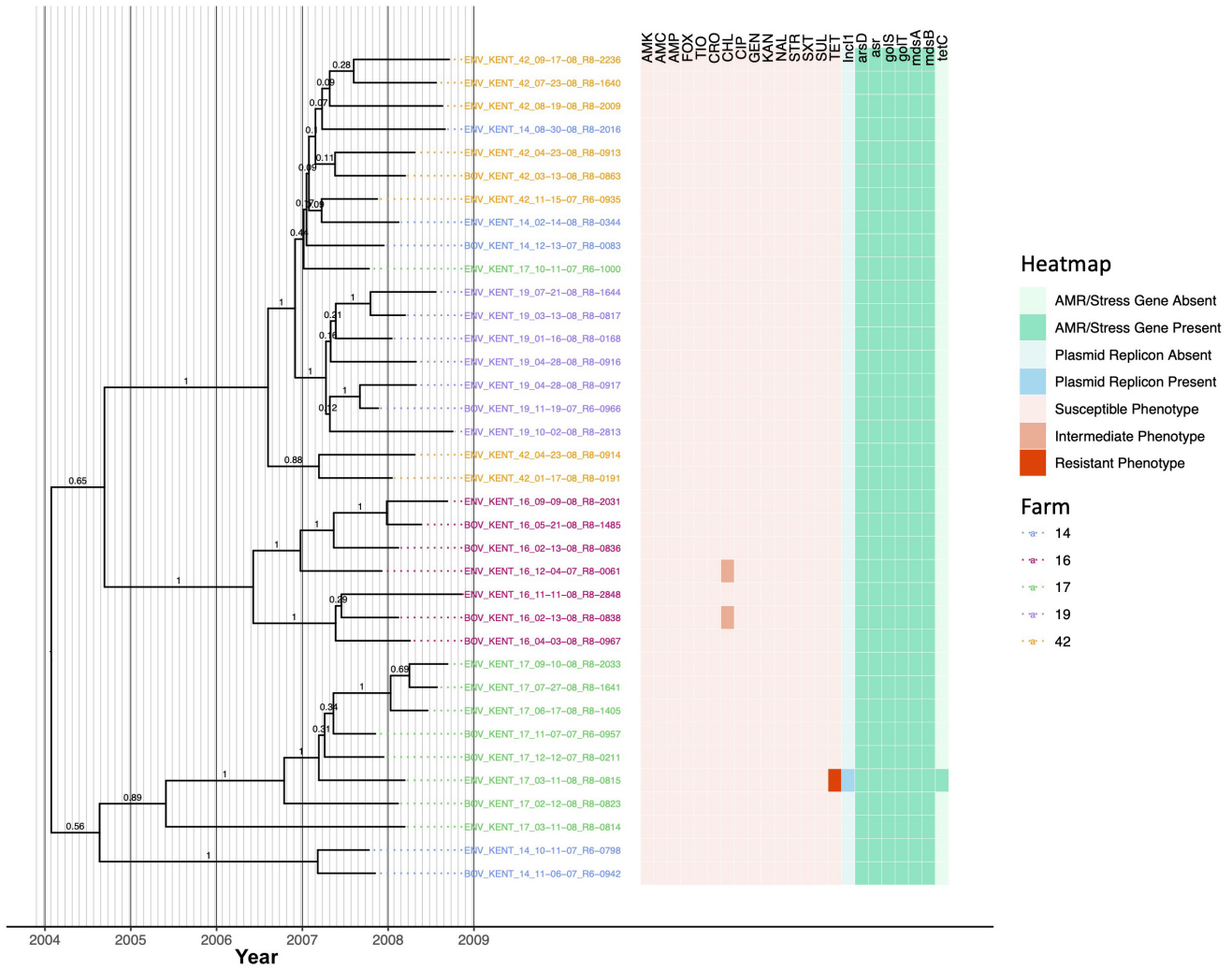

FIGURE 7 | Rooted, time-scaled maximum clade credibility (MCC) phylogeny constructed using core SNPs identified among 36 *Salmonella* Kentucky genomes isolated from subclinical bovine sources and the surrounding bovine farm environment. Tip label colors denote the ID of the farm from which each strain was isolated. Branch labels denote posterior probabilities of branch support. Time in years is plotted along the *X*-axis, and branch lengths are reported in years. The heatmap to the right of the phylogeny denotes (i) the susceptible-intermediate-resistant (SIR) classification of each isolate for each of 15 antimicrobials (obtained using phenotypic testing and NARMS breakpoints; orange); (ii) presence of plasmid replicons (detected using ABRicate/PlasmidFinder and minimum nucleotide identity and coverage thresholds of 80 and 60%, respectively; blue); (iii) presence of antimicrobial resistance (AMR) and stress response genes (identified using AMRFinderPlus and default parameters; green). Core SNPs were identified using Snippy. The phylogeny was constructed using the results of ten independent runs using a relaxed lognormal clock model, the Standard_TVMef nucleotide substitution model, and the Coalescent Bayesian Skyline population model implemented in BEAST version 2.5.1, with 10% burn-in applied to each run. LogCombiner-2 was used to combine BEAST 2 log files, and TreeAnnotator-2 was used to construct the phylogeny using common ancestor node heights. Abbreviations for the 15 antimicrobials are: AMK, amikacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; FOX, cefoxitin; TIO, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; SUL, sulfisoxazole; TET, tetracycline.

of three farms (four, five, and seven isolates from Farms 17, 35, and 62, respectively); all isolates were resistant to amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, streptomycin, sulfisoxazole, and tetracycline (**Figure 9**). All *S.* Newport genomes harbored IncA/C2 and ColRNAI plasmids, as well as streptomycin resistance genes *APH(3″)-Ib* and *APH(6)-Id* (i.e., *strAB*), beta-lactamase $bla_{\mathrm{CMY-2}}$, sulfonamide resistance gene *sul2*, and tetracycline resistance gene *tetA* (**Figure 9**). Notably, the *S.* Newport lineage circulating on each farm formed one of three separate clades (PP = 0.99–1.0) that evolved from a common ancestor that existed circa March/April 2004 (node age 2004.23, CA node height 95% HPD interval 2000.42–2007.85; **Figure 9**, **Table 3**, **Supplementary Figure 9**, and **Supplementary Table 5**).

The *S.* Newport lineages present on Farm 17 and Farm 62 were additionally resistant to chloramphenicol and kanamycin and possessed chloramphenicol and kanamycin resistance genes

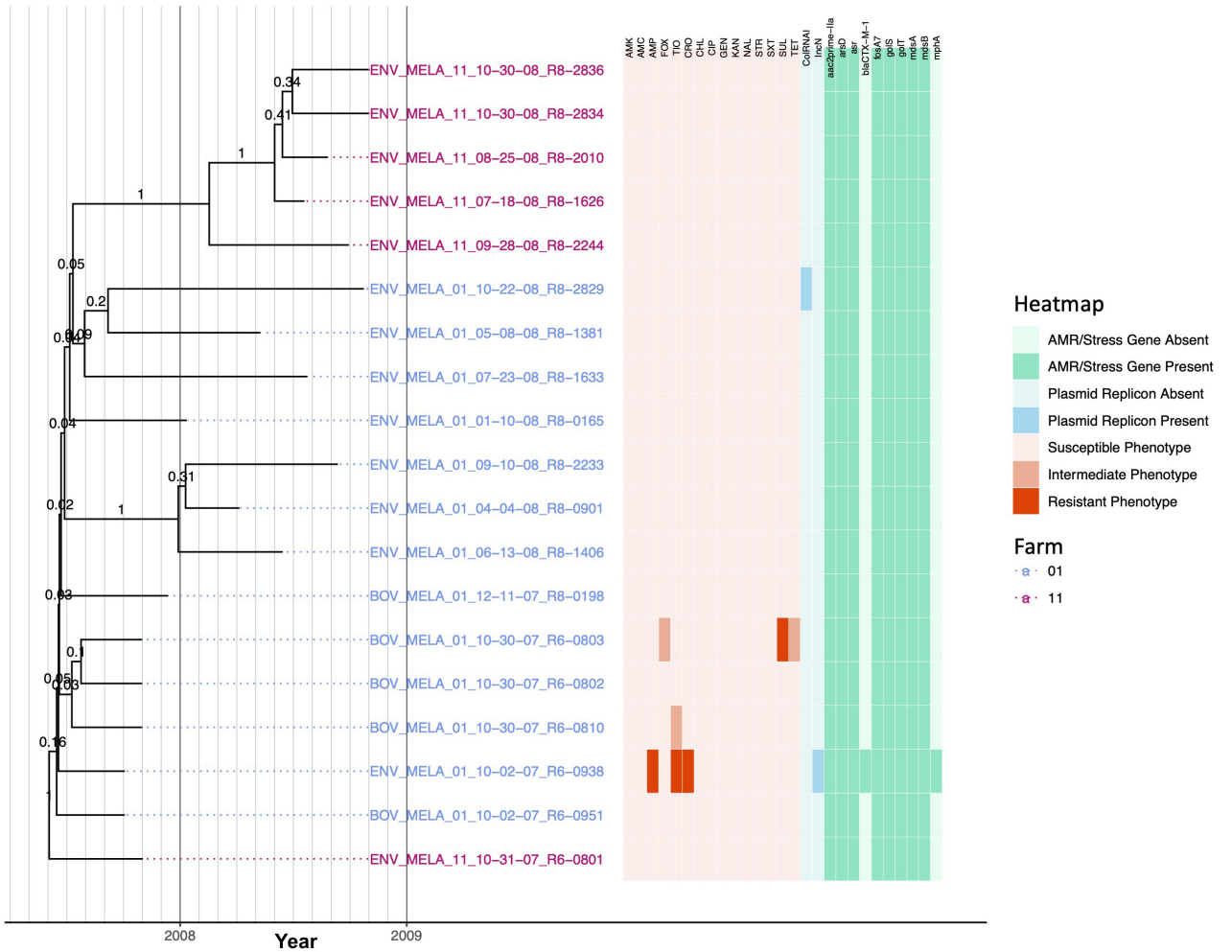

FIGURE 8 | Rooted, time-scaled maximum clade credibility (MCC) phylogeny constructed using core SNPs identified among 19 *Salmonella* Meleagridis genomes isolated from subclinical bovine sources and the surrounding bovine farm environment. Tip label colors denote the ID of the farm from which each strain was isolated. Branch labels denote posterior probabilities of branch support. Time in years is plotted along the *X*-axis, and branch lengths are reported in years. The heatmap to the right of the phylogeny denotes (i) the susceptible-intermediate-resistant (SIR) classification of each isolate for each of 15 antimicrobials (obtained using phenotypic testing and NARMS breakpoints; orange); (ii) presence and absence of plasmid replicons (detected using ABRicate/PlasmidFinder and minimum nucleotide identity and coverage thresholds of 80 and 60%, respectively; blue); (iii) presence and absence of antimicrobial resistance (AMR) and stress response genes (identified using AMRFinderPlus and default parameters; green). Core SNPs were identified using Snippy. The phylogeny was constructed using the results of ten independent runs using a strict clock model, the Standard_TPM2 nucleotide substitution model, and the Coalescent Bayesian Skyline population model implemented in BEAST version 2.5.1, with 10% burn-in applied to each run. LogCombiner-2 was used to combine BEAST 2 log files, and TreeAnnotator-2 was used to construct the phylogeny using common ancestor node heights. Abbreviations for the 15 antimicrobials are: AMK, amikacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; FOX, cefoxitin; TIO, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; SUL, sulfisoxazole; TET, tetracycline.

floR and *APH(3′)-Ia*, respectively (**Figure 9**). The Farm 17 and Farm 62 lineages evolved from a common ancestor predicted to have existed circa November/December 2005 (node age 2005.91, CA node height 95% HPD interval 2003.77–2007.85; **Figure 9** and **Supplementary Figure 9**). All members of the Farm 17 lineage additionally harbored a ColpVC plasmid and shared a common ancestor dated to circa August/September 2007 (node age 2007.65, CA node height 95% HPD interval 2007.29–2007.85; **Figure 9** and **Supplementary Figure 9**). The Farm 62 lineage, which did not possess the ColpVC plasmid, evolved from a common ancestor circa August/September 2008 (node age 2008.68, CA node height 95% HPD interval 2008.42–2008.78; **Figure 9** and **Supplementary Figure 9**).

Unlike the *S.* Newport lineages present on Farm 17 and Farm 62, the Farm 35 *S.* Newport lineage did not possess kanamycin resistance gene *APH(3′)-Ia* and was kanamycin-susceptible (**Figure 9**). The common ancestor of the Farm 35 *S.* Newport lineage was dated circa May 2008 (node age 2008.38, CA node height 95% HPD interval 2008.06–2008.55). All but one Farm 35 *S.* Newport isolates were additionally resistant to chloramphenicol and possessed *floR*;

TABLE 3 | Summary of within-serotype evolutionary analyses[a].

Serotype	Isolates	Core SNPs (Pairwise Range)[b]	Clock/Population Model[c]	Mean/Median Tree Height in Years (95% HPD Interval)[d]	Mean/Median Evolutionary Rate in Substitutions/Site/Year (95% HPD Interval)[e]
Anatum	15	337 (0–257)	Strict/Skyline	1484.9/1837.0 (549.6–1980.1)	1.67×10^{-7}/1.48×10^{-7} (6.92×10^{-11}–3.86×10^{-7})
Cerro	13	21 (0–12)	Strict/Skyline	2008.2/2008.4 (2007.6–2008.6)	9.11×10^{-7}/8.94×10^{-7} (3.06×10^{-7}–1.57×10^{-6})
Kentucky	36	102 (0–30)	Relaxed/Skyline	2004.1/2005.0 (2000.8–2006.8)	6.39×10^{-7}/6.34×10^{-7} (2.05×10^{-7}–1.07×10^{-6})
Meleagridis	19	27 (0–9)	Strict/Skyline	2007.4/2007.5 (2006.9–2007.7)	6.88×10^{-7}/6.66×10^{-7} (2.90×10^{-7}–1.12×10^{-6})
Newport	16	52 (0–38)	Relaxed/Skyline	2004.2/2004.6 (2000.4–2007.8)	9.02×10^{-7}/8.22×10^{-7} (2.64×10^{-7}–1.65×10^{-6})
Typhimurium (Copenhagen)	27	732 (0–634)	Relaxed/Skyline	1936.0/1943.0 (1864.7–1991.4)	1.07×10^{-6}/9.66×10^{-7} (2.84×10^{-7}–2.05×10^{-6})

[a] *See* ***Supplementary Table 5*** *for an extended version of this table; note that evolutionary rates may be higher than previously reported estimates for Salmonella populations isolated over a longer time frame, due to the small sample sizes and short temporal period characterized here (Moller et al., 2018).*
[b] *Number of core SNPs identified among all genomes within the serotype after removing recombination with Gubbins; the range of pairwise SNP differences between isolates was calculated using the dist.gene function in the ape package in R.*
[c] *The optimal model selected for the data set; can be a combination of a strict or lognormal relaxed molecular clock ("Strict" or "Relaxed," respectively) and a Constant Coalescent or Coalescent Bayesian Skyline population model ("CC" or "Skyline," respectively); see* ***Supplementary Table 5*** *for more details.*
[d] *The tree height parameter and its respective 95% highest posterior density (HPD) interval reported by Tracer.*
[e] *Corresponds to the clock Rate and rate.mean parameters estimated by BEAST2 for models using strict and lognormal relaxed molecular clock models, respectively, as reported by Tracer.*

BOV_NEWP_35_10-02-08_R8-2688 did not possess *floR* and was chloramphenicol-susceptible (**Figure 9**).

Each of Four Major Lineages Composed of *Salmonella* Typhimurium and Its O5-Copenhagen Variant Is Associated With One of Three New York State Dairy Farms

Twenty-seven bovine and farm environmental *S.* Typhimurium and *S.* Typhimurium Copenhagen isolates that encompassed five PFGE types (**Supplementary Table 1**) were isolated from three dairy farms (1, 10, and 16 strains isolated from Farm 17, 22, and 25, respectively). All isolates queried here shared a common ancestor that existed circa 1936 (node age 1935.62, CA node height 95% HPD interval 1864.84–1991.86; **Figure 10**, **Table 3**, **Supplementary Figure 10**, and **Supplementary Table 5**). Notably, the *S.* Typhimurium Copenhagen variant was polyphyletic (**Figure 10**), regardless of whether traditional or *in silico* (i.e., SeqSero2) methods had been used for serotype variant assignment. Additionally, the *S.* Typhimurium/*S.* Typhimurium Copenhagen isolates sequenced here showcased the most diverse AMR phenotypic profiles and AMR gene presence/absence profiles (**Figures 4**, **10**).

Isolates from Farm 25 were partitioned into two clades: one containing *S.* Typhimurium isolates, and one containing *S.* Typhimurium Copenhagen isolates (based on SeqSero2's *in silico* serotype assignments; **Figure 10**). Farm 25 isolates assigned to the *S.* Typhimurium Copenhagen variant (i) shared a common ancestor that existed circa December 2007/January 2008 (node age 2007.99, CA node height 95% HPD interval 2007.68–2008.21); (ii) were all resistant to ampicillin, kanamycin, streptomycin, sulfisoxazole, and tetracycline, with reduced susceptibility to additional antimicrobials observed sporadically; (iii) all possessed replicons for IncA/C2, IncFIB(AP001918), and IncFII(s) plasmids; and (iv) all possessed streptomycin resistance genes *aadA12*, *APH(3″)-Ib* and *APH(6)-Id* (i.e., *strAB*), beta-lactamase $bla_{\mathrm{TEM-1}}$, and antiseptic resistance gene *qacE* delta 1, with other AMR/stress response genes present sporadically (**Figure 10** and **Supplementary Figure 10**). Farm 25 isolates assigned to the *S.* Typhimurium clade shared a common ancestor that existed circa July 2007 (node age 2007.55, CA node height 95% HPD interval 2007.18–2007.78; **Figure 10** and **Supplementary Figure 10**). All Farm 25 *S.* Typhimurium isolates were resistant to cefoxitin; resistance to additional antimicrobials, along with presence of IncI1 plasmids and $bla_{\mathrm{CMY-2}}$, was observed sporadically (**Figure 10**).

The isolate from Farm 17 was predicted to belong to the *S.* Typhimurium Copenhagen serotype variant using SeqSero2 and shared a common ancestor with the *S.* Typhimurium isolates from Farm 22, which existed circa 2000 (node age 1999.92, CA node height 95% HPD interval 1991.72–2006.21; **Figure 10** and **Supplementary Figure 10**). Of the ten *S.* Typhimurium strains from Farm 22, seven were pan-susceptible (**Figure 10**). A bovine strain (BOV_TYPH_22_03−14−08_R8−0865) was resistant to ampicillin, ceftiofur, and ceftriaxone and was found to harbor IncI1 and IncI2 plasmids, as well as beta-lactamase $bla_{\mathrm{CTX-M-55}}$ (**Figure 10**). The remaining two bovine isolates were intermediately resistant to chloramphenicol and additionally resistant to either (i) amoxicillin-clavulanic acid, ampicillin, and sulfisoxazole, or (ii) tetracycline (**Figure 10**). Overall, isolates from Farm 17 and Farm 22 shared a common ancestor with the Farm 25 *S.* Typhimurium clade that existed circa 1988 (node age 1988.02, CA node height 95%

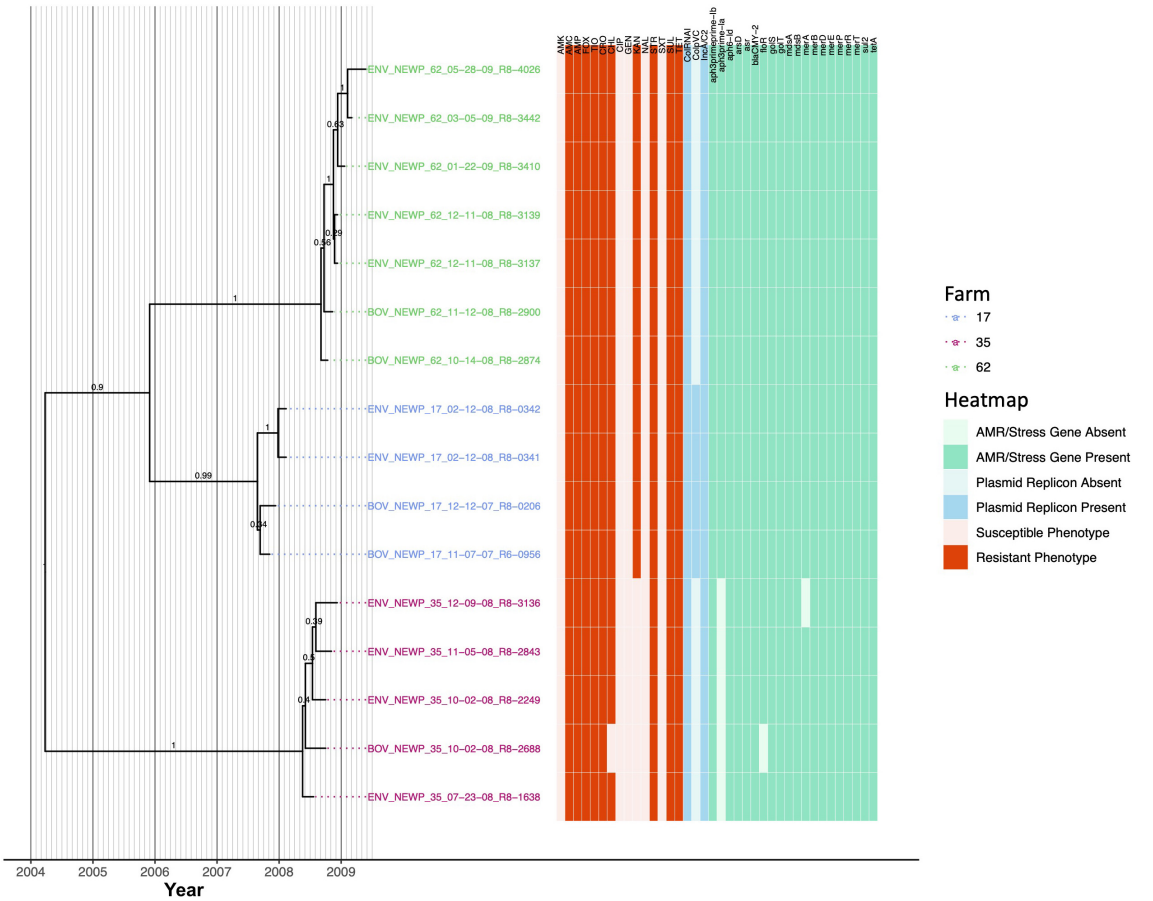

FIGURE 9 | Rooted, time-scaled maximum clade credibility (MCC) phylogeny constructed using core SNPs identified among 16 *Salmonella* Newport genomes isolated from subclinical bovine sources and the surrounding bovine farm environment. Tip label colors denote the ID of the farm from which each strain was isolated. Branch labels denote posterior probabilities of branch support. Time in years is plotted along the *X*-axis, and branch lengths are reported in years. The heatmap to the right of the phylogeny denotes (i) the susceptible-intermediate-resistant (SIR) classification of each isolate for each of 15 antimicrobials (obtained using phenotypic testing and NARMS breakpoints; orange); (ii) presence and absence of plasmid replicons (detected using ABRicate/PlasmidFinder and minimum nucleotide identity and coverage thresholds of 80 and 60%, respectively; blue); (iii) presence and absence of antimicrobial resistance (AMR) and stress response genes (identified using AMRFinderPlus and default parameters; green). Core SNPs were identified using Snippy. The phylogeny was constructed using the results of ten independent runs using a relaxed lognormal clock model, the Standard_TPM1 nucleotide substitution model, and the Coalescent Bayesian Skyline population model implemented in BEAST version 2.5.1, with 10% burn-in applied to each run. LogCombiner-2 was used to combine BEAST 2 log files, and TreeAnnotator-2 was used to construct the phylogeny using common ancestor node heights. Abbreviations for the 15 antimicrobials are: AMK, amikacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; FOX, cefoxitin; TIO, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; SUL, sulfisoxazole; TET, tetracycline.

HPD interval 1969.24–2002.47; **Figure 10** and **Supplementary Figure 10**).

DISCUSSION

Whole-Genome Sequencing Can Be Used to Monitor Pathogen Microevolution and Temporal Antimicrobial Resistance Dynamics in Animal Reservoirs

Cattle may act as a reservoir for *Salmonella* and may facilitate its transmission to other animals (Mentaberre et al., 2013; Wiethoelter et al., 2015) or humans, either through direct contact or via the food supply chain (Hoelzer et al., 2011; Cummings et al., 2012; Mughini-Gras et al., 2014; An et al., 2017; Gutema et al., 2019). Even outside of a bovine host, *Salmonella* can survive in the farm environment for a prolonged amount of time, making persistent strains a particularly relevant threat to animal and human health (Rodriguez et al., 2006; Cummings et al., 2010b; Gorski et al., 2011; Toth et al., 2011; Tassinari et al., 2019). This threat can be compounded when persistent strains are exposed to antimicrobials, as a number of studies have linked antimicrobial exposure to the emergence of AMR in different foodborne pathogens, including *Salmonella*, *Escherichia coli*, and *Campylobacter* (Boerlin et al., 2001; McDermott et al., 2002; Delsol et al., 2003; Dutil et al., 2010; Hoelzer et al., 2017).

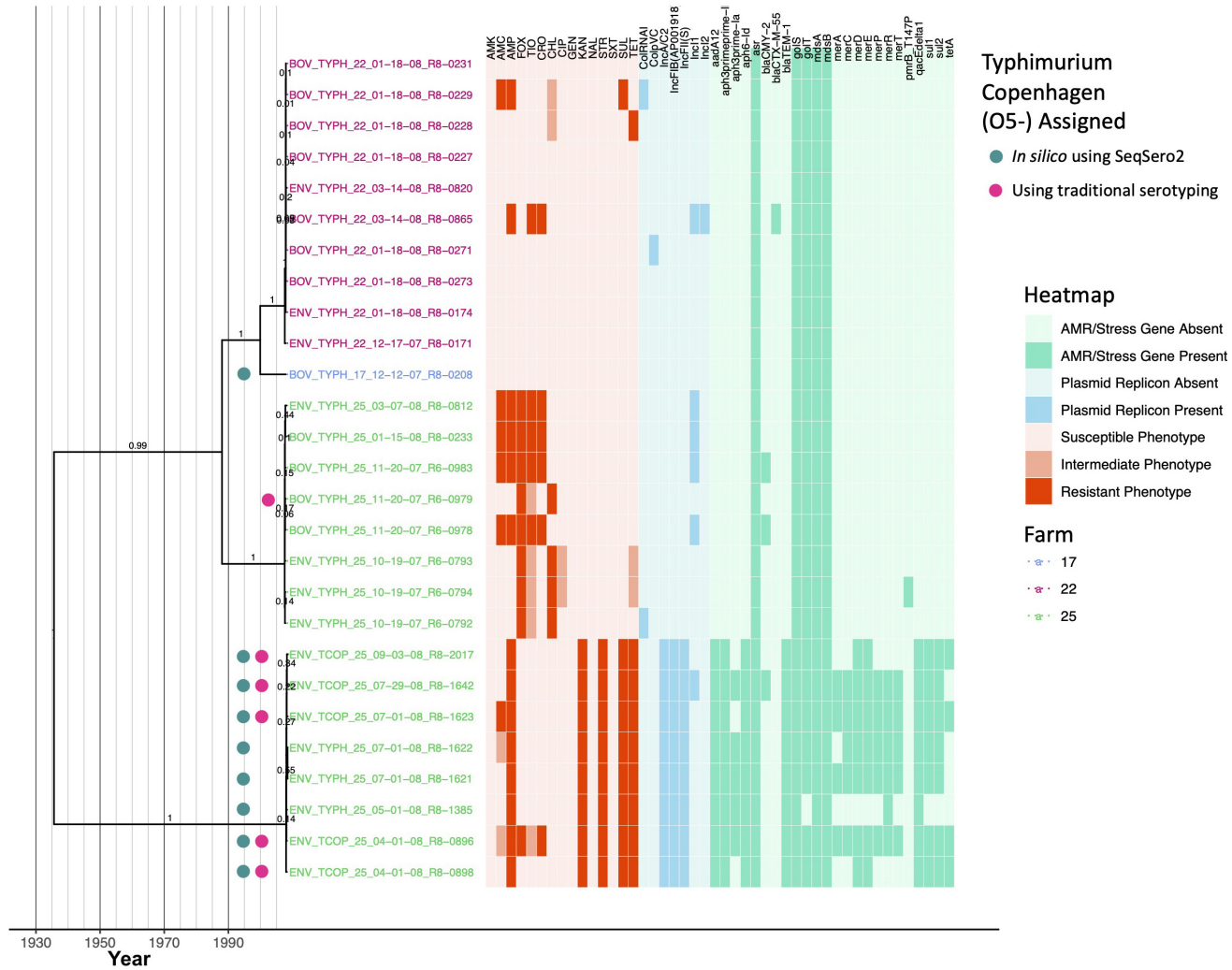

FIGURE 10 | Rooted, time-scaled maximum clade credibility (MCC) phylogeny constructed using core SNPs identified among 27 *Salmonella* Typhimurium and Typhimurium Copenhagen genomes isolated from subclinical bovine sources and the surrounding bovine farm environment. Tip label colors denote the ID of the farm from which each strain was isolated. Circles to the left of tip labels denote isolates that were assigned to the Typhimurium Copenhagen variant of *S.* Typhimurium using SeqSero2 (teal) and/or traditional serotyping (pink). Branch labels denote posterior probabilities of branch support. Time in years is plotted along the *X*-axis, and branch lengths are reported in years. The heatmap to the right of the phylogeny denotes (i) the susceptible-intermediate-resistant (SIR) classification of each isolate for each of 15 antimicrobials (obtained using phenotypic testing and NARMS breakpoints; orange); (ii) presence and absence of plasmid replicons (detected using ABRicate/PlasmidFinder and minimum nucleotide identity and coverage thresholds of 80 and 60%, respectively; blue); (iii) presence and absence of antimicrobial resistance (AMR) and stress response genes (identified using AMRFinderPlus and default parameters; green). Core SNPs were identified using Snippy. The phylogeny was constructed using the results of ten independent runs using a relaxed lognormal clock model, the Standard_TPM1 nucleotide substitution model, and the Coalescent Bayesian Skyline population model implemented in BEAST version 2.5.1, with 10% burn-in applied to each run. LogCombiner-2 was used to combine BEAST 2 log files, and TreeAnnotator-2 was used to construct the phylogeny using common ancestor node heights. Abbreviations for the 15 antimicrobials are: AMK, amikacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; FOX, cefoxitin; TIO, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; SUL, sulfisoxazole; TET, tetracycline.

However, AMR acquisition among pathogens in livestock environments is far from absolute; in the absence of selective pressures (e.g., antimicrobial exposure), some AMR traits may be associated with a fitness cost for a given organism (Melnyk et al., 2015; Hoelzer et al., 2017; San Millan and MacLean, 2017). Consequently, interventions or changes in farm management practices (e.g., limiting antimicrobial use for all or selected antimicrobials, targeted use of some antimicrobials) may lead to reduced selection of AMR bacteria (Aarestrup, 2015; Tang et al., 2017; Scott et al., 2018). As such, the dynamics of AMR acquisition and loss among livestock-associated bacterial pathogens are complex and influenced by a wide range of factors, including the antimicrobials and treatment regimens used, farm management practices, environmental conditions, and the biology of the pathogens themselves (Aarestrup, 2015;

Hoelzer et al., 2017; Davidson et al., 2018; Pereira et al., 2019; Clarke et al., 2020).

Using a WGS-based approach applied to serially sampled *Salmonella* strains isolated over a short time frame (i.e., less than 2 years), the study detailed here reveals that sporadic acquisition and loss of acquired AMR genes can occur within closely related populations over a short timescale. One particularly notable observation is represented by multiple, independent acquisitions of the beta-lactamase bla_{CMY} among *S.* Typhimurium and *S.* Typhimurium Copenhagen, as all bla_{CMY} acquisition events within this serotype group were confined to the 2000s. bla_{CMY} can confer resistance to cephalosporins, including (i) ceftriaxone, which has been used in human medicine since the early 1980s, and is used to treat invasive salmonellosis cases when fluoroquinolones cannot be used (e.g., for pediatric salmonellosis cases), and (ii) ceftiofur, which has been used in veterinary settings since the late 1980s to treat disease cases among dairy cattle and other animals (Hornish and Kotarski, 2002; Alcaine et al., 2005; Liebana et al., 2013; Yang et al., 2016; Carroll et al., 2017b, 2020a). Because bla_{CMY} often confers resistance to both ceftriaxone and ceftiofur, there has been concern that the use of ceftiofur in livestock can contribute to the dissemination of bla_{CMY} and thus yield bacterial populations that are co-resistant to ceftriaxone (Alcaine et al., 2005; Tragesser et al., 2006; Carroll et al., 2017b, 2020a).

Two independent bla_{CTX-M} acquisition events among *S.* Meleagridis and *S.* Typhimurium were additionally observed. bla_{CTX-M}, which also confers resistance to cephalosporins, was rarely detected in the United States in the 1990s (Lewis et al., 2007; Canton et al., 2012). However, bla_{CTX-M} rapidly increased in prevalence in the United States between 2000 and 2005 (Lewis et al., 2007; Canton et al., 2012), and there is evidence that bacterial populations associated with dairy cattle may have been affected as well. In a study of *E. coli* isolated from dairy cattle in the western United States, the prevalence of bla_{CTX-M} was found to have increased between 2008 and 2012 (Afema et al., 2018). The results of our study are congruent with these findings, as all observed bla_{CTX-M} acquisition events were estimated to have occurred in the 2000s.

Antimicrobial resistance loss events were additionally observed among the bovine-associated, MDR *S.* Newport isolates sequenced here. Prevalence of MDR *S.* Newport among humans increased rapidly in the United States within the late 1990s and early 2000s and was linked to cattle exposure, farm/petting zoo exposure, unpasteurized milk consumption, and ground beef consumption (Spika et al., 1987; Gupta et al., 2003; Karon et al., 2007). While chloramphenicol resistance is often a hallmark characteristic of MDR *S.* Newport, the MDR *S.* Newport lineage represented by an isolate in this study was chloramphenicol-susceptible and was predicted to have lost chloramphenicol resistance gene *floR* after 2008. These results indicate that even well-established MDR pathogens can still be subjected to temporal changes in AMR profile.

Due to the global burden that AMR pathogens impose on the health of humans and animals, numerous agencies have called for improved monitoring of pathogens and their associated AMR determinants along the food supply chain (World Health Organization, 2014, 2017; Centers for Disease Control and Prevention, 2019). The study detailed here showcases how WGS can be used to identify temporal changes in the resistomes of livestock-associated pathogens at the farm level. However, further sequencing efforts querying (i) a larger selection of *Salmonella* strains isolated from livestock on individual farms (ii) over a longer timeframe are needed to determine whether the AMR dynamics observed here are merely sporadic, or rather are indications of larger trends.

Bovine-Associated *Salmonella* Lineages With Heterogeneous Antimicrobial Resistance Profiles May Be Present Across Multiple Farms or Strongly Farm-Associated

Geography has been shown to play an important role in shaping bacterial populations (Achtman, 2008; Strachan et al., 2015), including some *Salmonella* lineages (Carroll et al., 2017b; Palma et al., 2018; Fenske et al., 2019; Liao et al., 2020). However, for some foodborne pathogens, including some *Salmonella* populations, global spread of lineages due to human migration and movement of food and animals can often obfuscate local phylogeographic signals (Wong et al., 2015; Llarena et al., 2016; The et al., 2016; Palma et al., 2018).

In the study detailed here, *Salmonella* lineages isolated from cattle and their associated environments on 13 separate farms in a confined geographic location (i.e., New York State) were found to vary in terms of the farm-specific signal they possessed; some lineages (i.e., *S.* Anatum, *S.* Newport, *S.* Typhimurium, *S.* Typhimurium Copenhagen, some *S.* Kentucky populations) were found to be strongly associated with a particular farm, while other lineages (i.e., *S.* Cerro, *S.* Meleagridis, some *S.* Kentucky populations) were distributed across multiple farms. Multiple scenarios may explain the existence of *Salmonella* lineages distributed across multiple farms, including movement of livestock, humans, pets, and/or wildlife (Skov et al., 2008; Hoelzer et al., 2011; Palma et al., 2018) or introduction via feed; however, additional metadata (e.g., farm geography, proximity to other farms in the study, and management practices) are needed to draw further conclusions. Even with limited metadata available, WGS data can provide important insights into *Salmonella* transmission and introduction on farms, as shown in this study. For example, for one farm (i.e., Farm 25), two *Salmonella* Typhimurium clonal groups were present (i.e., one representing Typhimurium and one representing Typhimurium Copenhagen), each of which shared a common ancestor dated circa 2007. WGS data can be used to identify time frames in which *Salmonella* lineages may have emerged in a given farm or region, which could help pinpoint root causes (e.g., changes in management practices that occurred around the predicted time of emergence).

While the characterization of additional, larger strain sets from more geographically diverse farms is essential, our data suggest that specific *Salmonella* clones may persist on a given farm. This suggests that WGS databases covering isolates from a large number of farms could be used to develop initial hypotheses

about farm sources of *Salmonella* strains. While such applications are tempting, it is crucial that these types of data are only used for initial hypothesis generation; rigorous, critical epidemiological investigations are essential before any conclusions regarding strain source are drawn.

In silico Serotyping of Bovine-Associated *Salmonella* Can Outperform Traditional Serotyping

Well into the genomic era, serotyping remains a vital microbiological assay that allows *Salmonella* isolates to be classified into meaningful, evolutionary units. Serotype assignments are used to facilitate outbreak investigations and surveillance efforts, construct salmonellosis risk assessment frameworks, and inform food safety and public health policy and decision-making efforts (Yoshida et al., 2016; Gutema et al., 2019). Importantly, serotyping is used worldwide to monitor salmonellosis cases among humans and animals, including cattle (Gutema et al., 2019; Centers for Disease Control and Prevention, 2020).

In this study, serotypes assigned using traditional phenotypic methods were compared to serotypes assigned using two *in silico* methods (i.e., SISTR and SeqSero2). Notably, both *in silico* serotyping approaches outperformed traditional *Salmonella* serotyping for this data set. Serotypes assigned using SISTR's cgMLST approach and/or SeqSero2 were congruent with the *Salmonella* whole-genome phylogeny and were able to resolve all un-typable, ambiguous, and incorrectly assigned serotypes (**Supplementary Table 1**). It is essential to note that the data set queried here is far too small and, thus, inadequate to formally benchmark these tools. Furthermore, all serotypes studied here were among the ten most frequently reported serotypes of *Salmonella* isolated from subclinical cattle between 2000 and 2017 (Gutema et al., 2019), indicating that they are well-represented in public databases and thus likely do not pose a significant challenge to *in silico* tools. However, the results observed here reflect observations made in several recent studies, which queried greater numbers of isolate genomes and/or a wider array of diverse *Salmonella* serotypes (Yachison et al., 2017; Ibrahim and Morin, 2018; Diep et al., 2019; Banerji et al., 2020; Uelze et al., 2020). In their analysis of 1,624 animal- and food-associated (i.e., non-human) *Salmonella* isolate genomes assigned to 72 serotypes, Uelze et al. (2020) reported that SISTR and SeqSero2 achieved the highest and second-highest accuracy of all tested *in silico Salmonella* serotype assignment tools, correctly serotyping 94 and 87% of isolates, respectively. However, unlike the results observed here, the authors note that neither tool outperformed traditional serotyping conducted by *Salmonella* reference laboratories. Similarly, in a study of 813 *Salmonella* isolates, SISTR outperformed the original version of SeqSero (i.e., SeqSero 1.0) with serotype prediction accuracies of 94.8 and 88.2%, respectively (Yachison et al., 2017).

With WGS data in hand, *in silico* serotyping is rapid, scalable, inexpensive, and reproducible (Uelze et al., 2020). Nevertheless, it is important to be mindful of the strengths and weaknesses of different *in silico* serotyping tools. In their benchmarking study, Uelze et al. (2020) recommended SISTR as the optimal contemporary tool for routine *in silico Salmonella* serotyping based on overall accuracy; however, they additionally report that the raw read mapping approach implemented in SeqSero2 (i.e., "allele mode") outperforms SISTR for prediction of monophasic variants. Banerji et al. (2020) did not assess the performance of SISTR on their data set, as it requires assembled genomes and not raw reads (another potential drawback if a high-quality assembly is not available or obtainable for an isolate of interest); however, they found that both SeqSero and MLST approaches misidentified monophasic variants, particularly among the important monophasic *S.* Typhimurium lineage. Among the bovine-associated *Salmonella* strains sequenced here, a combination of *S.* Typhimurium strains that possessed the O5 epitope, and those that did not (i.e., *S.* Typhimurium Copenhagen) was observed. Importantly, SISTR was unable to differentiate *S.* Typhimurium from *S.* Typhimurium Copenhagen, while SeqSero2 could, as reported previously (Ibrahim and Morin, 2018; Zhang et al., 2019). While the differentiation of *S.* Typhimurium from its O5- counterpart may not be essential for all microbiological applications, it is important to be aware of this limitation; *S.* Typhimurium Copenhagen has been responsible for outbreaks and illnesses around the world (Luceron et al., 2017; Tack et al., 2020) and can be multidrug-resistant, as demonstrated here and elsewhere (Frech et al., 2003; Tack et al., 2020).

While serotypes assigned *in silico* using SISTR and SeqSero2 are highly accurate and congruent, each tool has strengths and limitations; as such, an approach that utilizes both methods, such as the one employed here, may increase accuracy and minimize potential misclassifications. Results from other studies support this (Yachison et al., 2017; Banerji et al., 2020). For example, in an analysis of 520 primarily human-associated *Salmonella* isolate genomes, Banerji et al. (2020) found that serotypes assigned *in silico* using SeqSero showed 98% concordance with traditional serotyping and outperformed serotype assignment using seven-gene MLST. However, when SeqSero and seven-gene MLST were used in combination, *in silico* serotyping accuracy surpassed 99%, consistent with our results that a combination of SeqSero2 and cgMLST-based serotyping (as implemented in SISTR) improved *in silico* serotyping accuracy. Overall, the results provided here lend further support to the idea that *in silico* serotyping may eventually replace traditional serotyping as WGS becomes more widely used and accessible (Yachison et al., 2017; Banerji et al., 2020; Uelze et al., 2020).

Limitations of the *in silico* Antimicrobial Resistance Method Evaluation Presented Here, and Considerations for Future Antimicrobial Resistance Monitoring Efforts Among Livestock and Beyond

In addition to studying the microevolution and AMR dynamics of bovine *Salmonella* on a genomic scale, the study presented here compared results obtained from numerous *in silico* AMR characterization pipelines that attempt to replicate traditional

microbiological assays used to characterize AMR *Salmonella*. More specifically, each of the following tools was applied to the set of 128 bovine-associated *Salmonella* genomes sequenced here: (i) combinations of five *in silico* AMR determinant detection pipelines (i.e., ABRicate, AMRFinderPlus, ARIBA, BTyper, and SRST2) and one to five AMR determinant databases (i.e., ARG-ANNOT, CARD, MEGARes, NCBI, and ResFinder); and (ii) an *in silico* MIC prediction tool (i.e., PATRIC3).

Here, all AMR determinant detection pipelines and AMR determinant databases showed an extremely high degree of concordance; regardless of pipeline or database selection, all tools performed nearly identically on an SIR-prediction task relative to (i) "true" SIR classifications based on NARMS breakpoints and "true" MIC values obtained for a panel of 15 antimicrobials, and (ii) each other. A previous, small-scale (n = 111) WGS-based study of AMR *Salmonella* observed similarly high rates of concordance among several *in silico* AMR determinant detection tools (Cooper et al., 2020). However, in addition to its small sample size, this study also relied on *Salmonella* strains isolated from a single source (broiler chickens) in a single country (Canada) over an extremely short temporal range (December 2012–2013). Similarly, the study detailed here is not a formal benchmarking study, and it is essential that its numerous limitations are pointed out.

First and foremost, the study conducted here relied on WGS data from an extremely small sample of *Salmonella* isolates (n = 128) from a single source (dairy cattle and their surrounding farm environments) in a confined geographic area (New York State, United States) isolated over a short temporal range (fewer than 2 years). While all isolates were "unique" (i.e., each strain was isolated from a separate sampling event of a unique source), many isolates were highly similar at both the genomic and pan-genomic level (e.g., *S.* Cerro, *S.* Meleagridis), indicating that, in some cases, the same lineage was being sampled repeatedly over time. Consequently, this relatively miniscule sample is unrepresentative of AMR pathogens and, more specifically, *Salmonella* as a whole; readers should not infer the general superiority or inferiority of any AMR detection tool or database tested, and the results obtained here should not be extrapolated to external data sets.

Secondly, the data set queried here was heavily biased toward susceptible isolates. More than half of all isolates were pan-susceptible to the 15 antimicrobials included on the panel, and only 21 unique phenotypic SIR profiles were observed. Congruent with this, relatively little diversity was observed in terms of AMR gene profile (e.g., the AMRFinderPlus pipeline produced 20 unique AMR/stress response determinant presence/absence profiles among the 128 isolates sequenced here). This is not particularly surprising; numerous studies have shown that the resistomes of bovine-associated *Salmonella* tend to be less diverse than *Salmonella* isolated from humans (Afema et al., 2015; Carroll et al., 2017b), as well as some other animals (Mellor et al., 2019). Furthermore, the resistomes of *Salmonella* isolated from subclinical cattle, such as the isolates queried in this study, have been shown to be less diverse than the resistomes of *Salmonella* isolated from cattle showing clinical signs of disease (Afema et al., 2015).

The relative homogeneity of the subclinical *Salmonella* bovine resistome and bias toward antimicrobial-susceptible isolates have important implications for the AMR pipeline/database comparison conducted here. For this data set, stringent and conservative approaches are rewarded, as isolates that do not possess AMR determinants are more likely to be predicted to be susceptible. While it is possible that different AMR detection tools may perform better on WGS data from pathogens with more diverse resistomes, very few formal benchmarking studies of *in silico* AMR determinant detection tools currently exist (Hendriksen et al., 2019). The choice of AMR determinant database in combination with the choice of pipeline, on the other hand, can clearly affect AMR determinant identification in a critical way. For example, the ARG-ANNOT database (Gupta et al., 2014), a manually curated AMR determinant database first published in 2014, is not updated as frequently as other AMR databases (e.g., CARD, NCBI, and ResFinder). Since it was last updated in May 2018 (accessed May 25, 2020), at the time of our study, the database does not yet include three novel plasmid-mediated genes (*mcr-8*, *-9*, and *-10*) that can confer resistance to colistin, a last-resort antibiotic used to treat MDR and extensively drug resistant infections (Wang et al., 2018, 2020; Carroll et al., 2019). Similarly, versions of tools that rely on even older versions of this database would not be able to detect all members of the continuously growing repertoire of *mcr* genes. For the low-diversity subclinical bovine *Salmonella* resistomes queried here, the use of a smaller database was inconsequential, as reflected in the high congruency of all methods and databases observed here. For some studies, a manually curated database of AMR genes that is updated conservatively may possibly even be desirable, as such a database may yield less noise and improve interpretability and reproducibility. However, for pathogens with more diverse resistomes (e.g., human clinical isolates, isolates from geographic regions with different antibiotic use practices), the omission of critically important genes could be a disastrous flaw. Similar to our results, a large study (n = 6,242) querying NARMS isolates belonging primarily to the *S. enterica* species (n = 5,425) observed a high degree of concordance between NCBI's AMRFinder tool and ResFinder (this study was used to validate AMRFinder and the NCBI AMR determinant database) (Feldgarden et al., 2019). However, when differences between tools were observed, the vast majority (81%) were attributed to differences in database composition (Feldgarden et al., 2019; Hendriksen et al., 2019).

Thirdly, the small sample size (n = 128) and sparsity of AMR isolates available in this study limited the methods that could be used to formulate the AMR tool/database comparison. The approach used here was similar to the one used to validate the AMRFinder tool (Feldgarden et al., 2019) in that it relied on known AMR-determinant/AMR phenotype associations available in the literature (see Supplementary Table 4 of Feldgarden et al.) (Feldgarden et al., 2019). As such, the approach used here does not account for previously unobserved genotype/phenotype associations. Furthermore, different variants of the same AMR gene may

yield different AMR phenotypes; for example, some variants of the OXA beta-lactamases are able to confer resistance to cephalosporins, while others are not (Evans and Amyes, 2014). All AMR determinant detection pipelines tested here produced nearly identical genes calls among the 128 isolates sequenced here, and all detected AMR determinants were manually annotated in a consistent fashion; while the overall accuracy of all pipeline/database combinations tested here could likely be optimized if more accurate, data set-specific AMR genotype/phenotype associations could be derived, congruency among tools/databases would likely remain high.

Classifying bacterial pathogens into discrete SIR groups using AMR determinant detection methods is challenging, as it requires users to have a great degree of prior knowledge regarding the AMR determinants that are detected, the antimicrobials of interest, and the pathogen being studied. PATRIC3's MIC prediction tool (Nguyen et al., 2019) offers a promising departure from this framework, as it allows for the prediction of MIC values directly from WGS data. Interpreting the resulting *in silico* MIC values does not require any prior knowledge on the user end, and results can be harmoniously integrated into the SIR framework using clinical breakpoints. Among the *Salmonella* isolates sequenced here, SIR classification using PATRIC3 resulted in an overall accuracy of 93%. However, all of the limitations of this study described above for AMR determinant detection (e.g., small sample size, AMR sparsity bias, single-source, single geographic region, small temporal range) apply to *in silico* MIC prediction as well; for example, when "intermediate" resistance predictions produced via PATRIC3 are re-classified as "susceptible" (as was done for the AMR determinant detection approaches used here), PATRIC3's accuracy for this data set increases to 96.0% and is on par with all other AMR prediction methods tested here. Readers should thus interpret comparisons among these methods with caution.

Benchmarking and validating AMR detection and prediction tools is notoriously challenging (Feldgarden et al., 2019), and very few researchers have undertaken this task (Hendriksen et al., 2019). While high congruency may be observed among tools (Clausen et al., 2016), identification of a clear "optimal" method for *in silico* AMR characterization has remained elusive, as the few available benchmarking studies differ in terms of the tools tested, the AMR database(s) used, and the data set(s) chosen for benchmarking. Furthermore, the underlying WGS data can affect pipeline performance (Clausen et al., 2016; Feldgarden et al., 2019). For example, assembly quality has been shown to influence AMR determinant detection for methods that rely on assembled genomes (Clausen et al., 2016; Hendriksen et al., 2018, 2019). Thus, whether a read- or assembly-based method performs optimally can depend on a given data set (e.g., sequencing quality, the organism being studied). Another criticism of BLAST-based AMR gene detection methods among assembled genomes has been the choice of thresholds used for considering AMR determinants present or absent (Hendriksen et al., 2019). Here, no significant differences were observed between the accuracy of read-based ARIBA and SRST2 and assembly-based ABRicate, AMRFinderPlus, and BTyper. Additionally, for BLAST-based methods, a relatively wide range of optimal nucleotide identity and coverage values were found to maximize accuracy, with thresholds of 75% identity and 50–60% coverage adequate for most pipeline/database combinations. Overall, when selecting an *in silico* AMR characterization method, researchers should take into account not only practical considerations (e.g., whether reads or assembled genomes are available, the quality of reads and/or assembled genomes), but also the biology of the pathogen being studied (e.g., by querying organism-specific, AMR-conferring point mutations). To assess the robustness of *in silico* AMR predictions, researchers may additionally consider employing multiple *in silico* AMR characterization tools and/or databases in combination, as well as testing various AMR gene detection thresholds.

Finally, it is essential to note that accuracy estimates for *in silico* AMR characterization tools relative to gold-standard phenotypic methods are only as reliable as the phenotypic data they rely on. Previous studies of *Salmonella* that attempted to predict phenotypic AMR using *in silico* methods (McDermott et al., 2016; Cooper et al., 2020) have reported accuracy values between 98 and 100%. For this data set, the highest accuracy achieved was 97.4% (for SRST2/ARG-ANNOT). However, sensitivity (i.e., the ability of an *in silico* pipeline/database combination to correctly classify an isolate as phenotypically resistant to an antimicrobial) was lower for this study (71.8–84.4%) than sensitivity estimates calculated in a study of MDR *Salmonella* that included bovine isolates from New York State (97.2%) (Carroll et al., 2017b). As mentioned above, this could be due to the sparsity of AMR among isolates in this data set (i.e., predicting susceptible, rather than resistant phenotypes, is incentivized here). However, it is important to note that the AMR phenotypes of several isolates were highly incongruent with their respective AMR genotypes, regardless of the tool/database used for *in silico* AMR prediction. For example, one *S.* Cerro isolate (BOV_CERO_35_10-02-08_R8-2685) was reported to be phenotypically resistant to nine antimicrobials but did not possess any acquired AMR determinants known to produce this phenotypic AMR profile. A recent case study in which WGS and phenotypic methods were used to characterize *Salmonella* isolates from raw chicken identified numerous AMR genotype/phenotype discrepancies resulting in both false negative and false positive predictions for *in silico* methods (Zwe et al., 2020). In this case study, the authors attributed these discrepancies to heteroresistant *Salmonella* subpopulations (i.e., a subpopulation of bacteria that exhibits a range of susceptibility to a particular antimicrobial). The possibility that several heteroresistant *Salmonella* populations were characterized here cannot be discounted, as isolates underwent phenotypic AMR characterization and WGS separately (i.e., years apart). Other biological phenomena, such as plasmid loss during storage or culturing, or unknown/undetected resistance genes or mutations, could also contribute to discrepancies (Hendriksen et al., 2018). However, it is also possible that one or more incongruent isolates was mislabeled and/or mishandled during AMR phenotyping, genomic DNA extraction, and/or WGS. While removal of these isolates from the data set would increase overall prediction accuracy, the high congruency among AMR genotyping methods would be unaffected.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

LC designed and carried out all computational analyses. AB, AG, JS, KC, and RC collected, analyzed, and/or interpreted all microbiological data. LC and MW conceived the study and co-wrote the manuscript with input from all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.763669/full#supplementary-material

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Prevalence and Characteristics of Ceftriaxone-Resistant *Salmonella* in Children's Hospital in Hangzhou, China

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The non-Typhi *Salmonella* (NTS) infection is critical to children's health, and the ceftriaxone is the important empirical treatment choice. With the increase resistance rate of ceftriaxone in *Salmonella*, the molecular epidemiology and resistance mechanism of ceftriaxone-resistant *Salmonella* needs to be studied. From July 2019 to July 2020, a total of 205 NTS isolates were collected, 195 of which (95.1%) were cultured from stool, but 10 isolates were isolated from an extraintestinal site. Serogroup B accounted for the vast majority (137/205) among the isolates. Fifty-three isolates were resistant to ceftriaxone, and 50 were isolated from children younger than 4 years of age. The resistance rates for ceftriaxone, ciprofloxacin, and levofloxacin were significantly higher in younger children than the older children. The resistance genes in the ceftriaxone-susceptible isolates were detected by PCR, and ceftriaxone-resistant *Salmonella* were selected for further whole-genome sequencing. Whole-genome analysis showed that serotype Typhimurium and its monophasic variant was the most prevalent in ceftriaxone-resistant isolates (37/53), which comprised ST34 (33/53), ST19 (2/53), and ST99 (2/53), and they were close related in the phylogenetic tree. However, the other isolates were diverse, which included one Enteritidis (ST11), one Indiana (ST17), one Derby (ST40), four Kentucky (ST198), two Goldcoast (ST2529, ST358), one Muenster (ST321), one Virchow (ST359), one Rissen (ST469), one Kedougou (ST1543), two Uganda (ST684), and one Kottbus (ST8839). Moreover, CTX-M-55 ESBLs production (33/53) was found to be mainly responsible for ceftriaxone resistance, followed by $bla_{\text{CTX-M-65}}$ (12/53), $bla_{\text{CTX-M-14}}$ (4/53), $bla_{\text{CTX-M-9}}$ (2/53), $bla_{\text{CTX-M-64}}$ (1/53), $bla_{\text{CTX-M-130}}$ (1/53), and $bla_{\text{CMY-2}}$ (1/53). IS*Ecp1*, IS*903B*, IS *Kpn26*, IS*1F*, and IS*26* were connected to antimicrobial resistance genes transfer. In conclusion, the dissemination of ESBL-producing *Salmonella* isolates resulted in an increased prevalence of ceftriaxone resistance in young children. The high rate of multidrug resistance should be given additional attention.

Keywords: ceftriaxone resistance, children, China, extended-spectrum beta-lactamase, non-Typhi *Salmonella*

INTRODUCTION

Salmonella is a genus of Gram-negative bacteria of the family Enterobacteriaceae, and *Salmonella enterica subsp. enterica* is the most frequent pathogen triggering human sickness (Smith et al., 2016). *Salmonella* infection is officially called as salmonellosis, which causes stomach cramps, diarrhea, and fever. Salmonellosis is usually related to contaminated foods, including raw meat and fruits, unpasteurized milk and dairy products, and undercooked eggs (Wegener et al., 2003). Salmonellosis is a global health issue, with approximately 93 million cases of gastroenteritis and 155,000 associated deaths caused by *Salmonella* every year worldwide (Majowicz et al., 2010).

Serodiagnosis signatures were first recognized for classification in medical routine practice, and *Salmonella* spp. can be divided into *Salmonella* Typhi, *Salmonella* Paratyphi (A, B, or C), and non-Typhi *Salmonella* (NTS; Switt et al., 2009). In contrast to *Salmonella* Typhi and *Salmonella* Paratyphi that are rarely encountered outside endemic countries or in returning travelers, NTS has a high worldwide distribution. NTS infections are usually self-limiting, and antibiotics are now not indicated for uncomplicated infections. However, antibiotic therapy needs to be considered for populations at increased risk for invasive infections, such as neonates and elderly person, and antimicrobial treatment should also be modified or discontinued when a clinically plausible organism is identified (Shane et al., 2017).

Ceftriaxone is a third-generation cephalosporin with excellent activity against many Gram-negative microorganisms (Nahata and Barson, 1985) and is the empirical choice for *Salmonella* spp. treatment. With the misuse and overuse of antibiotics, as well as poor infection prevention and control, antibiotic resistance has accelerated over time, which threatens public health and clinical treatment. The most important resistance mechanism of ceftriaxone is producing AmpC β-lactamases, such as CMY and DHA, and extended spectrum β-lactamases (ESBLs), which include the TEM, SHV, CTX-M, VEB, and GES enzymes (Nahata and Barson, 1985).

In this study, we aimed to evaluate the molecular epidemiology of *Salmonella* spp. in children and to reveal the resistance mechanism and transmission pattern of ceftriaxone-resistant *Salmonella*. For the purpose, we collected the *Salmonella* spp. from Children's Hospital from July 2019 to July 2020, and antimicrobial susceptibility tests and seroagglutination tests were conducted for these isolates. After that, we analyzed the genome characteristics and phylogenetic relationship of ceftriaxone-resistant *Salmonella* spp. by whole-genome sequencing, moreover, analyzed the genetic background mediated the ceftriaxone resistance.

MATERIALS AND METHODS

Collection of Isolates and Clinical Information

In Children's Hospital of Zhejiang University School of Medicine from July 2019 to July 2020, all patients for whom clinical specimen were collected and found to host *Salmonella* were included in this study. For the routine work in the department of clinical laboratory, briefly, stool samples were inoculated onto blood agar (BIOIVD, Zhengzhou, China) and SS agar (Comagal, Shanghai, China) and cultured at 35°C for 18 h. Blood samples were cultured with a Bactec FX400 System (BD, NJ, United States), and the other types of samples were incubated on blood agar (BIOIVD, Zhengzhou, China). Then, the correct clones were picked and streaked to purity for the tests. Patients' information, including age, sex, and infection site, was obtained from the digital record system, and their names were masked. The age of children was calculated in decimal years by considering a month approximately 0.083 years, and a day approximately 0.0027 years. This study was approved by the Ethics Committee of Children's Hospital of Zhejiang University School of Medicine (2021-IRB-031).

Bacterial Isolate Identification and *Salmonella* Serotyping

All the isolates were identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry systems (Bruker Daltonics, Bremen, Germany). The serogrouping was conducted by a seroagglutination test (Tianrun, Ningbo, China), and serogroups A, B, C1, C2-C3, D1, and E1 were determined. These serogroups were also identified as O:2, O:4, O:7, O:8, O:9, and O:3, 10, respectively.

Antimicrobial Susceptibility Testing

Antimicrobial agent susceptibility was tested for all collected isolates, including against ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, levofloxacin, meropenem, and trimethoprim/sulfamethoxazole. *Escherichia coli* ATCC 25922 was used as the quality control. The agar dilution method was used following the guidelines of the Clinical & Laboratory Standards Institute (CLSI), and the breakpoint was interpreted according to the CLSI guidelines (M100 29th edition). The AST of colistin was determined by the micro broth dilution method, and a minimal inhibitory concentration (MIC) > 2 mg/L was considered resistant following European Committee on Antimicrobial Susceptibility Testing (AST; EUCAST) breakpoint interpretation.

Whole Genome Sequencing

Ceftriaxone-resistant *Salmonella* were selected for whole-genome sequencing, and genomic DNA was extracted with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The Illumina HiSeq X-Ten platform (Illumina, San Diego, CA, United States) was chosen for whole-genome sequencing with a 150 bp paired-end strategy, as previously described (Shi et al., 2020). Then, the reads were assembled by Shovill,[1] and unqualified contigs (coverage <10 or length < 200 bp) were eliminated from subsequent analysis.

[1]https://github.com/tseemann/shovill

Detection of the Multilocus Sequence Typing, Serovar, and Antimicrobial Resistance Genes

Detection of the multilocus sequence typing (MLST) and the Inc-type plasmid of the strain were screened and determined by using the MLST 2.0 server and Plasmid Finder 1.3 at the Center for Genomic Epidemiology,[2] respectively (Larsen et al., 2012; Carattoli et al., 2014), and the molecular serovar was identified by SISTR (Yoshida et al., 2016) on Pathogenwatch.[3] Monophasic *Salmonella* Typhimurium (1, 4, [5], 12: i: -) was classified as *Salmonella* Typhimurium (1, 4, [5], 12: i: 2).

For ceftriaxone-resistant isolates, antimicrobial resistance genes (ARGs) were identified with whole genome sequences (WGSs) by ABRicate with parameter identity >90% and minimum length > 60%,[4] and the National Center for Biotechnology Information (NCBI) bacterial antimicrobial resistance reference gene database was set as the reference (Feldgarden et al., 2019). The bla_{TEM}, $bla_{\text{CTX-M-1}}$ group, $bla_{\text{CTX-M-9}}$ group, and $bla_{\text{CMY-2}}$ among the ceftriaxone susceptible isolates were screened by polymerase chain reaction (PCR) and Sanger sequencing, and the primers used in this study are shown in **Supplementary Table S1**.

The Genetic Structure Surrounding of ESBLs

The contigs that contained ESBL genes were extracted and annotated by Prokka (Seemann, 2014), and particularly by ISfinder for identifying mobile genetic elements (MGEs; Siguier et al., 2006).[5] The transposons or translocatable units that were responsible for ESBL gene transfer were ascertained and their genetic structure was visualized.

Phylogenetic Relationship Analysis

All 53 ceftriaxone-resistant isolates were imported for phylogenetic relationship analysis based on their single-nucleotide polymorphisms (SNPs). The genome alignment was established by Snippy with default parameters,[6] and one internal strain, SM-1 (accession number: SAMN20422865), was used as a reference, and the SNPs numbers between each isolate were calculated by Snp-dists.[7] Fasttree (version 2.1.11) and generalized time-reversible model was used in phylogeny calculation, and the tree was illustrated and annotated by Evolview (Subramanian et al., 2019).

The Statistical Analysis

The comparison of antimicrobial resistance rates was performed by Fisher's exact, and $p < 0.05$ was considered as statistically significant.

[2]http://www.genomicepidemiology.org/
[3]https://pathogen.watch
[4]https://github.com/tseemann/abricate
[5]http://www-is.biotoul.fr
[6]https://github.com/tseemann/snippy
[7]https://github.com/tseemann/snp-dists

Nucleotide Sequence Accession Numbers

The WGS of ceftriaxone-resistant *Salmonella* spp. were deposited in the NCBI database under BioProject accession number PRJNA749852.

RESULTS

Patients and Strains

During the period of the current study, a total of nonduplicated 205 *Salmonella* isolates were obtained. The isolates from feces accounted for the vast majority (195/205), and the remaining 10 isolates were from several types of sterile body fluids. Six out of 10 were from blood samples in the gastroenterology department, respiratory department, neonatal department, and pediatric intensive care unit (PICU). One strain (SM-201) was isolated from pleural fluid and the other (SM-290) was isolated from abdominal pus (general surgery department). There were two isolates from subperiosteal pus (SM-103 and SM-301, orthopedics department). A total of 86.8% (178/205) of *Salmonella* isolates were from children less than 4 years of age in this study, and the median age of these children was 1.25 (IQR: 0.79 to 2.29), suggesting that younger children were the most potentially susceptible population.

Antimicrobial Susceptibility and Serotyping of *Salmonella* spp.

The seroagglutination test and AST were performed to explore the distribution of *Salmonella* serogroups and antimicrobial susceptibility in a tertiary children's hospital (**Table 1**). Fifty-three ceftriaxone-resistant isolates were found in these collected *Salmonella* isolates and resistance rate reached 25.9%, and the other antimicrobial resistance rates for chloramphenicol, ciprofloxacin, levofloxacin, ampicillin, and trimethoprim/sulfamethoxazole were 44.4% (91/205), 23.4% (48/205), 11.7% (24/205), 75.6% (155/205), and 37.1% (76/205), respectively. The multidrug resistance (MDR) rate in the current study was 37.6% (77/205), and there was no meropenem-resistant *Salmonella*. Interestingly, the resistance rates for ceftriaxone, ciprofloxacin, and levofloxacin were significantly higher in younger children (age < 1) (**Supplementary Figure S1**), and 94.3% (50/53) of ceftriaxone-resistant *Salmonella* isolates were distributed in children less than 4 years of age. Furthermore, the resistance rates for ciprofloxacin and levofloxacin in the ceftriaxone-resistant group were significantly higher than those in the ceftriaxone-susceptible group ($p = 0.0006$ and < 0.0001, respectively). For instance, the ciprofloxacin resistance rate was 41.5% (22/53) in the ceftriaxone-resistant group, compared with 17.1% (26/152) in the ceftriaxone-susceptible group; and the difference resistance rates were 34.0% (18/53, ceftriaxone resistant) vs. 3.9% (6/152, ceftriaxone susceptible) for levofloxacin.

Serogroup determination by seroagglutination testing showed that serogroup B was the most prevalent type (137/205) among these *Salmonella* isolates, followed by serogroup D1 (21/205), serogroup C1 (19/205), serogroup E1 (12/205), and serogroup C2-C3 (10/205). There were six isolates that could not

TABLE 1 | The clinical information, serogroup and antibiotic resistance rate of 205 *Salmonella* isolates.

	Age (<1)	Age (1–4)	Age (≥4)	Total
Isolates number	81	97	27	205
Age (medium, IQR)	0.75 (0.46, 0.9)	1.60 (1.25, 2.29)	5.58 (4.91, 8)	1.25 (0.79, 2.29)
Gender (male)	50.6 (41/81)	55.7 (54/97)	59.3 (16/27)	53.9 (111/205)
Resistance rate				
Ceftriaxone	33.3 (27/81)	23.7 (23/97)	11.1 (3/27)	25.9 (53/205)
Chloramphenicol	50.6 (41/81)	43.3 (41/97)	33.3 (9/27)	44.4 (91/205)
Ciprofloxacin	33.3 (27/81)	17.5 (17/97)	14.8 (4/27)	23.4 (48/205)
Levofloxacin	21.0 (17/81)	5.2 (5/97)	7.4 (2/27)	11.7 (24/205)
Meropenem	0 (0/81)	0 (0/97)	0 (0/27)	0 (0/205)
Ampicillin	77.8 (63/81)	71.1 (69/97)	85.2 (23/27)	75.6 (155/205)
SMZ	43.2 (35/81)	34.0 (33/97)	29.6 (8/27)	37.1 (76/205)
Serogroup				
B	64.2 (52/81)	76.3 (74/97)	40.7 (11/27)	66.8 (137/205)
C1	6.2 (5/81)	10.3 (10/97)	14.8 (4/27)	9.3 (19/205)
C2-C3	9.9 (8/81)	1.0 (1/97)	3.7 (1/27)	4.9 (10/205)
D	4.9 (4/81)	8.2 (8/97)	33.3 (9/27)	10.2 (21/205)
E1	8.6 (7/81)	3.1 (3/97)	7.4 (2/27)	5.9 (12/205)
Unindentified	6.2 (5/81)	1.0 (1/97)	0 (0/27)	2.9 (6/205)

be classified into a specific serogroup by the seroagglutination testing. One of these isolates, SM-233, was resistant to ceftriaxone, and further whole genome sequencing showed that SM-233 belonged to serovar Kedougou, which is serogroup G *Salmonella* (Rodriguez-Urrego et al., 2010). Serogroup B *Salmonella* isolates also showed the most prevalent ceftriaxone resistance.

The Molecular Characteristics of Ceftriaxone-Resistant *Salmonella* Isolates

Whole-genome sequencing data of ceftriaxone-resistant *Salmonella* isolates were utilized to further clarify their molecular characteristics and phylogenetic relationship. All the ceftriaxone-resistant *Salmonella* isolates were from stools except one from blood. The MLST results showed that ST34 was the predominant sequence type (33/53, 62.3%), and the others were diverse, including one ST11, one ST17, two ST19, one ST40, two ST99, four ST198, one ST2529, one ST321, one ST358, one ST359, one ST469, one ST1543, and two ST684. Furthermore, one novel ST (ST8839) was found with the MLST profile (*aroC* 844, *dnaN* 71, *hemD* 43, *hisD* 12, *purE* 190, *sucA* 20, *thrA* 18), and it was nearest to ST808, with the point mutation C102T in *aroC*.

Among these isolates, serovar Typhimurium and its monophasic variant were predominant, and 37 serovar Typhimurium and its monophasic variant isolates corresponded to more than one sequence type, which included ST19, ST34 and ST99. ST358 and ST2529 belonged to serovar Goldcoast. In the other serovars, there was a one-to-one relationship between the serovar and the MLST, for instance, serovar Kentucky corresponded to ST198, and serovar Uganda corresponded to ST684.

The phylogenetic tree showed that serovars, such as Enteritidis, Muenster, Rissen, Virchow, Indiana, Kedougou, and Kottbus, were differently positioned on the phylogenetic tree and unrelated (**Figure 1**). The results showed that the phylogenetic relationship among the same ST isolates was indistinguishable, such as among the ST684 serovar Uganda or among the ST198 serovar Kentucky. The mean number of SNPs between each pair of ST34 isolates was 52.5 ± 22.7, indicating that the relationships within the predominant ST strains were relatively close. There were 23 indistinguishable pairs of isolates in ST34 that has less than 5 SNPs. SM-90 (ST358) isolated from stool and SM-18 (ST2529) isolated from blood were nearly unrelated, although both of them belonged to the serovar Goldcoast.

Resistance Mechanisms of Ceftriaxone-Resistant *Salmonella* Isolates

To understand the ceftriaxone resistance mechanisms of these *Salmonella* spp. isolates, the antimicrobial resistance genes were analyzed. Almost all ceftriaxone resistant isolates were CTX-M-type ESBL producers, except one producer of CMY-2 β-lactamase, which is an AmpC cephalosporinase. In contrast, no CTX-M ESBLs or AmpC cephalosporinase genes were detected among the ceftriaxone-susceptible isolates, suggesting that those enzymes were possibly responsible for ceftriaxone resistance. Among the CTX-M-producing isolates, $bla_{\text{CTX-M-55}}$ (33/53) was the most prevalent, followed by $bla_{\text{CTX-M-65}}$ (12/53), $bla_{\text{CTX-M-14}}$ (4/53), $bla_{\text{CTX-M-9}}$ (2/53), $bla_{\text{CTX-M-64}}$ (1/53), and $bla_{\text{CTX-M-130}}$ (1/53). In particular, SM-55 harbored two different types of ESBLs, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-55}}$. TEM β-lactamases were mainly distributed (40/53) in ceftriaxone resistant isolates; however, the $bla_{\text{TEM-1}}$ gene was still detected in 62.5% (95/152) of ceftriaxone susceptible isolates, showing its limited effect on ceftriaxone resistance. Among the ceftriaxone-resistant isolates, 38 isolates harbored $bla_{\text{TEM-1}}$, and two isolates (SM-20 and SM-22) had $bla_{\text{TEM-215}}$, which were both ST684. Four isolates were $bla_{\text{OXA-1}}$ positive; eight isolates were $bla_{\text{OXA-10}}$ positive, and there were three isolates contained $bla_{\text{LAP-2}}$ (**Figure 2**).

Genetic environment analyses showed that the IS*Ecp1*-ESBL-IS*903B* structure was the most prevalent ESBL gene transfer. The CTX-M-1 group included CTX-M-55 and CTX-M-64, and the former had three different environments: IS*Ecp1*-$bla_{\text{CTX-M-55}}$-IS*903B*, IS*Ecp1*-$bla_{\text{CTX-M-55}}$-$bla_{\text{TEM-1}}$-IS*903B*, and IS*1F*-$bla_{\text{CTX-M-55}}$. Similarly, the gene structure of the latter was IS*Kpn26*-IS*Ecp1*-$bla_{\text{CTX-M-64}}$-IS*26*. The CTX-M-9 group contained CTX-M-9, CTX-M-14, CTX-M-65, and CTX-M-130. The genetic structures of $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-65}}$, and $bla_{\text{CTX-M-130}}$ were similar, which were mediated by IS*Ecp1* and IS*903B*, but $bla_{\text{CTX-M-9}}$ was located on the chromosome, which was still mediated by IS*Ecp1*. The gene upstream of CMY-2 was IS*Ecp1*, which was located on the IncI1-I (Alpha) type plasmid (**Figure 2**).

The Prevalence of *mcr*-1 in Ceftriaxone Resistant *Salmonella*

Interestingly, SM-66 and SM-212 both harbored the *mcr*-1 gene, which mediates resistance against colistin, the last-resort therapy choice for multidrug-resistant Gram-negative bacterial

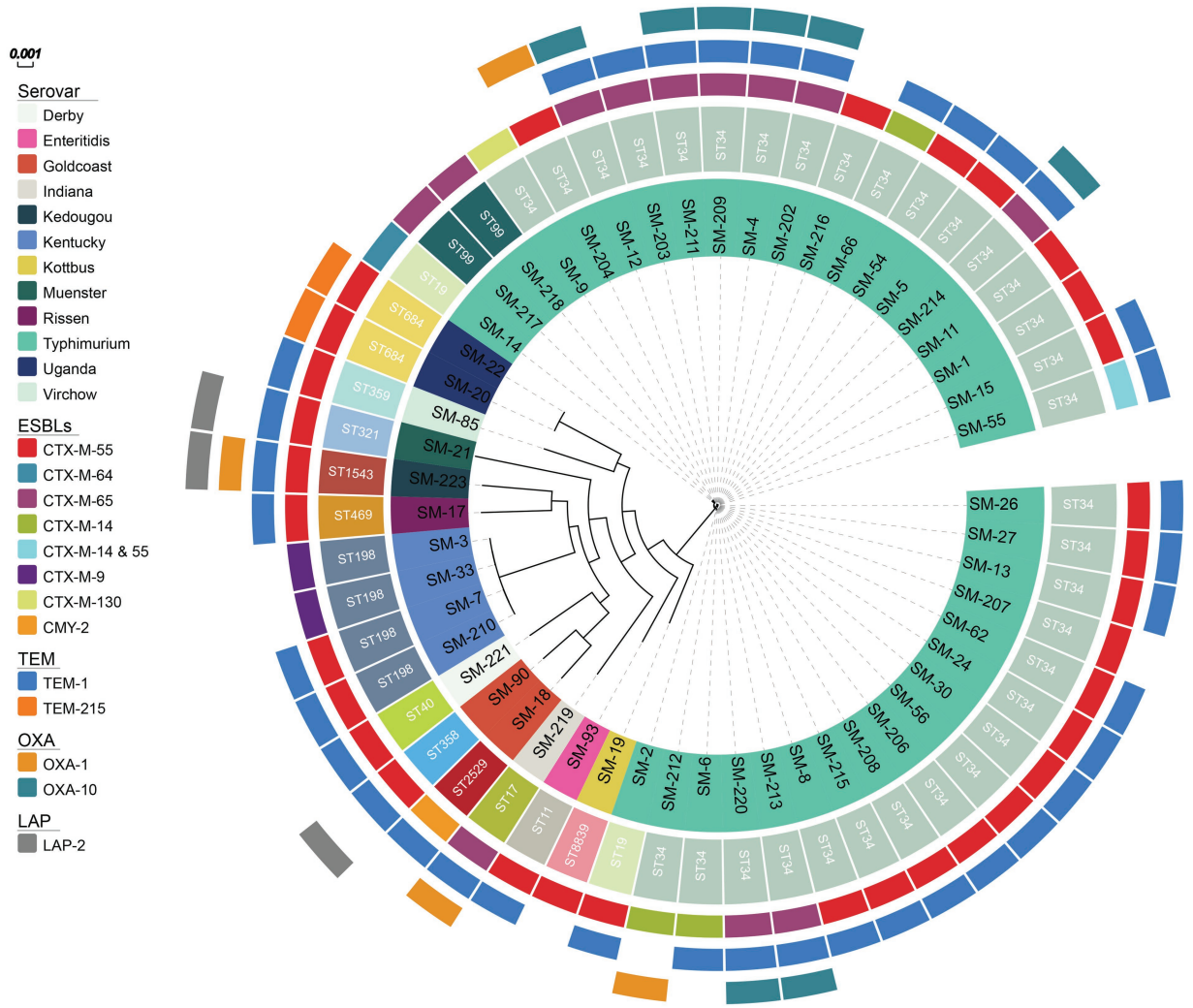

FIGURE 1 | The phylogenetic tree of 53 ceftriaxone-resistant *Salmonella* from the inside to the outside, each circle represents the ST type, serovar, ESBL genes, bla_{TEM}, bla_{OXA}, bla_{LAP}, respectively.

infection. The colistin MIC of isolate SM-212 (accession number: SAMN20422907) reached 8 mg/L; however, the colistin MIC of isolate SM-66 (accession number: SAMN20422894) was just 0.5 mg/L, remaining susceptible. WGS analysis showed that the deletion mutation (GTGGCGAGTGTTG) of *mcr*-1 in SM-66 was observed between nucleic acid positions 55 and 67. The plasmid type of these two *mcr*-1-harboring isolates was IncHI2, which is common plasmid groups carrying multidrug resistance determinants.

DISCUSSION

Gastroenteritis induced by NTS is usually self-limited, and antibiotics are usually not indicated for uncomplicated infection, as expected for children and immunocompromised patients (Pragasam et al., 2019). In the current study, in addition to gastrointestinal *Salmonella* infection, we found other invasive NTS (iNTS) infections, such as bloodstream infections, abdominal infection, and osteomyelitis, and even ceftriaxone-resistant *Salmonella* bloodstream infection, which caused an increased burden on clinical treatment. Chen's report indicated that iNTS would cause prolonged hospitalization, increased medical costs, and elevated mortality (Chen et al., 2012).

The recommendations for the treatment of acute gastroenteritis due to NTS vary (Haeusler and Curtis, 2013), and the empirical choice of treatment is usually ceftriaxone or ciprofloxacin, but ciprofloxacin and other fluoroquinolones have been restricted in children (Stahlmann and Lode, 2013). Because of the clear side effects, which make ceftriaxone very important in NTS infection treatment for children. However, ceftriaxone-resistant *Salmonella* incidence has been increasing in recent year, and research has shown that the resistance rates to ceftriaxone in 2019 were significantly higher than those in 2012 (14.3% vs. 4.1%, $p < 0.001$) (Chang et al., 2021), which presented increased

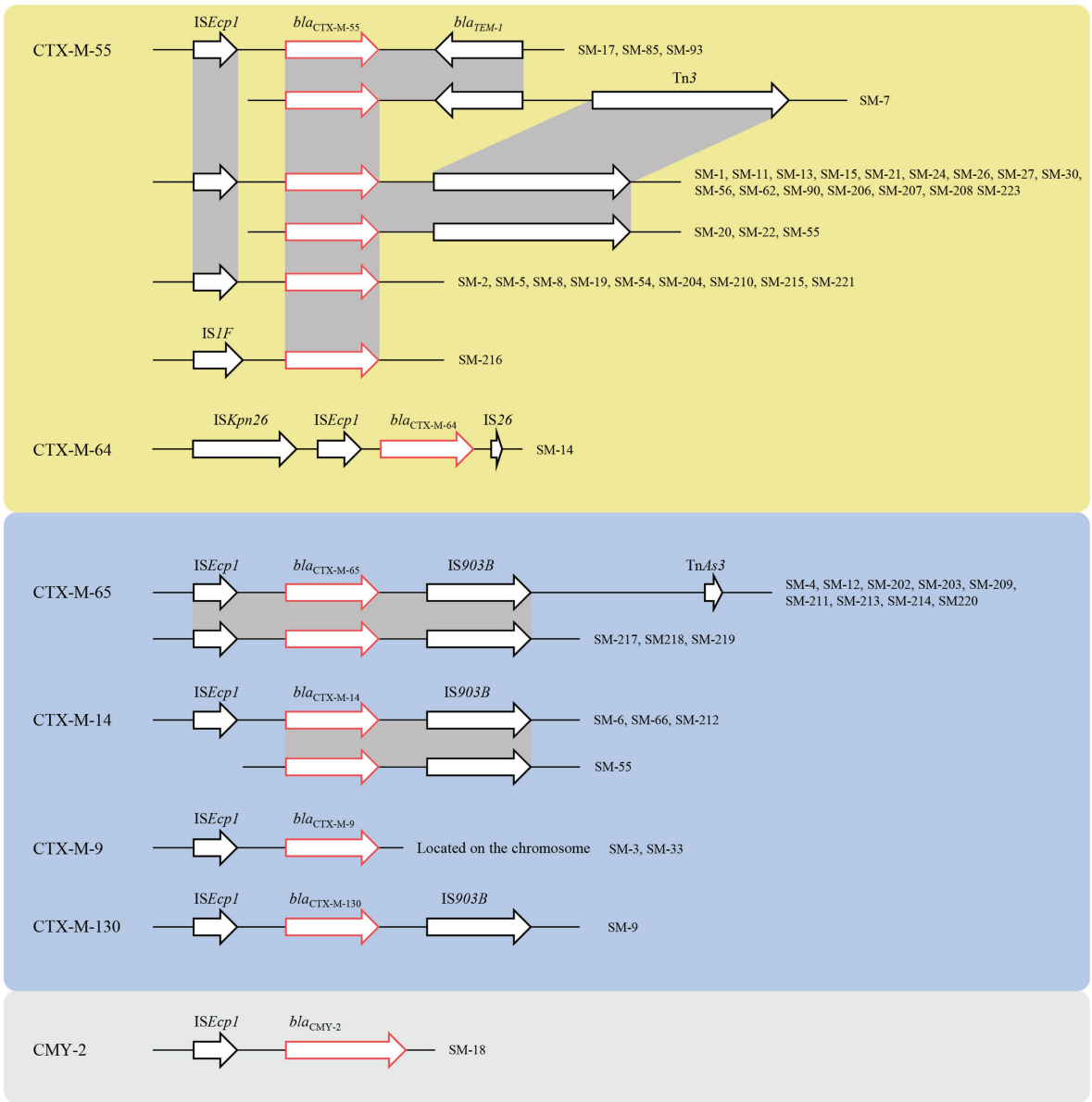

FIGURE 2 | The genetic environment of ESBL, the yellow region represented for CTX-M-1 group, the blue region represented for the CTX-M-9 group, and the grey region represented CMY-2. The red arrows represented for the resistance genes, and the black arrows represented for the mobile genetic elements (MGEs).

challenges to clinical treatment. The results of Chinet, an annual national surveillance in China, showed that the rate of resistance to ceftriaxone in 453 *Salmonella enterica* isolates was only 5.7% in 2020, and the rate of resistance to ceftriaxone in 498 *Salmonella* Typhimurium isolates was 25.2%.[8] In this study, the ceftriaxone resistance incidence was 25.9%. Previous research indicated that NTS isolates from children aged <5 years showed a higher prevalence of antimicrobial resistance than those from patients >5 years, with a similar elevated resistance rate to cephalosporins and quinolones (Liang et al., 2015), which is consistent with our results.

Ceftriaxone and ciprofloxacin had a tendency of co-resistance in this study; 40.7% (22/54) of ceftriaxone-resistant isolates also showed resistance to ciprofloxacin, and the percentage was higher than that in the ceftriaxone-susceptible group. The antimicrobial resistance genes ciprofloxacin and ceftriaxone are usually carried by plasmids, which facilitates the coexistence of these genes on the same plasmids. The results showed that strain SM-15 contained the $bla_{CTX-M-55}$ and *qnrS1* on the same contig, which indicated that these genes were located on the same plasmid. Paterson indicated that ESBL gene harboring plasmids likely carry genes for resistance to many other antimicrobial agents, such as aminoglycosides, trimethoprim, sulfonamides, tetracyclines, and chloramphenicol, which may be the reason

[8]http://www.chinets.com/

for MDR (Paterson, 2000). In these ceftriaxone-resistant *Salmonella* isolates, we found two *mcr*-1-positive isolates; interestingly, one of them was colistin susceptible, with a truncated *mcr*-1 allele. *mcr*-1-positive *Salmonella* isolates have been previously reported in Brazil (Rau et al., 2020), Spain (Trujillo-Soto et al., 2019), South Korea (Moon et al., 2021), and China (Lu et al., 2019), and the *mcr*-1 gene was found to be normally located on IncI2, IncHI2, and IncX4 plasmid types, respectively.

The serogroup of *Salmonella* is diverse worldwide. The majority serogroup of *Salmonella* in South India was serogroup B followed by E and C1/C2 (Pragasam et al., 2019), and the most prevalent ceftriaxone-resistant *Salmonella* serotype was serovar Heidelberg, followed by Newport in the United States (Medalla et al., 2016). In this study, we found that *Salmonella* Typhimurium represented the overwhelming majority of ceftriaxone-resistant *Salmonella*.

According to previous research, CMY-2 AmpC β-lactamase was the most important ceftriaxone resistance gene in *Salmonella* spp., followed by $bla_{\text{CTX-M-3}}$ (Su et al., 2005). However, in our study, only one isolate contained CMY-2, and no isolates produced CTX-M-3. $bla_{\text{CTX-M-55}}$ was the most common ESBL in this study, similar to a previous study in Shanghai (Li et al., 2021). The genetic structure of the ESBL gene was identical to that in a previous study. CTX-M and CMY-2 were in both the IS*Ecp1* and IS*903B* elements (He et al., 2021; Nguyen et al., 2021), and one CTX-M-64 was located in the IS*Kpn26*-IS*Ecp1*-$bla_{\text{CTX-M-64}}$-IS*26* structure. In this study, $bla_{\text{CTX-M-9}}$ was located on the chromosome, which was mediated by IS*Ecp1*, and became fixed. Maria et al. reported that the $bla_{\text{CTX-M-55/57}}$ gene can transfer to *E. coli*, while repeated attempts to transfer plasmids harboring the $bla_{\text{CTX-M-5}}$ and $bla_{\text{CTX-M-15}}$ genes failed (Sjolund-Karlsson et al., 2011), which makes the prevalence of $bla_{\text{CTX-M-55}}$ in our study explicable. Because ESBL genes are often carried by large plasmids (Rawat and Nair, 2010), we speculated that the plasmids carrying ESBL genes were transformed from other bacteria in the human intestinal tract, and further studies should be conducted to determine the origin and relationship of these resistance plasmids.

CONCLUSION

In this study, we investigated the epidemiology of *Salmonella* in the children's hospital in Hangzhou, China, which could supplement important local epidemiological data. Younger children were the potentially susceptible population for *Salmonella* infections, and ST34 *Salmonella* Typhimurium dominated the ceftriaxone-resistant strains. The major resistance mechanism of ceftriaxone-resistant *Salmonella* was producing CTX-M-type ESBLs, in which $bla_{\text{CTX-M-55}}$ was the most prevalent. The dissemination of these ESBLs was mediated by mobile elements, including IS*Ecp1* and IS*903B*. In the current study, we found the co-transfer tendency of cephalosporin- and quinolone-resistance, and the increased prevalence of ceftriaxone resistance and the high-rate multidrug resistance should be concerned.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA749852.

AUTHOR CONTRIBUTIONS

YJ and YY participated in the design of the study. QS, YY, and MZ collected the isolates and clinical information. PL and YY performed the antimicrobial susceptible testing and serotyping. QS and XH prepared for whole-genome sequencing and PCR. JQ and XH performed the *in silico* analysis. QS drafted the manuscript. YJ reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.764787/full#supplementary-material

Supplementary Table S1 | The primers used in this study.

Supplementary Figure S1 | The resistance rates for ceftriaxone, chloramphenicol, ciprofloxacin, levofloxacin, ampicillin, and SMZ in different age of children, $p<0.05$ was labelled.

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Extensive Drug-Resistant *Salmonella enterica* Isolated From Poultry and Humans: Prevalence and Molecular Determinants Behind the Co-resistance to Ciprofloxacin and Tigecycline

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The emergence of extensive drug-resistant (XDR) *Salmonella* in livestock animals especially in poultry represents a serious public health and therapeutic challenge. Despite the wealth of information available on *Salmonella* resistance to various antimicrobials, there have been limited data on the genetic determinants of XDR *Salmonella* exhibiting co-resistance to ciprofloxacin (CIP) and tigecycline (TIG). This study aimed to determine the prevalence and serotype diversity of XDR *Salmonella* in poultry flocks and contact workers and to elucidate the genetic determinants involved in the co-resistance to CIP and TIG. Herein, 115 *Salmonella enterica* isolates of 35 serotypes were identified from sampled poultry (100/1210, 8.26%) and humans (15/375, 4.00%), with the most frequent serotype being *Salmonella* Typhimurium (26.96%). Twenty-nine (25.22%) *Salmonella enterica* isolates exhibited XDR patterns; 25 out of them (86.21%) showed CIP/TIG co-resistance. Exposure of CIP- and TIG-resistant isolates to the carbonyl cyanide 3-chlorophenylhydrazone (CCCP) efflux pump inhibitor resulted in an obvious reduction in their minimum inhibitory concentrations (MICs) values and restored the susceptibility to CIP and TIG in 17.24% (5/29) and 92% (23/25) of the isolates, respectively. Molecular analysis revealed that 89.66% of the isolates contained two to six plasmid-mediated quinolone resistance genes with the predominance of *qepA* gene (89.66%). Mutations in the *gyrA* gene were detected at codon S83 (34.62%) or D87 (30.77%) or both (34.62%) in 89.66% of XDR *Salmonella*. The *tet*(A) and *tet*(X4) genes were detected in 100% and 3.45% of the XDR isolates, respectively. Twelve TIG-resistant XDR *Salmonella* had point mutations at codons 120, 121, and 181 in the *tet(A)* interdomain loop region. All CIP and TIG co-resistant XDR *Salmonella* overexpressed

ramA gene; 17 (68%) out of them harbored 4-bp deletion in the *ramR* binding region (T-288/A-285). However, four CIP/TIG co-resistant isolates overexpressed the *oqxB* gene. In conclusion, the emergence of XDR *S. enterica* exhibiting CIP/TIG co-resistance in poultry and humans with no previous exposure to TIG warrants an urgent need to reduce the unnecessary antimicrobial use in poultry farms in Egypt.

Keywords: XDR, *Salmonella enterica*, AcrAB efflux pump, ciprofloxacin, tigecycline

INTRODUCTION

Salmonella enterica subspecies *enterica* serovars are the leading cause of food-borne zoonoses worldwide, accounting for 93.8 million cases of gastroenteritis and 155000 deaths annually (Majowicz et al., 2010). *S. enterica* is common in chickens, turkeys, quails, pheasants, and other game birds and has been isolated at high percentages from commercially reared poultry. Hence, multiple *S. enterica* serovars originating from poultry have been considered the potential source of human salmonellosis through the consumption of contaminated bird meat (Hoelzer et al., 2011; Thomas et al., 2017; Anbazhagan et al., 2019). Furthermore, farms represent a direct hazard to public health (Hoelzer et al., 2011), while indirect infections occur through the contaminated food products from carrier birds harboring *Salmonella* in the vicinity of food production units (Thomas et al., 2017). The spread of multidrug-resistant (MDR, i.e., resistant to at least one agent in three or more antimicrobial categories) *Salmonella* usually stems from the unjustified use of antimicrobials particularly in the poultry industry. Thus, the potential risk to public health posed by transmission of MDR non-typhoidal *Salmonella* (NTS) from poultry (Anbazhagan et al., 2019) warrants the need for an integrative "One Health" approach for NTS surveillance among human, poultry, and animal populations. More alarming than the MDR is the recent emergence of the extensive drug-resistant [XDR, i.e., resistant to one or more agents in all antimicrobial categories except two or fewer from the worksheet for defining and categorizing the isolates according to Magiorakos et al. (2012)] *Salmonella* such as the previously reported *Salmonella* Indiana (Wang et al., 2017) and *Salmonella* Typhi (Klemm et al., 2018; Saeed et al., 2019). There have been scarce data on the prevalence of such threatening XDR isolates in humans and poultry. It is worthy to note that the available studies on the mechanisms of antimicrobial resistance in *S. enterica* and in particular the XDR isolates have focused on the resistance to a single antibiotic (Chen et al., 2017; Zhang et al., 2017) without deciphering the molecular rationale behind the emergence of cross-antibiotic resistance, including to unrelated drugs.

Fluoroquinolone (e.g., ciprofloxacin, CIP) resistance in *Salmonella* species is mainly attributed to chromosomal mutations or the existence of plasmid-mediated quinolone resistance (PMQR) determinants or both (Robicsek et al., 2006). The chromosome-mediated quinolone resistance is commonly arbitrated by two main mechanisms: (1) spontaneous point mutations in the quinolone-resistant determining regions (QRDRs) of topoisomerase subunits, mainly *gyrA* (Hopkins et al., 2005; Aldred et al., 2014; Wasyl et al., 2014) and (2) overexpression of the major efflux pump (AcrAB-TolC) in *Salmonella* efflux systems, which usually leads to reduced cellular drug accumulation (Zhang et al., 2017).

The expression of AcrAB efflux pump is known to be regulated by global regulators (Usui et al., 2013), including the *ramR* gene. *RamR* mutations could increase the expression of *ramA* and *acrAB* genes resulting in the appearance of efflux-mediated MDR phenotype (Abouzeed et al., 2008), including CIP resistance. Because the resistance of *Salmonella* to CIP is often associated with cross-resistance to other antimicrobials (e.g., tetracyclines, beta-lactams, and chloramphenicol) (Hopkins et al., 2005), seeking novel antimicrobials that could inactivate XDR *Salmonella* is becoming a top priority. In this regard, glycylcycline (e.g., tigecycline, TIG) has come out as a promising, novel, and last-resort broad-spectrum agent for managing XDR *Salmonella* infections. However, decreased susceptibility of *S. enterica* to TIG has been observed (Chen et al., 2017). It was hypothesized that TIG resistance may be attributed to three main tetracycline resistance mechanisms (Chopra and Roberts, 2001): (i) mutations within tetracycline-specific efflux proteins, mainly *tet*(A), (ii) mutations in ribosomal protection proteins, *tet*(M), and (iii) enzymatic inactivation, *tet*(X). Additional resistance mechanisms as AcrAB-TolC and OqxAB efflux pumps overexpression may be also involved in such resistance (Tuckman et al., 2000; Hentschke et al., 2010; Chen et al., 2017).

The available information on the resistance mechanisms of *Salmonella* to CIP and TIG suggests a complex mechanistic view that needs to be delineated.

This study was designed to determine the prevalence, serotype diversity, and antimicrobial resistance phenotypes of *S. enterica* isolated from poultry and contact workers. Moreover, we combined molecular approaches with data analyses to investigate the genetic determinants involved in the co-resistance of XDR *Salmonella* isolates to CIP and TIG. Further, we precisely pinpointed the mutational alterations in *ramRA* regulatory gene that lie behind the overexpression of AcrAB-TolC efflux pump, which could confer CIP and TIG co-resistance.

MATERIALS AND METHODS

Sampling

This study was conducted during the period from January 2019 to April 2020. A total of 1210 clinically diseased and recently dead 1-day to 4-week-old poultry including broiler chickens (n = 650), ducks (n = 150), pigeons (n = 160), quails (n = 130), and turkeys (n = 120) from 25 poultry flocks located in five

governorates in Egypt were sampled aseptically. Diseased birds were diarrheic, depressed, and weak with ruffled feathers. Flight inability was observed on pigeons and quails. Gross lesions including enteritis, enlarged liver with necrotic foci, perihepatitis, pericarditis, and unabsorbed yolk sac were observed in recently dead birds. The samples included fresh fecal dropping and cloacal swabs from diarrheic birds as well as liver, spleen, cecum, gall bladder, and yolk sac from recently dead birds. Fluoroquinolones, sulfonamides, penicillins, aminoglycosides, and tetracyclines were administered to the entire flocks for treatment. For prophylaxis, ESB3 (sulfaclozine 30%), lincomycin, oxytetracycline, and tylan premix (tylosin) were frequently used in poultry farms. Moreover, 375 human stool samples were collected from diarrheic poultry workers who were employed in the same flocks from which the poultry samples were obtained. The samples were collected in sterile separate containers and transported immediately in an icebox to the Microbiology Laboratory, Faculty of Veterinary Medicine, Zagazig University, for bacteriological analysis.

Ethics Approval and Consent to Participate

The study was approved by Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC) (approval number ZU-IACUC/2/F/12/2019). Written informed consent was obtained from the owners for the participation of their animals in this study. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable data included in this article.

Isolation and Identification of *Salmonella enterica*

Isolation of *S. enterica* was carried out according to ISO (ISO, 2017). Briefly, 1 g of each sample was suspended in 9 ml of buffered peptone water (BPW, Oxoid, United Kingdom) and incubated at 37°C for 18 ± 2 h. An aliquot of 0.1 ml of the pre-enrichment culture was inoculated into 10 ml of Rappaport Vassiliadis soy broth (RV; Oxoid, United Kingdom) then incubated at 42°C for 24 h. Selective plating was done on xylose lysine deoxycholate agar (XLD, Oxoid, United Kingdom) and salmonella shigella agar (SS; Oxoid, United Kingdom) followed by incubation at 37°C for 24 h. For biotyping, fresh colonies from each pure culture were examined for oxidase, methyl red, Voges–Proskauer, and citrate utilization tests as well as their characteristic reactions on triple sugar iron and lysine decarboxylase agar (Oxoid, United Kingdom) media. Serotyping of *Salmonella* isolates was conducted using commercially available antisera (Denka Seiken Co., Ltd., United Kingdom) according to the antigenic profile (Kauffmann, 1957). Polymerase chain reaction (PCR) of the *invA* gene was performed to confirm *Salmonella* identification (Oliveira et al., 2003). The reference strain *S. enterica* serovar Typhimurium ATCC® 14028™ was used for quality control.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibilities of all *Salmonella* isolates were evaluated against 24 commercially available antimicrobial agents (Oxoid, Hampshire, England, United Kingdom) using the disk diffusion method (Bauer et al., 1966). The tested antimicrobials were ampicillin (10 μg), amoxycillin-clavulanic acid (20/10 μg), ampicillin-sulbactam (20/10 μg), cefazolin (30 μg), cefuroxime (30 μg), ceftriaxone (30 μg), cefepime (30 μg), cefoxitin (30 μg), ertapenem (10 μg), imipenem (10 μg), meropenem (10 μg), doripenem (10 μg), gentamicin (10 μg), tobramycin (10 μg), amikacin (30 μg), nalidixic acid (30 μg), CIP (5 μg), tetracycline (30 μg), TIG (15 μg), fosfomycin (50 μg), chloramphenicol (30 μg), sulfamethoxazole-trimethoprim (23.75/1.25 μg), aztreonam (30 μg), and colistin (10 μg). The inhibition zone diameters were measured and interpreted according to Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (European Committee Antimicrobial Susceptibility Testing (EUCAST), 2018; Clinical Laboratory Standards Institute (CLSI), 2019). The multiple antibiotic resistance (MAR) indices were calculated as described elsewhere (Tambekar et al., 2006). The MDR and XDR *Salmonella* isolates were categorized according to Magiorakos et al. (2012). The analyzed antimicrobials were synchronized with veterinary guidelines (Plumb, 2015). Justification of the selected antimicrobial agents was for monitoring the XDR *Salmonella* isolates (Magiorakos et al., 2012) and with the end goal of public health concern, e.g., TIG, fluoroquinolones, carbapenems, aminoglycosides, and cephalosporins.

To determine the minimum inhibitory concentrations (MIC) of CIP against XDR *Salmonella* isolates, VITEK® 2 (bioMérieux, Marcy L'Étoile, France) testing was performed using AST-GN91 cards (SKU Number: 414780) according to the manufacturer's instructions. Interpretive correlation of the VITEK® 2 MIC results was applied using the Advanced Expert System (AES™) rules. Moreover, the MICs of TIG and colistin were determined using broth microdilution method, and the interpretive criteria were those reported in the above mentioned CLSI and EUCAST documents. The MIC breakpoints for CIP (≥1 mg/L) and TIG (>2 mg/L) were considered accordingly.

Phenotypic Detection of the Efflux Pump Activity

The efflux pump activity of XDR *Salmonella* isolates was determined using the ethidium bromide (EtBr) cartwheel method as described previously (Martins et al., 2011). In brief, trypticase soy agar (TSA, Oxoid, United Kingdom) plates containing 0.0 to 2.5 mg/L of EtBr (Sigma-Aldrich, Germany) concentrations were prepared on the same day of the experiment and kept away from light. Each XDR *Salmonella* isolate (approximately 10^8 CFU/ml) was streaked on an EtBr plate in a cartwheel pattern. The plates were wrapped in aluminum foil and incubated at 37°C overnight. The minimum concentration of EtBr that produced fluorescence of bacterial colonies under Accuris™ E3000 UV Transilluminator (Accuris Instruments, United States)

was recorded. A pan-susceptible *Salmonella* Tamale isolate generated during this study was used as a comparative control for fluorescence analysis. The capacity of each XDR *Salmonella* isolate to expel EtBr substrate was graded relative to the control isolate according to the following equation:

$$\text{Efflux activity index} = \frac{\text{MC}_{\text{EtBr}}\ (\text{XDR}) - \text{MC}_{\text{EtBr}}\ (\text{REF})}{\text{MC}_{\text{EtBr}}\ (\text{REF})}$$

where MC_{EtBr} (XDR) represents the minimum EtBr concentration that produces fluorescence of the XDR test isolate. Meanwhile, MC_{EtBr} (REF) indicates the minimum EtBr concentration that produces fluorescence of the reference isolate.

The MICs of CIP and TIG were determined by broth microdilution method in the presence of the carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 5 μg/ml; Sigma-Aldrich, United Kingdom) efflux pump inhibitor (EPI), which is known to dissipate the proton-motive force essential for the activity of resistance-nodulation division (RND) family efflux pumps. A significant efflux inhibition activity was considered when a ≥ 4-fold decrease in the MIC values was reported in the presence of EPI (Rushdy et al., 2013; Deng et al., 2014).

Detection of Plasmid-Mediated Quinolone Resistance Genes

Plasmid DNA was extracted from XDR *Salmonella* isolates using QIAprep Spin Miniprep Kits (Qiagen, Germany) following the manufacturer's recommendations. PMQR genes, including *qnrA*, *qnrB*, and *qnrS* were amplified through a multiplex PCR assay, while the *qepA*, *aac(6′)-Ib-cr*, *oqxA*, and *oqxB* genes were amplified by uniplex PCRs using previously published oligonucleotide primers (**Supplementary Table 1**) and cycling conditions (Robicsek et al., 2006; Cattoir et al., 2008; Kim et al., 2009; Lunn et al., 2010). Positive (*S. enterica* serovar Typhimurium ATCC® 14028™) and negative (a reaction mixture without DNA template) controls were included in each run.

Detection of Tetracycline Resistance Determinants

The plasmid-encoded *tet*(X1 to X5) genes conferring TIG resistance were amplified using multiplex PCR (Ji et al., 2020). The presence of *tet*(A), *tet*(B), and *tet*(M) genes were also examined (Van et al., 2008) using primer sequences depicted in **Supplementary Table 1**.

Detection of Mutations in *gyrA*, *tet*(A), and *ramRA* Genes

The QRDR of *gyrA*, *tet*(A), and *ramRA* genes were amplified using PCR followed by DNA sequencing using oligonucleotide primers listed in **Supplementary Table 1**. Genomic DNA was extracted from overnight cultures of the XDR *Salmonella* isolates using QIAamp DNA Mini kit (Qiagen, Germany) following the manufacturer's instructions. The PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen, Germany) and sequenced in an ABI 3130 automated DNA Sequencer (Applied Biosystems, United States) using the BigDyeR Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United States) following the supplier protocol. Nucleotide sequences were compared with those previously deposited at GenBank using Basic Local Alignment Search Tool (BLAST[1]). Alignment of the nucleotide sequences was performed using the MEGA6 program (Tamura et al., 2013). The amino acid sequences were deduced using the ExPASy (Expert Protein Analysis System) Translate Tool[2]. The respective regions of nucleotide and polypeptide sequences were analyzed for mutational changes by comparison with the complete genome of *S. enterica* serovar Typhimurium LT2 (GenBank accession number NC_003197).

Quantification of the Transcription Levels of Efflux Pump Genes

Quantitative PCR (qPCR) was used to determine the relative expression levels of *ramA*, *acrB*, and *oqxB* genes using previously published oligonucleotide primers (**Supplementary Table 1**). Total RNA was extracted from XDR *Salmonella* isolates using QIAamp RNeasy Mini kit (Qiagen, Germany) following the manufacturer's instructions. The relative quantification was done in triplicates using QuantiTect SYBR Green real-time PCR Kit (Qiagen, Germany) in MX3005P real-time PCR thermal cycler (Agilent, La Jolla, CA, United States) following the manufacturer's recommendations. Melting curve analysis was conducted to confirm the specificity of the tested assays. The 16S rRNA housekeeping gene was used as a normalizer (Fàbrega et al., 2016), and the fold change values were estimated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). A pan-susceptible *Salmonella* Tamale isolate was used as a comparative control.

Bioinformatics and Data Analyses

Fisher's exact test was used to determine if there were significant differences between the infection rates with *Salmonella* in the two age groups (i.e., 1–5 days and 1–4 weeks) in bird species. To visualize the overall distribution of the XDR *Salmonella* isolates based on their resistance patterns, a heatmap supported by hierarchical clustering (dendrogram) was generated (Kolde, 2019). To determine the significance of the association between a certain genetic marker and the resistance phenotype in *Salmonella* isolates, Fisher's exact test and odds ratio (confidence intervals = 95%) were estimated on contingency tables considering the presence of genetic markers as independent variables and the resistance phenotypes as dependent variables. These analyses were done using GraphPad Prism version 8 for Windows, San Diego, CA, United States[3]. To reveal the significance of the genetic markers as classifiers of the isolates as resistant or susceptible to a certain drug, we applied a random forest classification model using an ensemble of 500 trees. The optimal number of random train predictor variables was determined using the *tuneRF* R function at an "out of bag error," OOB (prediction error) = 0. The mean decrease in the Gini index was used as an indicator of variable importance (the higher the mean decrease in the Gini index, the more important

[1]www.ncbi.nlm.nih.gov/BLAST/

[2]https://www.expasy.org/

[3]www.graphpad.com

the marker). This analysis was done using R package "Random Forest" (Breiman, 2001) in the R environment (v. 3.6.2). To calculate the correlation, the raw data were converted into binary outcomes, and the correlation significance was determined (*p*-value significance level = 0.05). These analyses were done using R packages *corrplot* and *Hmisc* (Harrell, 2020). Binary distances between isolates of the same host were measured using dist function in R software. Non-metric multidimensional scaling was used to visualize the clustering of isolates belonging to different hosts based on binary distance. This analysis was done using VEGAN package and the function metaMDS in R software[4].

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the genes under study were deposited into the GenBank under the following accession numbers: MT725561–MT725589 for *gyrA*, MT725590–MT725614 and MT740093–MT740096 for *tet*(A), and MT743008–MT743036 for *ramRA* genes.

RESULTS

Prevalence and Serotypes of *Salmonella* Isolates in Poultry Flocks and Contact Workers

As shown in **Table 1**, out of 1210 diarrheic and recently dead poultry sampled, 100 (8.26%) were positive for *Salmonella* species. The prevalence of *Salmonella* was 11.54% (75/650), 6.67% (10/150), 3.13% (5/160), 3.85% (5/130), and 4.17% (5/120) in chickens, ducks, pigeons, quails, and turkeys, respectively. Meanwhile, 15 *S. enterica* isolates were isolated from 375 contact worker stool samples (4.00%). The recovered *Salmonella* isolates were confirmed based on conventional phenotypic and molecular identification methods. Statistical analysis revealed that the difference in the infection rates of all birds with *Salmonella* species was significant (*p*-value = 0.04), whereas it was non-significant among each poultry species (*p*-value > 0.05). Typical *Salmonella* colonies were pink with or without black centers on XLD agar, while white colonies with black centers were characteristic on SS agar medium. Biochemical reactions for presumptive identification of *Salmonella* isolates indicated that all tested isolates were positive for methyl red, Simmons' citrate, and oxidase tests and displayed characteristic reactions on triple sugar iron and lysine decarboxylase agar media, whereas the analyzed isolates were negative for indole and Voges–Proskauer tests. All *Salmonella* isolates were further confirmed by PCR detection of the *invA* gene (fragment size = 284 bp).

In all, 35 *Salmonella* serotypes were identified among the 115 isolates originating from poultry and humans using a classical agglutination assay. Regardless of the isolate source, the most frequent serotype was *Salmonella* Typhimurium (26.96%) followed by *Salmonella* Enteritidis (11.30%), *Salmonella* Infantis (6.96%), *Salmonella* Kentucky (5.22%), and *Salmonella* Newport (3.48%); other *Salmonella* serotypes were reported by lower frequencies (**Supplementary Table 2**). The distribution of *Salmonella* serotypes varied within host species. Typhoidal *Salmonella* serotypes Typhi and Paratyphi C were detected among the human isolates only (3/15; 20% each), whereas all the subspecies *enterica* of poultry origin were categorized as NTS. It was noted that *Salmonella* Typhimurium was the predominant serotype in chickens (22/75; 29.33%) followed by *Salmonella* Enteritidis (12/75; 16.00%). However, certain *Salmonella* serovars were reported and characterized only for ducks (*Salmonella* Derby and *Salmonella* Larochelle), pigeons (*Salmonella* Alfort and *Salmonella* Wingrove), turkeys (*Salmonella* Vejle and *Salmonella* Apeyeme), or quails (*Salmonella* Shangani and *Salmonella* Jedburgh).

Antimicrobial Susceptibilities and Selection of XDR Isolates

As depicted in **Supplementary Table 3**, the highest level of resistance was recorded for amoxycillin-clavulanic acid (99.13%) followed by cefazolin (94.78%), nalidixic acid (77.39%), cefoxitin (76.52%), tetracycline (74.78%), and cefepime (73.04%). More than 50% of the isolates exhibited resistance to each of tobramycin, cefuroxime, chloramphenicol, ceftriaxone, sulfamethoxazole-trimethoprim, and ampicillin. CIP and TIG resistance were observed in 51.30 and 24.35% of *S. enterica* isolates, respectively. However, the carbapenems including ertapenem, imipenem, meropenem, and doripenem displayed the maximum activity against the isolates (70.43, 79.13, 89.57, and 93.04%, respectively). Only one isolate of chicken origin (*Salmonella* Tamale; 0.87%), was pan-susceptible to the tested antimicrobials. According to the antimicrobial resistance profile, 73.9% (n = 85) of the isolates were MDR (MAR index = 0.13–0.71) and 25.22% (n = 29) were XDR (MAR index = 0.63–0.88), of which 24 originated from poultry and only 5 were of human origin (**Table 1**). *Salmonella* Typhimurium accounts for almost half of the XDR isolates (12/29; 41.38%), of which 8 (66.67%) were human isolates and 4 (33.33%) were of chicken origin.

As shown in **Table 2** and **Supplementary Table 4**, all XDR *Salmonella* isolates (n = 29) were CIP-resistant (MIC = 2–128 μg/ml), and 25 (86.21%) were TIG-resistant (MIC = 4–32 μg/ml). It is worthy of note that four (13.79%) XDR isolates were uniquely resistant to CIP (MIC = 4–8 μg/ml), while 25 isolates (86.21%) showed CIP/TIG co-resistance (**Figure 1**).

Phenotypic Detection of the Efflux Pump Activity

The efflux activity of XDR *Salmonella* isolates was assessed by testing the ability of the bacteria to pump EtBr out of the cell using the cartwheel test. Fluorescence of *Salmonella* isolates which grew as a confluent mass along a radial line of TSA plates containing increasing concentrations of EtBr was reported. The minimum concentration of EtBr and the efflux activity index for each *Salmonella* isolate are illustrated in **Table 2**. The fluorescence of tested *Salmonella* was lower than that produced by the control (pan-susceptible *Salmonella* Tamale), which fluoresced at 0.25 μg/ml EtBr. Two chicken isolates (2/29; 6.90%) began

[4]https://www.r-project.org

TABLE 1 | *Salmonella* prevalence and XDR isolates recovered from clinically diseased, recently dead birds, and contact poultry workers.

Host	Examined no.	Infected no. (prevalence %)	1–5 days old			1–4 weeks old			*p*-value[e]
			Examined no.	*Salmonella*-positive (%)[a]	XDR isolates (%)[b]	Examined no.	*Salmonella*-positive (%)[a]	XDR isolates (%)[b]	
Poultry	Broiler chicken (650)	75 (11.54)	280	40 (14.29)	8 (20.00)	370	35 (9.46)	9 (25.71)	0.06
	Duck (150)	10 (6.67)	40	5 (12.50)	0 (0.00)	110	5 (4.55)	3 (60.00)	0.13
	Pigeon (160)	5 (3.13)	65	3 (4.62)	1 (33.33)	95	2 (2.11)	0 (0.00)	0.39
	Quail (130)	5 (3.85)	50	3 (6.00)	2 (66.67)	80	2 (2.50)	1 (50.00)	0.37
	Turkey (120)	5 (4.17)	55	4 (7.27)	0 (0.00)	65	1 (1.54)	0 (0.00)	0.17
	Total (1,210)	100 (8.26)	490	55 (11.22)	11(20.00)	720	45 (6.25)	13 (28.89)	0.04*
Poultry workers	375	15 (4.00)	5 XDR (1.3%[c], 33.3%[d])						

XDR, extensive drug resistant.
[a]The percentages were calculated according to the total number of examined birds in each poultry species.
[b]The percentages were calculated according to the total number of recovered Salmonella isolates from each poultry species.
[c]The percentage was calculated according to the total number of examined poultry workers.
[d]The percentage was calculated according to the total number of infected poultry workers.
[e]p-values obtained by Fisher's exact test refer to the differences between young (1–5 days) and old (1–4 weeks) birds infected with Salmonella.
Asterisk (*) indicates a significant value.

to fluoresce at an EtBr concentration of 2.5 μg/ml, whereas 6 isolates from various poultry types fluoresced at 2 μg/ml EtBr. The remaining *Salmonella* isolates (n = 21) fluoresced at EtBr concentration range of 0.5–1.5 μg/ml. As shown in **Table 2**, the MIC values of CIP were reduced by one–threefold in the presence of CCCP in 25 out of 29 (86.21%) CIP-resistant isolates; among them, only 5 isolates (17.24%) showed reverse CIP resistance patterns (MIC = 0.5 μg/ml), while one–fourfold decrease in the TIG MICs was reported in 23 out of 25 (92%) TIG-resistant isolates. Based on these results, we continued the study by further molecular evaluation of the contribution of different mechanisms to the resistance to CIP and TIG, known to be substrates for the efflux pump system, in the 29 XDR and EtBr cartwheel positive *Salmonella* clinical isolates.

Contribution of Plasmid-Mediated Quinolone Resistance Determinants and *gyrA* Mutations in Fluoroquinolone Resistance

The existence of PMQR genes, *qnrA*, *qnrB*, *qnrS*, *qepA*, *oqxA*, *oqxB*, and *aac(6′)-lb-cr*, was examined in XDR *Salmonella* using conventional PCRs. Data analyses revealed that three XDR *Salmonella* isolates (10.34%) did not have any of the studied PMQR genes, while none of the isolates harbored all the PMQR genes. The majority of the isolates (89.66%) contained two to six PMQR genes. The *qepA* gene was the most frequent PMQR determinant (89.66%) followed by *qnrS* (62.07%), *qnrA* (55.17%), and *qnrB* (27.59%). Six isolates (20.69%) were tested positive for both *oqxA* and *oqxB* genes, whereas one isolate (3.45%) was tested positive for each of *oqxA* and *oqxB* genes. The *aac(6′)-lb-cr* was not detected in any of the tested *Salmonella* isolates (**Table 3**).

DNA sequencing of the QRDRs of *gyrA* gene revealed specific stepwise point mutations in the topoisomerase target gene; those were associated with nalidixic acid and CIP resistance in 26 out of 29 (89.66%) XDR *Salmonella* isolates. As presented in **Table 3**, single mutations in *gyrA* gene either at S83 (9/26; 34.62%) or D87 (8/26; 30.77%) were detected in either low (MIC = 2 μg/ml; 5/29), moderate (MIC = 4–16; 8/29), or high (MIC = 32–128 μg/ml; 4/29) levels of CIP resistance. However, nine XDR isolates (CIP MIC = 16–64 μg/ml) were associated with double mutations at both positions. No mutation was reported in the QRDR of *gyrA* among three *Salmonella* isolates that showed nalidixic acid resistance and low-level CIP resistance (MIC = 2 μg/ml). The amino acid substitution at S83F was the most frequent mutation being detected in 23.08% of the isolates followed by D87Y (19.23%).

As depicted in **Tables 2** and **3**, *Salmonella* isolates (code Nos. 3, 11, and 29) contained double amino acid substitutions at S83 and D87 in QRDR of *gyrA* along with four to six PMQR determinants that had elevated MIC values for CIP (32–64 μg/ml). However, isolates of code Nos. 19 and 21 harbored single amino acid substitutions in QRDR of *gyrA* gene (S83V and D87Y, respectively) as well as six PMQR genes with CIP MICs of 4 and 128 μg/ml, respectively. Some isolates (code Nos. 15, 16, and 20) had up to three PMQR genes with no mutations in *gyrA* gene and showed a low level of CIP resistance (MIC = 2 μg/ml). On the other hand, other isolates of code Nos. 13, 23, and 27 had a single amino acid substitution, and no or just one PMQR gene showed moderate (MIC = 4–8 μg/ml) or low (MIC = 2 μg/ml) levels of CIP resistance. These results suggest that the acquisition of *gyrA* mutations as well as PMQR determinants were not the primary cause of the resistance to nalidixic acid and CIP in *S. enterica* serovars, and additional resistance mechanisms, such as enhanced efflux pump activity, may be involved.

Impact of *tet* Genes and Mutations of the *tet*(A) Gene on TIG Resistance

The *tet*(A) and *tet*(B) genes were detected in 29 (100%) and 18 (62.07%) of the XDR *Salmonella* isolates, whereas *tet*(X4) gene was detected in only one isolate (3.45%) (code No. 18, **Tables 2**, **3**), and *tet*(M) gene was not detected. To determine

TABLE 2 | Phenotypic characterization of XDR *Salmonella* isolates recovered from poultry and human origins.

Isolate no.	Isolate code	Serovar	Source	Antimicrobial resistance pattern[a]	MAR index	MIC (μg/ml)[b]		Efflux Pump activity		MIC (μg/ml)	
						CIP	TIG	MC_{EtBr} (μg/ml)	Index[c]	CIP + CCCP	TIG + CCCP
1	H1	Typhimurium	Human stool	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, IPM, TOB, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.79	16	4	1	3	4	0.5
2	C1	Typhimurium	Chicken muscle	AMC, SAM, CZ, CXM, CRO, FEB, FOX, CN, TOB, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.75	32	4	1	3	16	1
3	D1	Untypable	Duck cloacal swab	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, IPM, TOB, NA, CIP, TE, TIG, FOS, C, SXT, CT	0.75	64	16	2	7	16	2
4	C2	Magherafelt	Chicken liver	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, TOB, NA, CIP, TE, TIG, FOS, C, SXT, CT	0.71	16	4	1	3	2	0.5
5	C3	Typhimurium	Chicken spleen	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, CN, TOB, NA, CIP, TE, FOS, C, ATM, CT	0.71	4	2	0.5	1	0.5	2
6	C4	Takoradi	Chicken cecum	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, CN, TOB, NA, CIP, TE, TIG, FOS, C, SXT, CT	0.75	32	8	1.5	5	8	2
7	C5	Labadi	Chicken gall bladder	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, CN, TOB, NA, CIP, TE, TIG, FOS, C, SXT, ATM	0.75	64	16	2.5	9	8	1
8	C6	Typhimurium	Chicken liver	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, CN, AK, NA, CIP, TE, TIG, FOS, C, SXT, ATM	0.75	2	4	1	3	0.5	2
9	Q1	Jedburgh	Quail dropping	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, TOB, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.75	64	8	1.5	5	8	1
10	Q2	Alfort	Quail liver	AM, AMC, SAM, CZ, FEB, FOX, IPM, TOB, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.71	64	16	2	7	16	2
11	C7	Typhimurium	Chicken muscle	AM, AMC, CZ, CXM, CRO, FEB, FOX, CN, TOB, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.75	32	8	1.5	5	4	1
12	C8	Typhimurium	Chicken spleen	AM, AMC, CZ, CXM, FEB, FOX, IPM, TOB, NA, CIP, TE, TIG, FOS, SXT, ATM, CT	0.67	16	16	2	7	8	2
13	D2	Untypable	Duck muscle	AM, AMC, SAM, CZ, FEB, FOX, IPM, ETP, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.71	8	4	1	3	2	1
14	C9	Blegdam	Chicken muscle	AMC, CZ, CXM, CRO, FEB, FOX, CN, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.67	2	4	0.5	1	0.25	0.5
15	C10	Infantis	Chicken liver	AMC, CZ, CXM, CRO, FOX, IPM, CN, TOB, NA, CIP, TE, TIG, FOS, C, SXT, CT	0.67	2	4	0.5	1	2	2
16	C11	Enteritidis	Chicken egg yolk	AMC, CZ, CXM, CRO, FEB, FOX, AK, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.67	2	4	0.5	1	0.5	1
17	H2	Typhimurium	Human stool	AM, AMC, CZ, CXM, CRO, FEB, FOX, AK, NA, CIP, TIG, FOS, C, SXT, ATM, CT	0.67	16	8	1.5	5	4	2
18	H3	Paratyphi C	Human stool	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, CN, TOB, AK, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.83	2	4	0.5	1	2	4

(Continued)

TABLE 2 | (Continued)

Isolate no.	Isolate code	Serovar	Source	Antimicrobial resistance pattern[a]	MAR index	MIC (μg/ml)[b]		Efflux Pump activity		MIC (μg/ml)	
						CIP	TIG	MC_{EtBr} (μg/ml)	Index[c]	CIP + CCCP	TIG + CCCP
19	H4	Typhimurium	Human stool	AM, AMC, CZ, CXM, CRO, FEB, FOX, CN, TOB, AK, NA, CIP, TE, FOS, C, SXT, ATM, CT	0.75	4	0.5	0.5	1	2	0.5
20	C12	Typhimurium	Chicken muscle	AMC, SAM, CZ, FEB, FOX, CN, TOB, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.67	2	4	1	3	0.5	2
21	C13	Bardo	Chicken liver	AM, AMC, CZ, CXM, CRO, FEB, FOX, IPM, ETP, MEM, DOR, AK, NA, CIP, TE, TIG, FOS, C, SXT, ATM, CT	0.88	128	32	2.5	9	32	2
22	C14	Sandiego	Chicken cecum	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, CN, TOB, NA, CIP, TE, TIG, FOS, C, SXT, CT	0.75	32	16	2	7	8	2
23	C15	Typhimurium	Chicken liver	AM, AMC, CZ, CXM, CRO, FEB, FOX, CN, AK, NA, CIP, TE, FOS, C, SXT, ATM, CT	0.71	4	0.5	0.5	1	4	0.5
24	C16	Magherafelt	Chicken cecum	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, IPM, ETP, MEM, DOR, TOB, NA, CIP, TE, TIG, FOS, C, SXT, CT	0.88	32	16	2	7	4	4
25	Q3	Jedburgh	Quail liver	AM, AMC, CZ, CXM, CRO, FEB, FOX, CN, TOB, NA, CIP, TE, TIG, FOS, SXT, ATM, CT	0.71	64	16	2	7	32	2
26	P1	Wingrove	Pigeon liver	AM, AMC, SAM, CZ, CXM, CRO, FEB, FOX, TOB, NA, CIP, TE, TIG, FOS, C, SXT, ATM	0.71	2	4	0.5	1	0.5	4
27	D3	Untypable	Duck muscle	AMC, SAM, CZ, CXM, FEB, FOX, CN, TOB, NA, CIP, TE, TIG, FOS, C, SXT, CT	0.67	2	4	1	3	2	2
28	H5	Typhimurium	Human stool	AM, AMC, SAM, CZ, CRO, FOX, IPM, ETP, MEM, DOR, CN, TOB, NA, CIP, TE, FOS, C, SXT, CT	0.79	8	1	0.5	1	4	1
29	C17	Typhimurium	Chicken muscle	AMC, SAM, CZ, CRO, FOX, CN, TOB, NA, CIP, TE, TIG, FOS, C, SXT, CT	0.63	32	8	1.5	5	16	4

XDR, extensive drug resistant; MAR, multiple antibiotic resistance; MIC, minimum inhibitory concentration; MC_{EtBr}, minimum ethidium bromide concentration; AM, ampicillin; AMC, amoxycillin-clavulanic acid; SAM, ampicillin-sulbactam; CZ, cefazolin; CXM, cefuroxime; CRO, ceftriaxone; FEB, cefepime; FOX, cefoxitin; IPM, imipenem; ETP, ertapenem; MEM, meropenem; DOR, doripenem; CN, gentamicin; TOB, tobramycin; AK, amikacin; NA, nalidixic acid; CIP, ciprofloxacin; TE, tetracycline; TIG, tigecycline; FOS, fosfomycin; C, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; ATM, aztreonam; CT, colistin; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; H, human; C, chicken; D, duck; Q, quail; P, pigeon; CLSI, Clinical and Laboratory Standards Institute.

[a]*Susceptibility of Salmonella isolates to colistin (CT) was determined using the broth microdilution method. Colistin MIC range was 4–32 μg/ml for resistant Salmonella isolates, while it was 0.5–2 μg/ml for those that were colistin sensitive (Nos. 7, 8, and 26).*

[b]*MICs for CIP and TIG were interpreted according to the relevant CLSI document.*

[c]*The efflux pump index provided the range of efflux activity, which was reported for each strain in comparison with a pan-susceptible Salmonella Tamale control isolate (assigned a value of 0.25 μg/ml).*

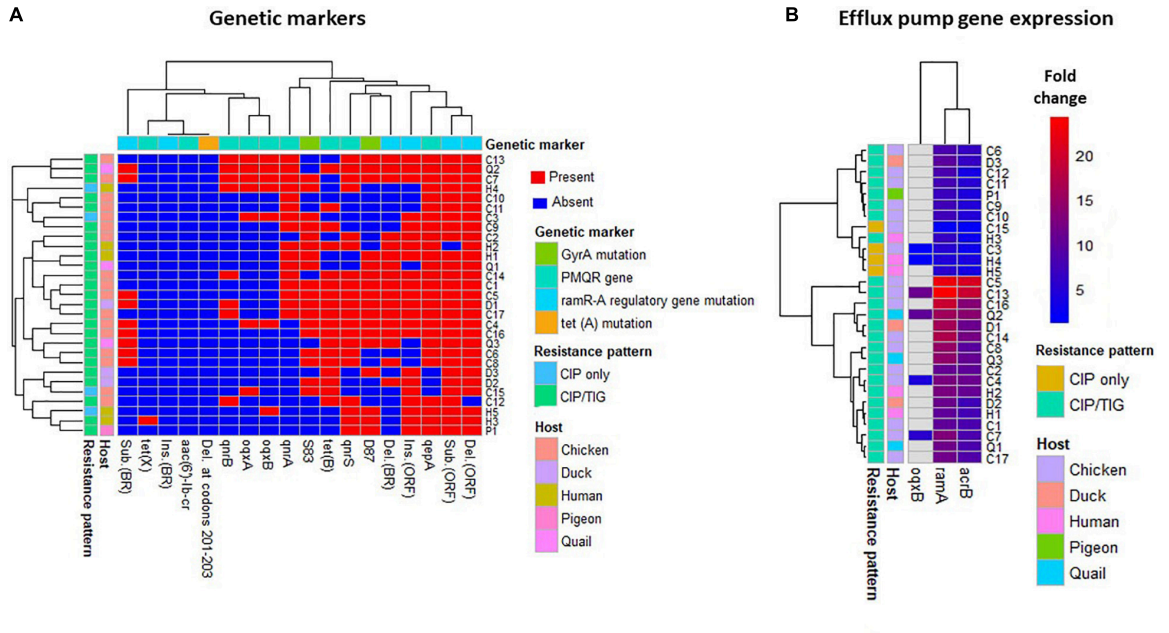

FIGURE 1 | Heatmap supported by hierarchical clustering (dendrogram) depicting the overall distribution of genetic markers **(A)** and efflux pumps gene expression **(B)** in the 29 XDR *Salmonella* isolates. The isolates are color-annotated based on their hosts (C, chicken; D, duck; H, human; P, pigeon, and Q, quails) and resistance pattern [i.e., to CIP, ciprofloxacin (n = 4) and to both CIP and TIG (n = 25)]. The genetic markers are color-annotated based on their type. BR, binding region; ORF, open reading frame.

whether *tet*(A)-positive isolates had mutations in the *tet*(A) interdomain loop region, the amplicons (402 bp) from all isolates were sequenced. As shown in **Tables 2**, **3**, comparisons of *tet*(A) gene sequences of *Salmonella* isolates with that of the wild type *Salmonella* Typhimurium reference strain (accession number NC_003197) revealed two-point mutations in the interdomain loop region of the efflux pump at codons 120 and 121 in eight TIG-resistant (MIC = 4–16 μg/ml) and one TIG-sensitive (MIC = 2 μg/ml) isolates. Moreover, a point mutation at codon 181 was observed in three isolates (MIC = 4–16 μg/ml); this important residue is located near the transmembrane domains of the efflux pump (codons 201–203) where mutations accumulated in these domains have a functional role in the efflux of TIG.

RamRA Mutations Induce Efflux Genes Expressions and Confer Relevant Resistance

To investigate the impact of non-target mutations on the expression of the AcrAB-TolC efflux system and consequently on the level of CIP and TIG resistance, DNA sequencing of the *ramRA* regulatory locus of the 29 XDR *Salmonella* isolates was performed then compared with the wild-type *ramRA* gene of *Salmonella* Typhimurium reference strain (accession number NC_003197). As depicted in **Table 3** and **Supplementary Figure 1**, screening for non-target mutations of the *ramRA* region revealed various amino acids alterations and frameshift mutations. In *ramR* gene open reading frame (ORF), the amino acid substitution $F_{145-150} \rightarrow$ L and the nucleotide deletion at G_{64}-A_{72} were the predominant mutations, being detected in 28 (96.5% each) XDR isolates, followed by the insertion at the nucleotide T319–326 (62.07%). Meanwhile, deletion at T_{-288}/A_{-285} was the highest detected mutation (17/29; 58.5%) in *ramR* binding region (BR). As shown in **Supplementary Table 5**, deletions in ORF and BR of *ramR* gene were found in 96% (24/25) and 68% (17/25) of the co-resistant XDR isolates, respectively, whereas amino acids substitutions in both regions were found in 96 and 36% of these isolates, respectively. It is interesting that the in-frame 4-bp deletion of T_{-288}/A_{-285} dominated in *ramR* BR of the 17 (68%) co-resistant XDR *Salmonella* isolates displaying 2- to 32-fold and 2- to 4-fold increased resistance to CIP and TIG, respectively, than the non-mutated isolates.

Upregulation of *ramA*, *acrB*, and *oqxB* Efflux Pump Genes in XDR *Salmonella* Isolates

To elucidate the role of enhanced efflux activity in CIP and TIG resistance, the relative expression levels of the global transcriptional regulator *ramA* and the transporter gene *acrB* of the AcrAB-TolC efflux system were assessed via qPCR. As shown in **Table 3**, the relative mRNA levels of *ramA* gene were maximal (exceeded 10-fold; range = 10.0–24.2, median = 12.8) in 17 out of 25 CIP/TIG co-resistant *Salmonella* isolates (68%) when compared to the pan-susceptible *Salmonella* Tamale control isolate (assigned a value of 1). Of these 17 co-resistant *Salmonella* isolates, 9 showed high expression levels for the *acrB* gene (range = 10.73–20.9; median = 11.8). None of the isolates that were uniquely resistant to CIP (n = 4) exhibited this overexpression in both genes. To further confirm the role of

TABLE 3 | Existence of plasmid-mediated quinolone resistance genes, *tet* genes, mutations in the target [*gyrA*, *tet*(A)] and regulatory (*RamR-A*) genes, and expression of efflux pump genes (*ramA* and *acrB*) in XDR *Salmonella enterica* serovars showing ciprofloxacin and/or tigecycline resistance.

Isolate no.	Serovar	PMQR pattern	*tet* genes	Target gene mutation[a]		*RamR-A* regulatory gene mutation[a]		Expression level			Accession number[b]
				gyrA	*tet*(A)	*ramR*/ramR	*ramR* binding site	*ramA*	*acrB*	*oqxB*	
1	Typhimurium	*qnrA*; *qepA*	*tet*(A)	S83A; D87V	–	F146L; Del G_{69}–A_{72}; Ins (3 nt) C_{235}	T-97C; Del T_{-288}/A_{-285}	10.04	7.61	ND	MT725561; MT725590; MT743008
2	Typhimurium	*qnrA*; *qnrS*; *qepA*	*tet*(A); *tet*(B)	S83Y; D87R	L121P	F146L; Ins (3 nt) G_{484}; Ins (3 nt) C_{235}; Del A_{67}–G_{69}	T-97C; Del T_{-288}/A_{-285}	10.10	8.09	ND	MT725562; MT725591; MT743009
3	Untypable	*qnrA*; *qnrB*; *qnrS*; *qepA*	*tet*(A); *tet*(B)	S83T; D87R	L121T	G186R; F149L; D104G; N87S; H82R; Ins (7 nt) G_{321}; Ins (5 nt) C_{252}; Del T_{65}–G_{69}	T-97C; V28M; Del T_{-288}/A_{-285}	16.33	10.84	ND	MT725563; MT725592; MT743010
4	Magherafelt	*qnrS*; *qepA*	*tet*(A)	S83L	–	F148L; Ins (7 nt) G_{319}; Ins (3 nt) C_{235}; Del G_{69}–A_{72}	T-97C; Del T_{-288}/A_{-285}	10.55	9.92	ND	MT725564; MT725593; MT743011
5	Typhimurium	*qnrA*; *qepA*; *oqxA*; *oqxB*	*tet*(A)	S83F	L121P	F148L; Ins (6 nt) T_{319}; Ins (3 nt) C_{235}; Del G_{69}–A_{72}	T-97C	5.10	2.83	1.4	MT725565; MT725594; MT743012
6	Takoradi	*qnrS*; *qepA*; *oqxA*; *oqxB*	*tet*(A); *tet*(B)	S83F; D87Y	F120S; L121A	G186R; F149L; D104G; N87S; H82R; Ins (7 nt) G_{321}; Ins (5 nt) C_{252}; Del T_{65}–G_{68}	V28M; Del T_{-288}/C_{-285}	12.21	11.12	4.3	MT725566; MT725595; MT743013
7	Labadi	*qnrA*; *qnrS*; *qepA*	*tet*(A); *tet*(B)	S83V; D87S	L121P	F149L; A102V; N87S; H82R; Ins (7 nt) G_{324}; Ins (5 nt) C_{252}; Del T_{65}–G_{69}	I40L; V28M; Del T_{-288}/A_{-285}; Del G_{-52}/A_{-49}	22.68	20.90	ND	MT725567; MT725596; MT743014
8	Typhimurium	*qnrS*; *qepA*	*tet*(A); *tet*(B)	S83Y	–	G182R; F145L; G109S; L72F; Del G_{307}–C_{309}; Del G_{251}–C_{254}; Del G_{64}–G_{69}	T-97C; I40L	8.11	6.62	ND	MT725568; MT725597; MT743015
9	Jedburgh	*qnrA*; *qepA*	*tet*(A)	S83L; D87N	–	F145L; S84G; G182R; Del C_{307}–G_{309}; Del C_{254}–A_{257}; Del T_{65}–G_{69}	T-97C; Del T_{-288}/A_{-285}; Del T_{-89}/T_{-87}	11.35	8.12	ND	MT725569; MT725598; MT743016
10	Alfort	*qnrA*; *qnrB*; *qnrS*; *qepA*; *oqxA*; *oqxB*	*tet*(A); *tet*(B)	D87F	G181D	G186R; F149L; D104G; N87S; H82R; Ins (7 nt) G_{321}; Ins (5 nt) C_{252}; Del T_{65}–G_{69}	V28M; Del T_{-288}/A_{-285}	15.86	13.66	10.1	MT725570; MT725599; MT743017
11	Typhimurium	*qnrA*; *qnrB*; *qnrS*; *qepA*; *oqxA*; *oqxB*	*tet*(A)	S83F; D87A	G181D	G186R; F149L; D104G; N87S; H82R; Ins (7 nt) G_{321}; Ins (5 nt) C_{252}; Del T_{65}–G_{69}	T-97C; V28M; Del T_{-288}/A_{-285}	12.80	8.24	5.4	MT725571; MT725600; MT743018
12	Typhimurium	*qnrS*; *qepA*	*tet*(A); *tet*(B)	S83E	–	G182R; F145L; G109S; L72F; Del G_{251}–C_{254}; Del T_{65}–G_{69}; Del G_{307}–C_{309}	I40L; Del T_{-288}/A_{-285}; Del T_{-89}/T_{-87}	14.83	9.51	ND	MT725572; MT740093; MT743019
13	Untypable	–	*tet*(A); *tet*(B)	S83F	–	F148L; Ins (7 nt) G_{319}; Ins (3 nt) C_{235}; Del G_{69}–A_{72}	T-97C; Del T_{-288}/A_{-285}	10.91	7.44	ND	MT725573; MT725601; MT743020
14	Blegdam	*qnrA*; *qepA*	*tet*(A); *tet*(B)	S83F	–	F149L; Ins (7 nt) G_{321}; Ins (5 nt) C_{252}; Del T_{65}–G_{69}	T-97C	7.11	4.38	ND	MT725574; MT725602; MT743021
15	Infantis	*qnrA*; *qepA*	*tet*(A)	–	L121P	F145L; Del G_{69}–A_{72}; Del G_{251}–C_{254}; Del G_{307}–C_{309}	T-97C	7.31	4.68	ND	MT725575; MT725603; MT743022
16	Enteritidis	*qnrA*; *qepA*	*tet*(A); *tet*(B)	–	–	F145L; Del G_{69}–A_{72}; Del G_{251}–C_{254}; Del G_{307}–C_{309}	T-97C	7.61	5.20	ND	MT725576; MT725604; MT743023

(Continued)

TABLE 3 | (Continued)

Isolate no.	Serovar	PMQR pattern	*tet* genes	Target gene mutation[a]		*RamR-A* regulatory gene mutation[a]		Expression level			Accession number[b]
				gyrA	*tet*(A)	*ramR*/ramR	*ramR* binding site	*ramA*	*acrB*	*oqxB*	
17	Typhimurium	*qnrA*; *qnrS*; *qepA*	*tet*(A); *tet*(B)	S83F	–	Ins (6 nt) C_{411}; Ins (3 nt) G_{377}; Del G_{307}–G_{309}; Del G_{251}–C_{254}; Del G_{69}–A_{72}	Del T_{-288}/A_{-285}	12.19	10.73	ND	MT725577; MT725605; MT743024
18	Paratyphi C	*qnrS*; *qepA*	*tet*(A); *tet*(X4)	D87Y	–	F148L; Ins (7 nt) G_{319}; Ins (3 nt) C_{235}; Del G_{69}–A_{72}	–	6.21	2.50	ND	MT725578; MT725606; MT743025
19	Typhimurium	*qnrA*; *qnrB*; *qnrS*; *qepA*; *oqxA*; *oqxB*	*tet*(A)	S83V	–	F145L; Del G_{307}–C_{309}; Del C_{254}–A_{257}; Del G_{69}–A_{72}	T-97C	4.60	3.97	1.1	MT725579; MT725607; MT743026
20	Typhimurium	*qnrB*; *qnrS*; *qepA*	*tet*(A); *tet*(B)	–	–	F150L; N88S; H83R; Ins (7 nt) G_{326}; Ins (5 nt) C_{257}; Ins (5 nt) G_{64}	–	8.32	3.84	ND	MT725580; MT725608; MT743027
21	Bardo	*qnrA*; *qnrB*; *qnrS*; *qepA*; *oqxA*; *oqxB*	*tet*(A)	D87Y	–	F147L; Ins (4 nt) A_{319}; Ins (3 nt) C_{235}; Del G_{69}–A_{72}	T-97C; Del T_{-288}/A_{-285}; Del G_{-52}/A_{-49}	24.20	19.23	10.9	MT725581; MT740094; MT743028
22	Sandiego	*qnrA*; *qnrB*; *qnrS*; *qepA*	*tet*(A); *tet*(B)	D87A	L121P	G186R; F148L; G112S; Ins (3 nt) T_{520}; Ins (7 nt) G_{319}; Ins (3 nt) C_{235}; Del G_{69}–A_{72}	T-97C; Del T_{-288}/A_{-285}; Del A_{-89}/T_{-87}	16.85	11.77	ND	MT725582; MT725609; MT743029
23	Typhimurium	*oqxA*	*tet*(A); *tet*(B)	S83I	–	F148L; Ins (7 nt) G_{319}; Ins (3 nt) C_{235}; Del G_{69}–A_{72}	–	1.6	1.1	ND	MT725583; MT725610; MT743030
24	Magherafelt	*qnrS*; *qepA*	*tet*(A); *tet*(B)	S83A; D87Y	–	F149L; N87S; H82R; Ins (3 nt) G_{493}; Ins (7 nt) G_{321}; Ins (5 nt) C_{252}; Del T_{65}–G_{69}	T-97C; V28M; Del T_{-288}/A_{-285}	19.25	13.22	ND	MT725584; MT725611; MT743031
25	Jedburgh	*qnrS*; *qepA*	*tet*(A); *tet*(B)	D87A	L121p	G182R; F145L; G109S; L72F; Del G_{307}–C_{309}; Del G_{251}–C_{254}; Del T_{65}–G_{69}	T-97C; I40L; Del T_{-288}/A_{-285}	14.18	10.74	ND	MT725585; MT725612; MT743032
26	Wingrove	*qnrS*; *qepA*	*tet*(A)	D87S	G181D	F147L; Ins (6 nt) C408; Del G_{307}–C_{309}; Del G_{251}–C_{254}; Del T_{65}–G_{69}	T-97C	6.66	4.09	ND	MT725586; MT740095; MT743033
27	Untypable	–	*tet*(A); *tet*(B)	D87G	L121p	F149L; A102V; N87S; H82R; Ins (7 nt) G_{321}; Ins (5 nt) C_{252}; Del T_{65}–G_{69}	T-97C; C-109T	9.44	6.42	ND	MT725587; MT725613; MT743034
28	Typhimurium	*qnrS*; *qepA*, *oqxB*	*tet*(A)	D87Y	–	F149L; N87S; H82R; Ins (7 nt) G_{321}; Ins (5 nt) C_{252}; Del T_{65}–G_{68}	T-97C	5.41	3.82	ND	MT725588; MT740096; MT743035
29	Typhimurium	*qnrA*; *qnrB*; *qnrS*; *qepA*	*tet*(A); *tet*(B)	S83L; D87G	–	F148L; Ins (7 nt) G_{319}; Ins (3 nt) C_{235}; Del G_{69}–A_{72}	T-97C; Del T_{-288}/A_{-285}	11.33	8.74	ND	MT725589; MT725614; MT743036

PMQR, plasmid-mediated quinolone resistance; Ins, insertion; Del, deletion; nt, nucleotide; ND, not detected.

Isolate numbers in bold were sensitive to tigecycline and resistant to ciprofloxacin. Other isolates exhibited co-resistance to ciprofloxacin and tigecycline. All isolates were nalidixic acid resistant.

[a]*Mutations were detected after comparison with the respective gene in the complete genome of S. enterica serovar Typhimurium LT2 (GenBank accession number NC_003197).*

[b]*GenBank accession numbers were assigned for gyrA, tet(A), and ramR-A genes, respectively.*

OqxAB efflux pump, we examined the relative expression levels of the *oqxB* gene in the *oqx*AB-positive isolates. Six isolates overexpressed the *oqxB* (range = 1.1–10.9; median = 4.85), that was more pronounced in four XDR isolates. Taking together the increased MIC values of CIP (2- to 32-fold) and TIG (2- to 16-fold), MAR and efflux activity indices (**Table 2**), and the maximal *ramA* and *acrB* expression levels for the above mentioned 17 CIP/TIG co-resistant isolates (**Table 3**), it is well suggested that enhanced AcrAB-TolC efflux pump activity was the most likely mechanism underlying CIP/TIG co-resistance in XDR *S. enterica* serovars.

Importance of Different Genetic Markers for the Co-resistance of *Salmonella* to Ciprofloxacin and Tigecycline

Figure 2 shows the ranked significance of each genetic marker as a contributor to the occurrence of certain resistance patterns. The random forest classification model (**Figure 2A**) showed that *ramA* gene expression and deletion in *ramR* BR were the top genetic markers differentiating the CIP/TIG co-resistant isolates ($n = 25$) from those that showed resistance to CIP only ($n = 4$). The odds ratio calculation and Fisher's exact test applied on the 25 co-resistant XDR isolates (**Figure 2B**) showed that the expression of *ramA* and *acrB* genes and deletion in the *ramR* BR were importantly (odds ratio = infinite) and significantly ($p < 0.05$) associated with the appearance of this phenotype.

Associations Among Various Genetic Markers

The correlations among pairs of the genetic markers are shown in **Figure 3**. In the 25 co-resistant XDR *Salmonella* isolates, certain PMQR genes were significantly positively correlated as seen in *qnrB-qnrA* and *qnrS-qepA* ($r = 0.5$ each). In the four isolates that were uniquely resistant to CIP, the highest positive correlation was found between *ramA* expression and the deletion in its BR ($r = 1$; $p < 0.05$), followed by an intermediate positive significant correlation between some members of PMQR genes. The insertion and substitution in *ramR* gene ORF were highly negatively correlated ($r = -0.5$; $p = 0.02$).

Regarding the isolation source, XDR *Salmonella* isolates belonging to human and poultry hosts were largely overlapped based on the resistance phenotypes and molecular characterization of the isolates (**Figure 4**). This is numerically evidenced by binary distance measured between pairs of isolates (**Supplementary Table 6**) belonging to different hosts or based on the average distances among hosts (0.3).

DISCUSSION

Salmonellosis is one of the most frequent food-borne zoonoses globally, and the consumption of contaminated poultry meat is considered the main source of NTS infections in humans (Antunes et al., 2016). A tremendous increase in the antimicrobial resistance in *S. enterica* poses a significant global concern. While resistance to CIP in *Salmonella* isolates has been intensively studied, limited data are available on how resistance to CIP could confer resistance to other unrelated antimicrobials such as TIG, which is the last-resort anti-*Salmonella* drug. In the current study, we exploited molecular and data analysis approaches to directly investigate the molecular rationale behind the occurrence of co-resistance to CIP and TIG in XDR *Salmonella* isolates recovered from poultry and contact workers in Egypt.

The prevalence of *Salmonella* in chickens (11.54%) and pigeons (3.13%) in this study was lower than that reported in Northern China, where 20% of 1- to 4-week-old broiler chickens and 21.82% of pigeons were positive for *Salmonella*. Meanwhile, the prevalence among 1- to 4-week-old ducks (4.51%) (Wang et al., 2020) was nearly similar to that reported in our study (6.67%). The observed *Salmonella* prevalence among turkeys (4.17%) was lower than that previously reported in German fattening flocks (10.3%) (Käsbohrer et al., 2013). In contrast to the reported prevalence of *Salmonella* among quails (3.85%), a very high rate (75%) has been declared in Brazil (de Freitas Neto et al., 2013). These variations may be attributed to the geographical areas, climatic conditions, poultry species or breed, sample types, and disparity in sampling procedure and *Salmonella* isolation protocol. Serological identification of *Salmonella* isolates ($n = 115$) demonstrated that *Salmonella* Typhimurium (26.96%) and *Salmonella* Enteritidis (11.30%) were the most frequent serotypes in poultry and contact workers, which is consistent with previous reports from China (Wang et al., 2020), Egypt (Gharieb et al., 2015), and some European countries (Osimani et al., 2016).

Our data showed high resistance to amoxycillin-clavulanic acid (99.13%), cefazolin (94.78%), nalidixic acid (77.39%), cefoxitin (76.52%), tetracycline (74.78%), and cefepime (73.04%). Modest resistance rates were observed for CIP and TIG (51.30 and 24.35%, respectively). Plausible explanations of resistance are the indiscriminate use of antibiotics both in human and poultry husbandry, in addition to the easy accessibility to antibiotics in many countries worldwide, notably in Egypt. In contrast, lower resistance rates were observed for ertapenem (7.83%), imipenem (19.13%), meropenem (5.22%), and doripenem (2.61%) as there is no history of using the carbapenems for prevention or treatment in poultry farms in Egypt.

While MDR *Salmonella* represented the majority of our isolates (73.9%), we rather focused our analyses on the XDR isolates ($n = 29$; 25.22%) from poultry ($n = 24$), and contact workers ($n = 5$) because of the scarcity of data on this particular *Salmonella* phenotype in Egypt [despite its presence in other countries, e.g., *Salmonella* Typhi in Pakistan (Klemm et al., 2018; Saeed et al., 2019) and *Salmonella* Indiana in China (Wang et al., 2017)] and due to its seriousness in cases of outbreaks, as compared to MDR strains.

In the present study, we attempted to elucidate the co-resistance mechanism contributed to 25 CIP/TIG co-resistant *Salmonella* isolates. Our results revealed that the CCCP EPI was able to reverse the CIP and TIG resistance patterns for some examined *Salmonella* isolates. Therefore, we could not exclude the prospect that the AcrAB-TolC efflux pump might play a role in CIP/TIG resistance. In accordance with

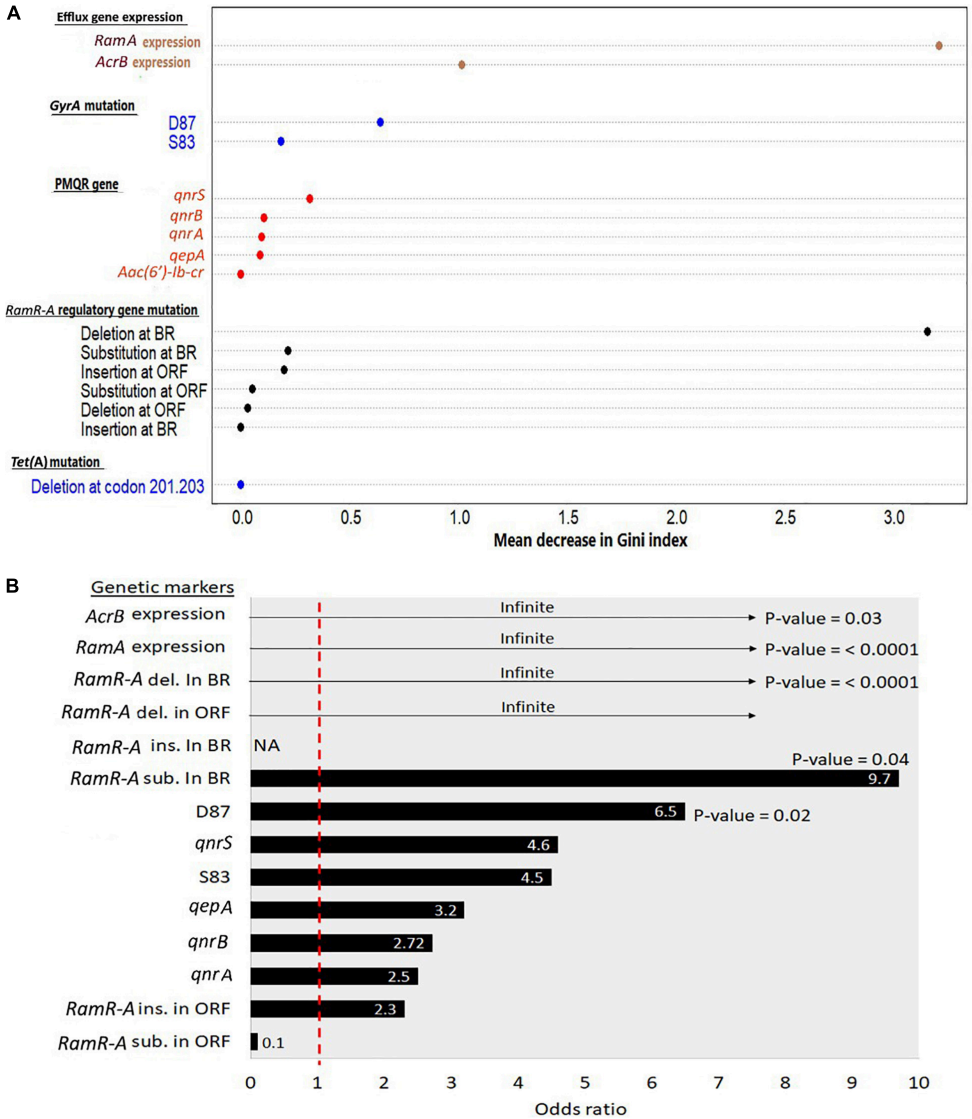

FIGURE 2 | Ranked significance of genetic markers in the XDR *Salmonella* isolates that showed co-resistance to ciprofloxacin and tigecycline (n = 25). **(A)** Random forest classification analyses showing the importance of genetic markers in differentiating the co-resistant XDR *Salmonella* isolates from those that were resistant to CIP only (n = 4). The X-axis shows the mean decrease in Gini index for each marker. The higher the mean decrease in the Gini index, the more important the marker. **(B)** Significance of association of genetic markers and the co-resistance phenotype. The X-axis shows the odds ratio (at confidence intervals = 95%) of the respective genetic features that are shown on the Y-axis. Numbers within the bars refer to odds value. Numbers beneath the bars show the significance of association (p-value based on Fisher's exact test at a cutoff level of 0.05) between the occurrence of certain genetic features and the appearance of the co-resistance to CIP and TIG in the 25 XDR isolates. The odds ratios that were shown as headed arrow indicate an infinite (∞) or maximum odds. The odds ratio of 1 (the vertical dashed red line) indicates no contribution of the respective genetic feature to the occurrence of the co-resistant phenotype. Del, deletion; Sub, substitution; Ins, insertion; BR, binding region; ORF, open reading frame.

these findings, reduction in CIP and TIG MICs have been documented while using the CCCP EPI in previous studies (Deng et al., 2014; Zhong et al., 2014; Razavi et al., 2020). The twofold or greater decrease in the MIC levels for CIP- and TIG-resistant *Salmonella* isolates affirmed the involvement of active efflux components (Marimón et al., 2004). In a previous study, the CIP MICs decreased by twofold and those of nalidixic acid decreased by fivefold in the presence of EPIs, establishing the inclusion of an efflux pump in conversing quinolone resistance (Keddy et al., 2010). However, no complete reversion of the CIP resistance phenotype was detected with CCCP EPI in the work of Rushdy et al. (2013) implying the contribution of other mechanisms to this resistance, mainly, mutations in the target genes.

Bacterial resistance to fluoroquinolones is usually mediated by the acquisition of PMQR determinants (Robicsek et al., 2006) or mutations in bacterial DNA gyrase, particularly *gyrA* (Hopkins et al., 2005; Wasyl et al., 2014) or active efflux

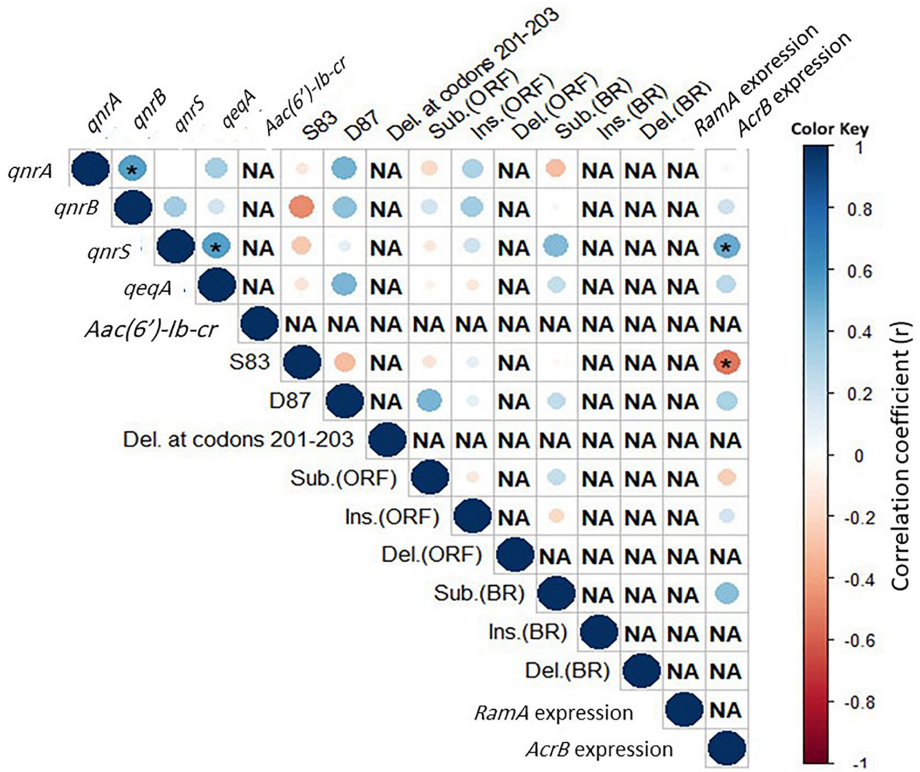

FIGURE 3 | Correlation among genetic features in the co-resistant XDR *Salmonella* isolates (n = 25). Asterisks (*) indicate a significant correlation at a 0.05 p-value. The color scale refers to the correlation coefficient (blue and red colors indicate negative and positive correlations, respectively).

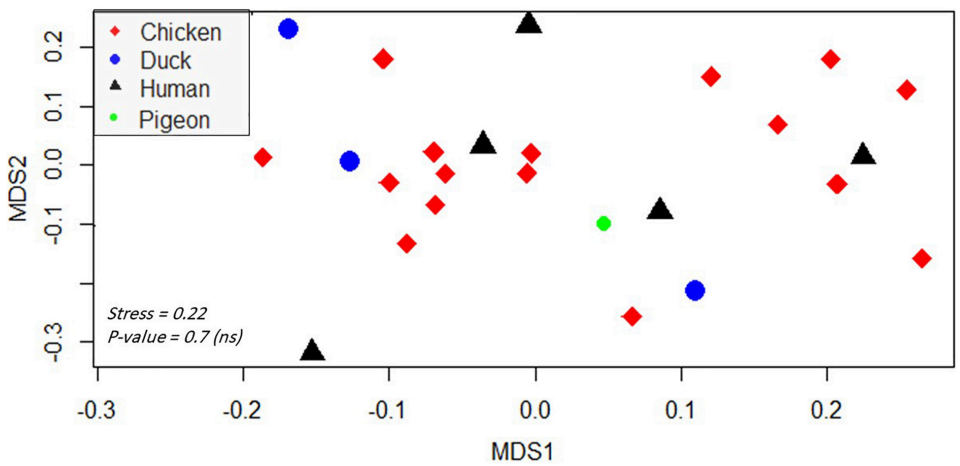

FIGURE 4 | Non-metric multidimensional scaling showing the clustering and overlap of various isolates belonging to different hosts. Different hosts are color- and shape-coded. Stress refers to the performance of the multidimensional scaling. The p-value was calculated by PERMANOVA test and refers to the significant clustering of the groups. A p-value < 0.05 indicates that isolates were not significantly clustered based on their hosts (thus there is an overlap among hosts). The abbreviations of codes are described in **Table 2** in detail.

(Zhang et al., 2017). Herein, PMQR determinants were found in 27 XDR *Salmonella* isolates resistant to CIP (MICs ranging from 2 to 128 μg/ml), indicating the frequent occurrence of transferable quinolone resistance (Wasyl et al., 2014). This corresponds to the recent findings that all *Enterobacteriaceae* strains with high and intermediate resistance phenotypes to CIP (MICs = 1.5–512 μg/ml) harbored one or more PMQR genes (Kotb et al., 2019). This differs from the results of Szabó et al. (2018) who found that PMQR-positive *Enterobacteriaceae* strains were susceptible or intermediately

resistant to CIP (MIC values between 0.06 and 1 mg/L). Notably, chromosomal copies of PMQR genes such as *oqxAB*, *aac(6′)-Ib-cr*, and *qnr*S genes have recently been discovered in CIP-resistant *Salmonella* strains (Chang et al., 2021). Thus, the presence of PMQR genes on the chromosome should not be excluded.

Previous results showed that fluoroquinolone resistance was attributed to amino acids substitutions at S83 or D87 codons within *gyrA* subunit, and these substitutions were not specific to certain *Salmonella* serovars (Robicsek et al., 2006; Chen et al., 2017). Our study revealed 16 different amino acids substitutions at both codons in 26 XDR *S. enterica* isolates representing 16 serovars, which were not associated with specific *Salmonella* serovar or CIP MIC value. Although quinolone resistance in *Salmonella* is firstly attributed to point mutations in the *gyrA* gene, other mutations of *gyrB* and *parC* genes may exist especially in higher-level resistance to fluoroquinolones. Oethinger et al. (2000) stated that the presence of *gyrA* mutations in the absence of active AcrAB efflux did not confer CIP resistance. This study's shortcoming is not detecting *gyrB*, *parC*, and *parE* mutations; whether mutations within these genes also contribute to CIP resistance here is unknown. The distribution of *gyrA* mutations in the 25 co-resistant XDR isolates did not follow a specific pattern, and they were present in the other 4 XDR isolates showing resistance to CIP. This agrees with the results of random forest classification (i.e., having a low mean decrease in Gini index) and odds values.

Here, plasmid-encoded *tet*(X4) gene was detected in only one out of the 29 (3.45%) XDR *Salmonella* isolates (MIC = 4 μg/ml). Consistent with our results, the mobile *tet*(X) gene was recently detected in diverse pathogens resulting in TIG resistance, which is considered a public health concern (Li et al., 2020). Tuckman et al. (2000) stated that *tet*(A) mutations could induce TIG resistance in *S. enterica* isolates. Considering mutations in *tet*(A) gene, sequence analysis indicated that the point mutations of codons 120, 121, and 181 in 12 *tet(A)*-carrying isolates differed from the frameshift mutations of codons 201, 202, and 203 that reduced the sensitivity to TIG (Hentschke et al., 2010). Therefore, the contribution of these mutations to TIG resistance needs further investigation.

According to Linkevicius et al. (2016), the chemical change in position C-9 of the *tet*(B) efflux transporter gene cannot expel TIG out of the cytoplasm. In this study, the MICs of TIG for *tet*(B) positive isolates (n = 18) ranged from 0.5 to 16 μg/ml. TIG resistance in such *tet*(B)-carrying isolates may be attributed to *tet*(A) mutations or upregulation of *ramA*, *acrB*, and *oqxB* efflux pump genes (**Table 3**). A previous study indicated that the MIC for *tet*(B) positive *S. enterica* isolates (n = 13/49) ranged from 0.064 to 0.5 mg/L (Akiyama et al., 2013). They concluded that the mutations in *tet*(A) gene promote a low-level resistance of *S. enterica* isolates to TIG (MIC ranged from 0.19 to 3 mg/L), and additional resistance mechanisms such as *ramA* and *ramR* mutations could increase the TIG MIC to reach the resistance breakpoint.

Since the *ramRA* region have been reported to play a major role in the regulation of *ramR* and *acrB* genes expression in *Salmonella* and, consequently, in the efflux-mediated decreased susceptibility to antimicrobials (Abouzeed et al., 2008; Fàbrega et al., 2016), we investigated how mutations of *ramRA* could influence the transcription level of *ramR* and *acrB* genes and how this is linked to the occurrence of CIP/TIG co-resistance in XDR *Salmonella*. While this mechanism has been shown to play roles in *Salmonella* resistance to CIP (Fàbrega et al., 2016) or TIG (Chen et al., 2017), limited data are available on its contribution to the concurrent *Salmonella* resistance to these two drugs. A reason behind this information shortage could be the fact that TIG is a newly launched drug that has not yet received much attention. Another reason could be the lack of studies on XDR *Salmonella*, where such resistance phenotype infrequently occurs. In our analyses, deletions in *ramR* BR and *ramA* overexpression existed in 17 XDR co-resistant isolates. In addition, the random forest classification model, odds ratio, and Fisher's exact test lend further support that these two determinants were the top two mechanisms occurring in the co-resistant XDR isolates. These data are consistent with a previous report (Fàbrega et al., 2016), although it is an *in vitro* study and involves MDR isolates. This suggests the importance of these two determinants as characteristic features of those co-resistant isolates (**Figures 2A,B**) and might also hint at a link between both markers as shown previously (Fàbrega et al., 2016). Coupling our data with the knowledge that *ramR* binds, through its BR, directly to the promoter of the *ramA* gene and thus controls its expression (Baucheron et al., 2012) puts forward the assumption that deletions in the *ramR* BR could impair its binding efficiency, leading to overexpression of *ramA* gene, and that the constitutive or concurrent occurrence of these two events are important for the occurrence of co-resistance to CIP and TIG in XDR *Salmonella.* This also in turn could enhance the expression of *acrB*, albeit to a low extent, and, thus, the increased MICs, MAR, and efflux activity indices. It is worth noting that although certain mutations (e.g., nucleotide deletions and amino acid substitutions in *ramR* ORF) were significantly associated with the appearance of the co-resistant phenotype and present in 96% of the 25 co-resistant isolates, respectively, their importance as determinants for such co-resistance is questionable because they also were present in the other four XDR isolates (**Figure 1** and **Table 3**). While deletions in *ramRA* region have been linked to *acrAB* overexpression and could confer CIP (Abouzeed et al., 2008; Kehrenberg et al., 2009; Fàbrega et al., 2016) and TIG (Hentschke et al., 2010) resistance in *Salmonella*, it does not seem to be the case for the co-resistant isolates as *acrB* overexpression existed in only 36% of the co-resistant isolates.

Our analyses also pinpointed specific novel mutations at *ramR* BR in the co-resistant XDR isolates. This included deletion at T_{-288}/A_{-285}, which was detected in 68% of the co-resistant isolates (odds ratio = infinite and p-value of Fisher's exact test $\leq$ 0.0001), suggesting the importance of this particular mutation in inducing such phenotype. We could not compare our results to others due to the scarcity of data on isolates exhibiting co-resistance to CIP and TIG. Similar results have also highlighted the role of *ramA* overexpression in the efflux-mediated MDR phenotype in *Salmonella* species (Fàbrega et al., 2016) and the positive correlation with the increased MICs of the antibiotics and expression of AcrAB efflux pump (Abouzeed et al., 2008). It is worthy of note that the genetic

alterations reported in our study differed from those reported in the aforementioned studies and confirmed the hypothesis that sequence alterations may occur at various positions in the *ramRA* gene (Kehrenberg et al., 2009). Therefore, these mutations may have switched on the transcription of the efflux pump genes constitutively and subsequently triggered the appearance of CIP/TIG co-resistance phenotype of XDR *Salmonella*. Wong et al. (2015) reported that oqxAB, an RND efflux pump, is one of several endogenous efflux systems found in *Klebsiella pneumoniae* and *Enterobacter* species, with a role functionally comparable to that of *acrAB* in other *Enterobacteriaceae* members. A recent research reported that the oqxAB-bearing plasmid might cause *Salmonella* Typhimurium to develop a TIG-resistant phenotype. This phenomenon was probably related to the overexpression of MDR efflux pumps AcrAB-TolC and OqxAB (Chen et al., 2017). In this study, overexpression of the *oqxB* gene in four CIP/TIG co-resistant isolates indicated that the OqxAB efflux pump might be also involved in CIP and TIG co-resistance in XDR *Salmonella*. This is consistent with the findings of Zhong et al. (2014) that AcrAB-TolC efflux pump plays an important role in TIG resistance in *K. pneumoniae* strains with MICs of 8 μg/ml, whereas both the AcrAB-TolC and OqxAB efflux pumps contributed to the TIG resistance in strains with an MIC of 16 μg/ml.

This study highlights the prevalence of *S. enterica* serovars in poultry flocks and their contact workers, particularly of the alarming XDR phenotype. The knowledge gained from this study is highly relevant in the field of antimicrobial resistance in *Salmonella* in many aspects. First, our data are deemed as an initial step in delineating the molecular rationale behind co-resistance of *Salmonella* to CIP/TIG, opening doors for the experimental validation of the proposed role of AcrAB-TolC and OqxAB efflux pumps in TIG and CIP resistance. Second, it makes it possible to understand the emergence of TIG resistance in the absence of respective selection pressure, which could be seen clinically in cases where TIG-resistant isolates emerge in *Salmonella*-infected humans or animals with no previous exposure to TIG (Hentschke et al., 2010; Chen et al., 2017).

This is the first report that concludes a variety of XDR *Salmonella* serotypes circulate in poultry flocks and their contact workers in Egypt, with *Salmonella* Typhimurium having the highest frequency. Enhanced efflux pump activity, in particular the overexpression of *ramA*, plays a fundamental role in *acrAB* overexpression and facilitates the efflux-mediated CIP/TIG co-resistance with no previous exposure to TIG. These genetic alterations suggest a potential public health concern possibly associated with the poultry-to-human transfer of resistant bacteria on farms using the antimicrobials for treatment or non-therapeutic use or both.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC) (approval number ZU-IACUC/2/F/12/2019). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC) (approval number ZU-IACUC/2/F/12/2019). Written informed consent was obtained from the owners for the participation of their animals in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

NA and YT contributed equally to the conception and design of the study and participated with RG in the application of classical microbiological techniques. AE carried out all PCR assays and sequencing approaches and participated in the analysis of the sequences. MS performed the bioinformatics, established the figures, and participated with MAS in statistical analyses of the data. RG, AE, MS, EK, MAS, and AA conceived the study and participated in the design. NA and YT carried out the sequence analysis and participated in the data analysis. NA, YT, and MS wrote the initial draft of the manuscript. All authors revised the manuscript critically for important intellectual content. All authors gave the final approval of the version to be published.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.738784/full#supplementary-material

Supplementary Figure 1 | Frequency of different mutations in the ramR-A regulatory gene as identified in the 2- investigated isolate's group (those resistant to CIP or to both CIP and TIG).

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Genetic Characterization of *mcr-1*-Positive Multidrug-Resistant *Salmonella enterica* Serotype Typhimurium Isolated From Intestinal Infection in Children and Pork Offal in China

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With the rapid emergence of plasmid-mediated colistin resistance gene *mcr-1*, the increased resistance of *Salmonella* has attracted extensive attention. This study reports on 11 multidrug-resistant *Salmonella enterica* serovar Typhimurium strains harboring *mcr-1* in China. They all presented resistance to colistin, and additionally, one that was isolated from a child's stool sample was also resistant to ceftriaxone and azithromycin. We screened 1454 strains of *Salmonella* for *mcr-1* gene through PCR, and these strains are all preserved in our laboratory. Antimicrobial sensitivity analysis was carried out for the screened *mcr-1* positive strains. Genetic polymorphism analysis of *S.* Typhimurium was performed by using the Pulsed-Field Gel Electrophoresis (PFGE). The plasmids harboring *mcr-1* were identified by S1-PFGE and southern blotting. Plasmid conjugation assays were used to analyze the transferability of colistin resistance. The plasmids harboring *mcr-1* were characterized by sequencing and bioinformatic analysis. Eleven *S.* Typhimurium strains harboring *mcr-1* with colistin resistance (MICs 4μg/ml) were detected, which were isolated from children and pig offal in China. All of them were multidrug-resistant strains. PFGE results revealed that the strains isolated from different samples or locations have identical genotypes. S1-PFGE and southern blotting experiments showed that three plasmids of different sizes (33, 60, and 250 kb) all carried the *mcr-1* gene. The plasmid conjugation assays revealed that *Salmonella* acquired *mcr-1* harboring plasmids by horizontal transfer. Sequencing and plasmid type analysis revealed that these plasmids were types IncX4, IncI2, and IncHI2. Among them, IncX4 and IncI2 plasmids had extremely similar backbones and contained one resistant gene *mcr-1*. IncHI2 plasmid contained multiple resistant genes including bla_{CTX-M}, *oqxB*, *sul*, *aph*, *aadA,* and bla_{TEM}. We identified 11 *mcr-1* harboring *S.* Typhimurium strains

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in China and described their characteristics. Our findings indicate that the *mcr-1* gene can effectively spread among intestinal bacteria by horizontal transfer of three types of plasmids. Moreover, the IncHI2 plasmid can also mediate the transfer of other drug resistance genes. These results reveal that constant surveillance of *mcr-1* harboring *S* Typhimurium is imperative to prevent the spread of colistin resistance.

Keywords: plasmid, colistin, *Salmonella* typhimurium, multidrug-resistant (MDR), bioinformatic analysis

INTRODUCTION

The rise of multidrug-resistant (MDR, resistance to three or more classes of antimicrobials) bacteria poses a serious threat to public health (Kumar et al., 2014; Jain et al., 2020). *Salmonella* is one of the common pathogens that can cause bacterial intestinal infections and diarrhea in developed and developing countries (Lokken et al., 2016). *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), one of the most prevalent serovars of *Salmonella*, is regularly linked to human infections and is frequently reported to be associated with human infections in several industrialized countries (Gomes-Neves et al., 2012), which can result in gastroenteritis and bacteremia. For clinical therapy of *Salmonella* infection, fluoroquinolones, azithromycin, and cephalosporins have been indicated. However, the extensive use of antibacterial medicines has resulted in the emergence of *S.* Typhimurium being resistant to antibiotics (Zhu et al., 2017; Wang Y. et al., 2020).

Polymyxin, a colistin antibiotic, acts as the last-line defense against severe infections caused by broad-spectrum active gram-negative bacteria (Lu et al., 2019). Additionally, colistin resistance has developed in *S.* Typhimurium, involving a variety of mechanisms. The plasmid-mediated colistin resistance gene *mcr-1* was first discovered in *E. coli* in China in 2015 (Liu et al., 2016), and has been the subject of research attention due to the *mcr-1* gene's ability to spread horizontally between bacteria. The colistin resistance gene *mcr-1* in the IncI2 plasmid encodes a phosphoethanolamine transferase, which is the modification of the lipid A and provides adequate protection from colistin. Multiple plasmids were used to propagate the colistin resistance gene *mcr-1*, including IncHI1, IncHI2, IncI2, IncX4, IncF, IncFI, IncFII, and IncP (Zurfluh et al., 2017; Touati and Mairi, 2021). These findings indicate that horizontal transfer of multiple resistance genes in the intestine bacteria may result in bacterial resistance. This study performed a screening analysis for the *mcr-1* gene of *S.* Typhimurium, which was preserved in the laboratory. The present study aimed to characterize the *S.* Typhimurium harboring *mcr-1* plasmids isolated from patients and food.

MATERIALS AND METHONDS

Bacterial *mcr-1* Gene Screening, Serotyping

To clarify the epidemic situation of the colistin resistance gene *mcr-1* in critical areas in China, we detected 1454 *S.* Typhimurium strains stored in our laboratory. All *S.* Typhimurium strains were isolated from stool samples of patients and food in markets, which were collected from Shanghai City (1046), Guangdong Province (209), and Guangxi Province (199) from 2006 to 2018, respectively. These strains were strictly identified by biochemical tests (API 20E system; bioMérieux Vitek, Marcy-L'Etoile, France) and serotyped on slides by microtiter agglutination tests for O and H antigens (SSI, Copenhagen, Denmark) according to the manufacturer's instructions. We screened all historical *S.* Typhimurium strains for *mcr-1* gene by PCR using the published primers sequence according to a study by Liu et al. (2016).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was designed using broth microdilution in Sensititre Gram Negative AST Plates for *Salmonella* strains (Thermo Fisher Scientific, Inc., West Sussex, United Kingdom) including 14 different antimicrobials: ceftriaxone (CRO), tetracycline (TE), ceftiofur (XNL), cefoxitin (FOX), gentamicin (GEN), ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CI), trimethoprim/sulfamethoxazole (SXT), sulfisoxazole (SX), nalidixic acid (NAL), streptomycin (SM), azithromycin (AZI), and amoxicillin/clavulanic acid 2:1 ratio (AUG2). The susceptibility to polymyxin is to use the dye WST (Dojindo Molecular Technologies, Inc., Japan) by a microbial viability assay kit. A reference strain of *Escherichia coli* ATCC 25922 strain was performed in the test as quality control (Wang et al., 2017).

Pulsed-Field Gel Electrophoresis (PFGE), S1-PFGE, and Southern Blotting

Genomic polymorphism analysis of *Salmonella* strains was performed using the pulsed field gel electrophoresis (PFGE) after a slight modification of the pulseNet standardized PFGE protocol for *Salmonella* (Ribot et al., 2006). To study the relationship between strains, *mcr-1*-negative *S.* Typhimurium Bacteria at different times in the same location and at the same time in other regions were used as the reference bacteria in the laboratory. These isolates were digested with *Xba*I (Takara, Dalian, China) at 37°C, and the *Salmonella enterica* var. Braendrup H9812 strain was used as the reference. Electrophoresis performed on a CHEF MAPPER variable angle system (Bio-Rad, California, America) with the parameters set at 2.16–63.8 s for 19 h performed following previously described methods (Liu et al., 2018). The plasmid profiles were characterized by S1-PFGE. The endonuclease S1 nuclease (Takara, Dalian, China) was used to digest at 37°C, and electrophoresis running set at 0.22–26.29 s for 15 h. The images were captured by a Gel Doc 2000 system (Bio-Rad), and imported into the BioNumerics software

(v6.0) database for further processing and analysis. The southern blotting with digoxigenin-labeled *mcr-1* probe using published primer sequences (Liu et al., 2016) was performed to membrane transfer, molecular hybridization, and probe detection following a previously reported method (Zou et al., 2015).

Plasmid Conjugation Assays

To verify the *mcr-1* positive plasmid's transfer capacity, plasmid conjugation experiments were performed by utilizing a standard *E. coli* J53 as the recipient, and the *mcr-1* positive *S.* Typhimurium strains as donors. The donor bacteria cultured overnight were mixed with the recipient bacteria in a ratio of 1:3 and harvested, re-suspended in 80μL. The mixture was incubated for mating at 37°C for 12–18 h in 5 ml LB liquid broth. Then a Muller-Hinton agar (BD Biosciences, San Jose, CA) plate containing 100 mg/L sodium azide and 2 mg/L polymyxin B was to a selective medium for *E. coli* J53 transconjugants. Putative transconjugants were confirmed by antimicrobial susceptibility testing and detection of *mcr-1* with PCR.

Whole Genome Sequencing and Bioinformatic Analysis

Using Next-Generation Sequencing (NGS), we sequenced plasmids of *S.* Typhimurium harboring the *mcr-1* gene. DNA was extracted from the overnight cultured strains using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The mate-pair library was constructed by nucleic acid protein analyzer Qsep100 to obtain DNA fragments (not less than 500 bp, not more than 800 bp) and sequenced by MiSeq sequencer. The raw reads were assembled into draft continuous sequences (contigs) by Newbler (Arredondo-Alonso et al., 2018) and NxTrim (O'Connell et al., 2015), and then spliced with Cytoscape's GapFiller. Complete plasmid genomes were annotated using the online annotation server RAST. Identification of insertion sequence (IS), plasmid replicons, and resistance genes were performed by ISfinder,[1] PlasmidFinder,[2] and ResFinder (Bortolaia et al., 2020), respectively. Multiple plasmids were compared by Mauve, Brig, and CLC Genomics Workbench. The circled figure of multiple plasmids for comparison was drawn by DNAploter (Roberts et al., 2008).

RESULTS

Antimicrobial Susceptibility Testing of *S.* Typhimurium Harboring *mcr-1*

Among the 1454 strains of *S.* Typhimurium maintained in our laboratory, 11 strains harboring colistin resistance gene *mcr-1* were identified. Eight strains were isolated from the feces of children under the age of five, while the remaining strains were isolated from pork offal. These strains exhibited multidrug resistance, including polymyxin (MICs 4μg/ml). Additionally, the majority (63.6%) of *S.* Typhimurium harboring *mcr-1* were resistant to the third-generation cephalosporins. Notably, one of them exhibited co-resistant to azithromycin and third-generation cephalosporins (**Table 1**).

Pulsed Field Gel Electrophoresis, Plasmid Profiling, and Southern Blotting

We studied the PFGE results of 11 *mcr-1*-positive *S.* Typhimurium strains and 12 *mcr-1*-negative *S.* Typhimurium strains. The majority of these (10/11) belong to ST34, while one (S55) belongs to ST19. In total, 13 distinct PFGE genotypes were discovered among 23 strains using the 85% cutoff (**Supplementary Figure 1**). The *mcr-1* positive strains were distributed in 10 different genotypes. Furthermore, numerous isolates obtained from diverse samples and provinces were identified as belonging to the same genotype. For instance, *S.* Typhimurium harboring *mcr-1* strains isolated from patient samples and food in Shanghai and the strains isolated from patients in Zhejiang and Henan were in the same cluster. S1-PFGE analysis showed that two of 11 *mcr-1* positive *S.* Typhimurium carried two plasmids, while the remaining eight carried single plasmid (**Supplementary Figure 2**). Southern blotting revealed that all of them carried one plasmid harboring *mcr-1* (**Supplementary Figure 2**).

Plasmid Conjugation Assays

To determine the transferability of the plasmid harboring *mcr-1*, plasmid conjugation assays were performed using 11 *mcr-1* positive *S.* Typhimurium as donors and *E. coli* J53 as the recipient. Five of eleven recipients tested positive for the resistance gene *mcr-1* via PCR amplification and sequencing analysis. Five plasmids harboring *mcr-1* were identified (pS49, pS51, pS52, pS55, pS56). Three were IncHI2 and two were IncI2 plasmids (**Supplementary Figure 1**). The MIC of colistin for the transconjugants (the *E. coli* J53 harboring *mcr-1* gene) was increased to 4 μg/ml, which was significantly more than the colistin resistance levels of the original J53 strains (which have MIC values of 0.125 μg/ml). It can be speculated that the transconjugants acquire the donor strains' colistin resistance gene.

The Complete Sequence of Plasmid Harboring *mcr-1*

We sequenced the plasmid of 11 *S.* Typhimurium strains. As a control, we downloaded five highly comparable plasmids harboring *mcr-1* from *Salmonella* strains for comparison (at above 95% coverage and above 99% identity) from NCBI (full name and cited reference). Three IncX4, two IncI2, and six IncHI2 plasmids harboring the *mcr-1* gene were identified by analyzing the plasmid sequences. Plasmid sequence comparison revealed that the lengths of the three IncX4 plasmids were approximately 33 kb and the sequences were completely identical; the lengths of the two IncI2 plasmids were 59,233 and 60,454 b, respectively, and the sequence differences were within 1.3 kb, and the 6 IncHI2 plasmid sequences were around 220-250 kb in length and the length variance was less than 2.8 kb. The three IncX4 plasmids with the identical sequence had a typical

[1] https://www-is.biotoul.fr/search.php

[2] https://cge.cbs.dtu.dk/services/PlasmidFinder/

TABLE 1 | Characteristic of 11 *mcr-1*-positive MDR *S.* Typhimurium.

Strain no.	Antibiogram[a]	Results of sequencing for 11 *mcr-1*-positive plasmids				
		Plasmid name	Size of *mcr-1* plasmid (b)	Type of *mcr-1* plasmid	Drug-resistant gene	IS types
S49	CRO,EFT,AMP,GEN, SM,SX,CHL,COL	pS49	222,291	IncHI2	*mcr-1*, bla_{CTX-M}, *aac*, *floR*, *aph*, *fosA*	ISApll
S51	CRO,EFT,AMP,GEN,SM, SX,SXT,CHL,TE,COL	pS51	249,475	IncHI2	*mcr-1*, bla_{CTX-M}, *sul*, *oqxA*, *oqxR*, *dfrA*, *floR*, *oqxB*, *aadA*, *aph*, *aac*, *fosA*, *cml*	ISApll
S52	CRO,EFT,AMP,GEN, SM,SX,CHL,TE,COL	pS52	249,043	IncHI2	*mcr-1*, bla_{CTX-M}, *oqxB*, *sul*, *aph*, *aadA*, *fosA*, *floR*, *aac*, *cml*, *oqxR*	ISApll
S53	CRO,EFT,AMP,GEN, SM,SX,CHL,COL	pS53	228,926	IncHI2	*mcr-1*, bla_{CTX-M}, *oqxA*, *sul*, *oqxR*, *aadA*	ISApll
S54	CRO,EFT,AMP,GEN, SM,SX,CHL,COL	pS54	222,880	IncHI2	*mcr-1*, bla_{CTX-M}, *aac*, *sul*, *aph*, *floR*, *fosA*	ISApll
S55	FOX,AUG2,CRO,EFT, AMP,SM,SX,SXT, AZ,CHL,TE,COL	pS55	59,233	IncI2	*mcr-1*	None
S56	COL	pS56	60,454	IncI2	*mcr-1*	None
S60	AMP,NAL,GEN,SM, SX,SXT,CHL,TE,COL	pS60	33,308	IncX4	*mcr-1*	IS26
S67	AMP,NAL,SM, SX,TE,COL	pS67	33,308	IncX4	*mcr-1*	IS26
S69	AMP,NAL,GEN,SX, SXT,CHL,TE,COL	pS69	33308	IncX4	*mcr-1*	IS26
S70	CRO,EFT,AMP,GEN, SM,SX,CHL,TE,COL	pS70	223,256	IncHI2	*mcr-1*, bla_{CTX-M}, *aac*, *sul*, *aph*, *floR*, *fosA*	ISApll

[a]*CRO, ceftriaxone; TE, tetracycline; XNL, ceftiofur; FOX, cefoxitin; GEN, gentamicin; AMP, ampicillin; CHL, chloramphenicol; CI, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; SX, sulfisoxazole; NAL, nalidixic acid; SM, streptomycin; AZI, azithromycin; AUG2, amoxicillin/clavulanic acid 2:1 ratio.*

IncX4 backbone and were extremely similar to pNG14043 from *Salmonella* in Taiwan (at above 99% homology). The IS26 was upstream of resistance gene *mcr-1* in our isolates. The IncX4 plasmids had only the resistance gene *mcr-1* and no other identifiable resistance genes (**Figures 1**, **2**). In contrast to pHNSHP45, three IncX4 plasmids lacked an ISApl1 insertion element upstream of *mcr-1* but had an IS26 insertion element. The IncI2 plasmids were similar to pHNSHP45 by *E. coli* strains from Shanghai in July 2013. The sequence of *mcr-1* on these plasmids was identical to pHNSHP45. However, two IncI2 plasmids lacked an ISApl1 insertion element upstream of *mcr-1*, and an IS683 region was found to be missing in all two IncI2-type plasmids isolated in this study (**Figures 2**, **3**). Unlike the IncX4 and IncI2 plasmids, IncHI2 plasmids exhibited the most genetic diversity (**Figures 2**, **4**). Compared with the pHNSHP45-2 plasmid, these IncHI2 plasmids with the common backbone were 250 kb in length. All the IncHI2 plasmids contained a single copy of *mcr-1*, and the sequence surrounding *mcr-1* shared 100% sequence identity. However, these plasmids contain numerous variable resistance genes, integrons, and ISs. The reference plasmid contained a variety of resistance genes, including bla_{CTX-M}, *oqxA*, *oqxB*, *oqxR*, *sul*, *aph*, *aadA*, *dfrA*, *floR*, *aac*, *fosA*, *hph*. The IncHI2 plasmids we investigated had different resistance genes and insert sequences with the reference plasmid (**Table 1**). Compared to the reference plasmid, several drug-resistant genes were missing, including *oqxA, oqxB, oqxR, sul*, *aadA* in three IncHI2 plasmids (pS49, pS54, pS70) and *dfrA12* in the five IncHI2 plasmids (pS49, pS52, pS53, pS54, pS70) (**Figure 4**). Five of six IncHI2 plasmids contained the insertion sequence ISApl1 on the upstream of *mcr-1*, but another plasmid lacked ISApl1 around *mcr-1* (**Figure 2**).

DISCUSSION

Salmonella is a widespread zoonotic pathogen that can cause human food poisoning and diarrhea (Ling et al., 2020). In general, food poisoning and diarrhea caused by non-typhoidal *Salmonella* (NTS) are self-limited. However, if the patient is young children, older people, and people with weak immune systems, antibiotic therapy will be preferred with multi-drug resistant *Salmonella* infection if the patient is a young child, elderly, or has a weak immune system. Studies have demonstrated that the multidrug resistance rate of *Salmonella* increased to 40% in the last decade of the twentieth century (Elbediwi et al., 2020). Resistance to fluoroquinolones, azithromycin, and third-generation cephalosporins in NTS species has been reported from numerous countries in the world (Tack et al., 2020; Appiah et al., 2021). Among a large number of *Salmonella* serotypes, *S.* Typhimurium and the rapid growth of multidrug-resistant has been a subject of concern globally (Huang et al., 2020). Colistin is considered as a last-line therapy for multidrug-resistant *S.* Typhimurium infection based on its prevalence and

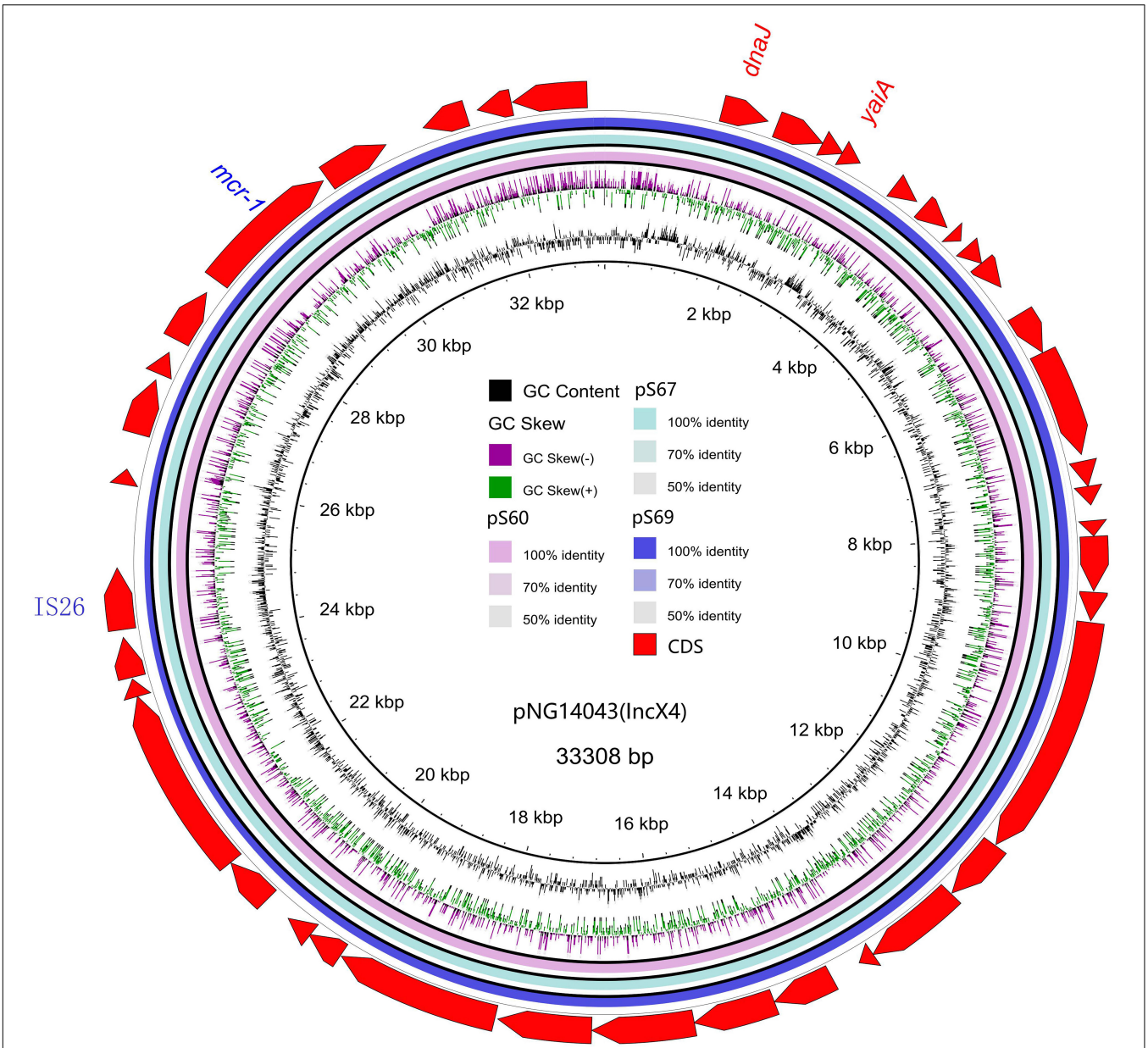

FIGURE 1 | Sequencing alignment of *mcr-1* harboring IncX4 plasmids. The *mcr-1* harboring plasmid pNG14043 with GenBank no. KY120364 which was isolated from *S.* Typhimurium in Taiwan was used as refrence plasmid (black circle). The outmost circle in red arrows denots the annotations of refrence plasmid. The figure shows the extremly high degree of homology of the four *mcr-1* harboring IncX4 plasmids. Detailed information of *mcr-1* location of plasmids is provided in **Figure 2**.

has been listed as a significant antibiotic by WHO since 2015 (Li et al., 2017). As colistin is widely used, bacteria have developed resistance to colistin. 37 *Salmonella* strains were identified harboring the *mcr-1* gene among 12,053 *Salmonella* strains collected from diarrhea outpatients under surveillance (Lu et al., 2019), and our finding appeared consistent with previous studies in Shanghai. We identified 11 *mcr-1*-positive strains among 1454 strains of *S.* Typhimurium (0.76%). Notably, eight strains of *S.* Typhimurium harboring *mcr-1* were isolated from the feces of children under the age of five (**Supplementary Table 1**). This observation is consistent with the finding of Luo et al. (2020), in which the majority of the *Salmonella* infection occurs in children under the age of five and patients with inadequate immunity. All *mcr-1* harboring *S.* Typhimurium strains from various sources were resistant to multiple antibiotics. In total, 63.6% of them were resistant to colistin and third-generation cephalosporin. Moreover, one of these strains was isolated from children under 5 years old was resistant to colistin, azithromycin, and third-generation cephalosporins. Considering the important role of azithromycin and third-generation cephalosporins in clinical treatment, this causes concern. ST19 and ST34 were common genotypes in *S.* Typhimurium (Zhang et al., 2021). The MDR

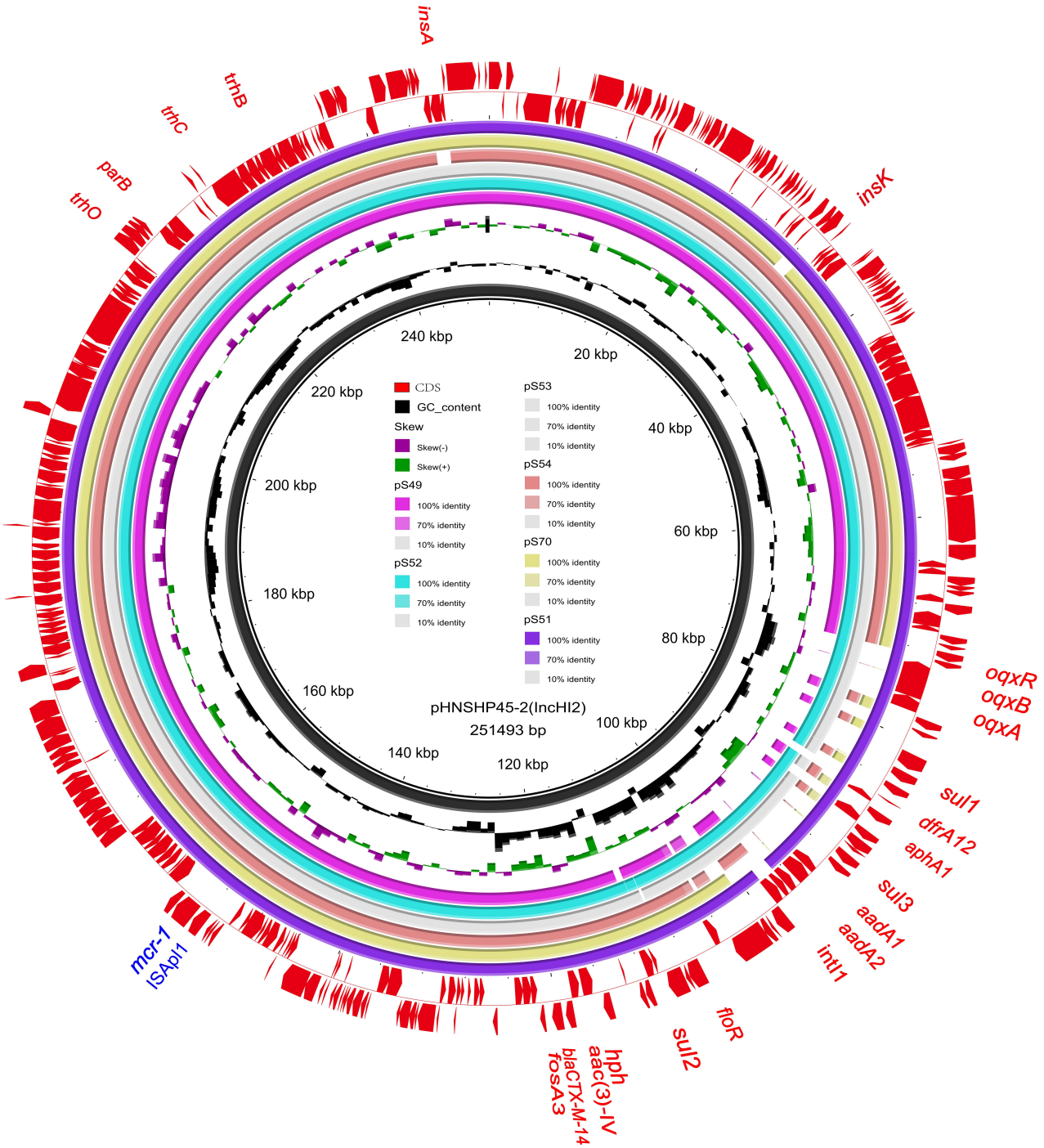

FIGURE 2 | Genetic context of 11 plasmids surrounding the *mcr-1* gene. In CDS, red and blue arrows represent *mcr-1* and IS, respectively, black arrows present plasmid bone. The light blue and orange shaded regions indicate genetic regions that show the direct and reverse nucleotide identity homology between different segments (> 99%).

ST34 *S.* Typhimurium has become a threat to public health due to its carriage of *mcr-1* and *mcr-3* (Biswas et al., 2019), and has been frequently detected in human clinical samples and food samples in China (Sun et al., 2014). The PFGE results indicated that *S.* Typhimurium that were isolated from various samples and provinces had identical genotypes. For example, *S.* Typhimurium harboring *mcr-1* strains isolated from children and food in Shanghai were clustered with the *S.* Typhimurium in Zhejiang and Henan strains. This result indicated that *S.* Typhimurium was prevalent in a number of regions in China.

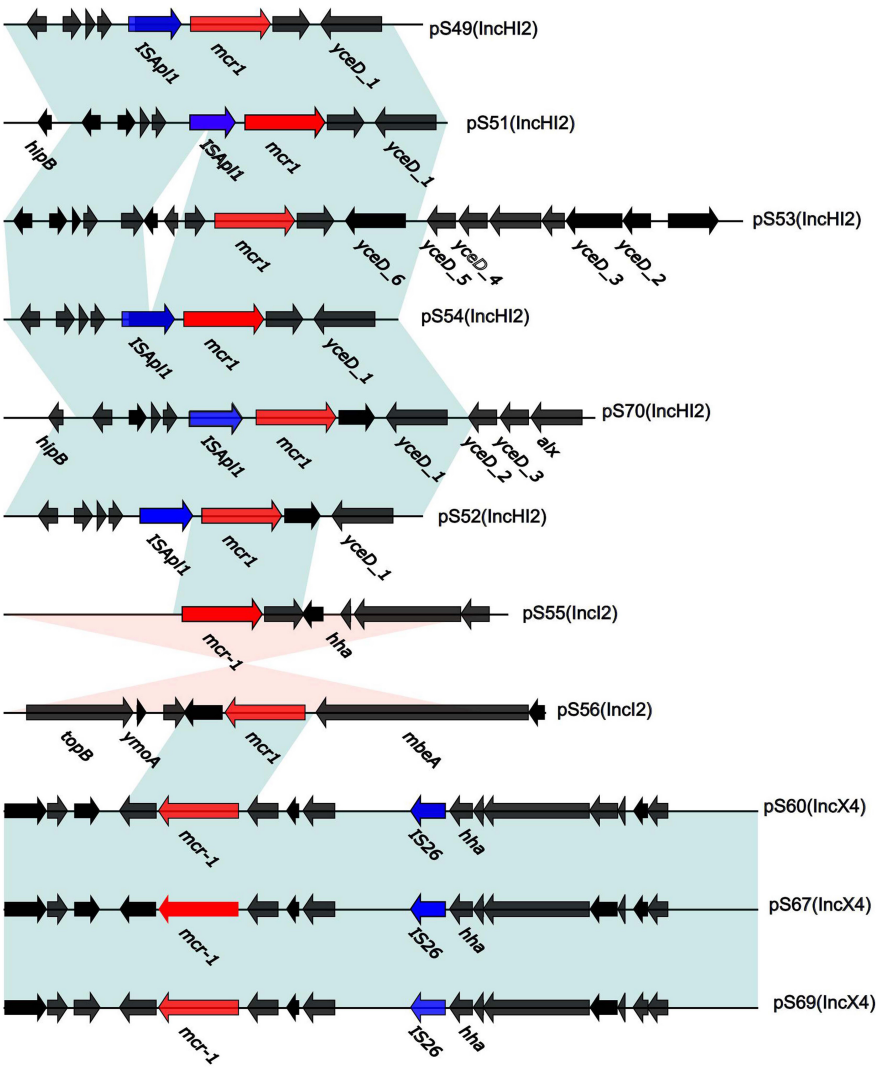

FIGURE 3 | Sequencing alignment of *mcr-1* harboring IncI2 plasmids. The first *mcr-1* harboring plasmid, pHNSHP45 with GenBank no. KP347127 which was isolated from E. coli strains from Shanghai in July, 2013 was used as refrence plasmid (black circle). The outmost circle in red arrows denots the annotations of refrence plasmid. The IS683 and ISApl1 are absent in two IncI2 plasmids in this study. Detailed information of *mcr-1* location of plasmids is provided in **Figure 2**.

Thus, the monitoring of multidrug-resistant *S.* Typhimurium strains should significantly prevent their spread.

Plasmids play a vital role in the acquisition of colistin resistance caused by drug resistant genes (McGann et al., 2016). In 2016, China reported the first case of Plasmid-mediated colistin resistance in the form of *mcr-1* (Liu et al., 2016). Colistin resistant gene *mcr-1* was widely spread in animals, the environment, and food in a number of nations and areas throughout the world by plasmid horizontal transfer (Zurfluh et al., 2017; Huang et al., 2020; Wang Z. et al., 2020; Touati and Mairi, 2021). During plasmid transfer, the plasmid harboring *mcr*-1 exhibited significant diversity in terms of antibiotic resistance patterns, incompatibility groups, and genetic content (Touati and Mairi, 2021). In our study, three types of plasmids (IncI2, IncX4, and IncHI2) harboring *mcr-1* were identified from 11 S. Typhimurium strains. The first reported *mcr-1* gene was identified in an IncI2 Plasmid. IncI2 and IncX4 plasmids, which promote *Salmonella* resistance, are the two major types of plasmids spreading globally (Arredondo-Alonso et al., 2018). IncHI2 plasmids are well-known for their role in clinically significant antibiotic resistant genes (Hammad et al., 2019). According to a previous study on *Enterobacteriaceae* (Zingali et al., 2020), IncHI2 plasmid (216–280 kb) is the fifth most common plasmid family containing a multidrug resistance region. it is also one of the major plasmid groups harboring *mcr-1* gene variants. The present study observed a coexistence of plasmids harboring *mcr-1* and multiple drug resistance genes, including *oqxB*, bla_{TEM}, and bla_{CTX} resistance genes. These genes in drug-resistant plasmids were one of the significant factors for the decreased sensitivity of colistin, quinolones, and third-generation cephalosporins. The rapid spread of antibiotic resistance in a particular area was caused by IncHI2 plasmids

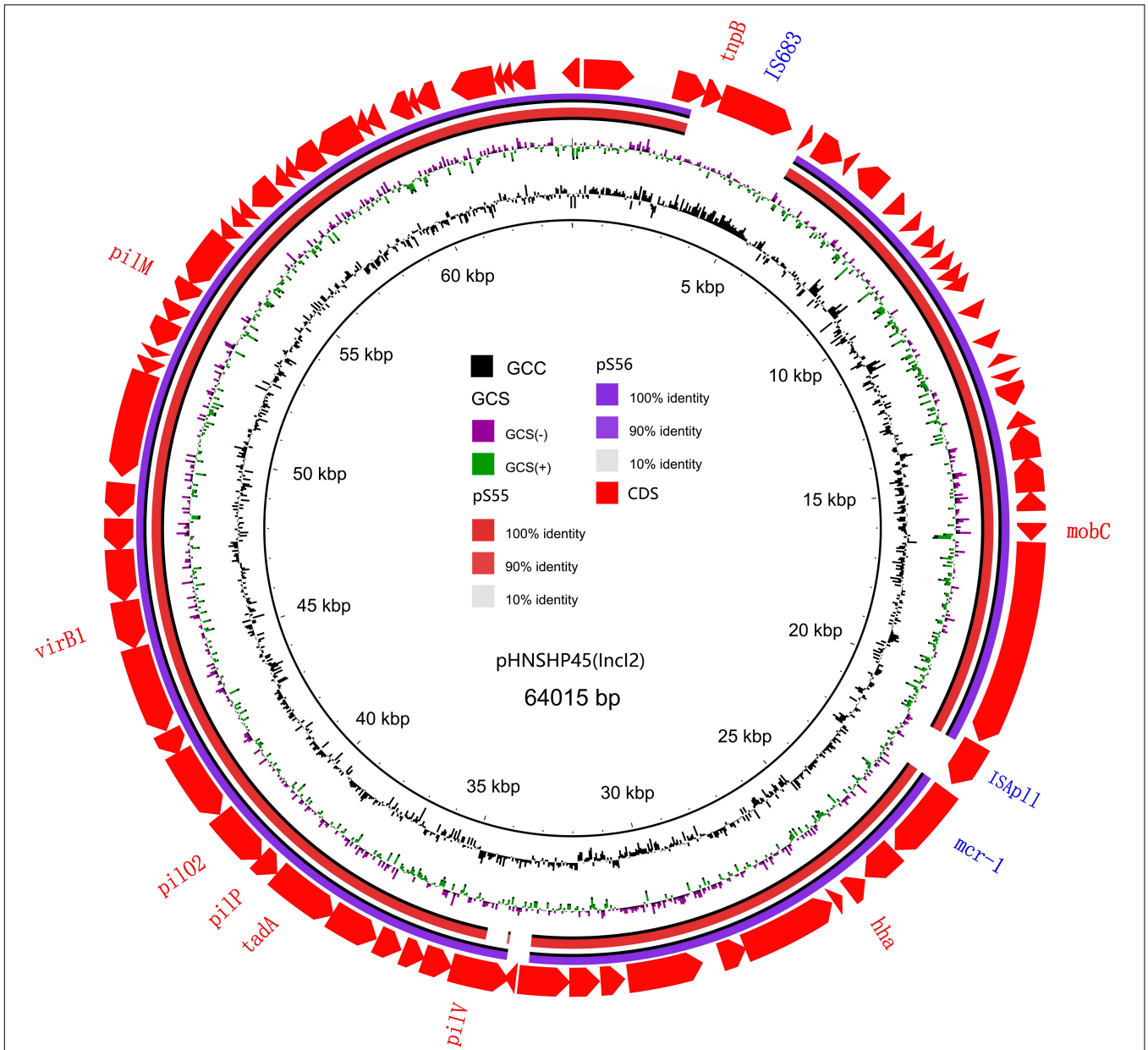

FIGURE 4 | Sequencing alignment of *mcr-1* harboring IncHI2 plasmids. pHNSHP45-2 with GenBank no. KU341381 which was isolated from E. coli strains was used as refrence plasmid (black circle). The outmost circle in red arrows denots the annotations of refrence plasmid. Among the six IncHI2 plasmids, pSH51, pS52, and pS53 exhibit sequence with the refrence sequence, others are low sequence homology. An MDR region is exhibieted in the five IncHI2 plasmids. Detailed information of *mcr-1* location of plasmids is provided in **Figure 2**.

transfer carrying multiple drug resistance genes between bacteria. MDR IncHI2 plasmids containing *mcr-1* are widely distributed in human pathogens and are the efficient vector for the transmission of *mcr-1* and other drug-resistant genes (Hammad et al., 2019). Whether MDR IncHI2 plasmids can alter the *Salmonella* resistance phenotype and disseminate rapidly is concerning.

The insertion sequence (IS) family in the bacterial genome with a widely variable DNA sequence in nature is also one of the significant modes for resistance gene transmission between bacterial pathogens. The 11 plasmids are classified into distinct patterns based on the existence of IS elements and the connection with *mcr-1* sites. In our study, IS26 elements are closely related to the IncX4 type plasmid, while the ISApl1 element is closely related to the IncHI2 type plasmid. ISApl1 is consistently associated with the *mcr-1* gene and the *mcr-1* gene cassette can be inserted into a variety of genetic loci in different plasmids. Five of the six IncHI2 plasmids contained the ISApl1 insertion sequence located upstream of *mcr-1*, and only one plasmid lacked ISApl1 around *mcr-1*. The IncHI2 backbone structure is considered to be stable (Garcia-Fernandez and Carattoli, 2010), and the presence

of different resistance genes in these plasmids is probably due to the acquisition of different mobile genetic elements (Cain and Hall, 2012). As such, the existence of ISApl1 in our study offers a potential hotspot for involving novel antibiotic resistant genes.

CONCLUSION

The present study describes the genetic characterization of *mcr-1*-positive multidrug-resistant *S.* Typhimurium isolated from intestinal infection in children and pork offal in China. Our results indicated that *mcr-1*-positive *S.* Typhimurium strains were multidrug-resistant, and one strain was additionally resistant to ceftriaxone and azithromycin. Three types of plasmids harboring *mcr-1* have respective characteristics regarding IS and resistance genes. Plasmids harboring *mcr-1* and other resistance genes confer resistance to colistin and other multiple antibiotics. Therefore, the findings of this study are critical to estimating the transmission of *mcr-1* and monitoring the international epidemic.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

JL, SQ, and HS designed the study. HZ wrote the main manuscript. YX, BL, and XX participated in the specimen collection and revised the manuscript. YH and LW contributed to the bioinformatics data analysis. JX, XD, XH, and LJ participated in data collection. XD, CY, HL (9th author), HL (10th author), HW, and MY performed the experiments. YJ critically revised important knowledge content. YJ, SQ, and HS gave final approval of the version to be submitted. All authors made substantial contributions to preparation and submission of the manuscript.

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SUPPLEMENTARY MATERIAL

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Antimicrobial Resistance Dynamics in Chilean *Shigella sonnei* Strains Within Two Decades: Role of *Shigella* Resistance Locus Pathogenicity Island and Class 1 and Class 2 Integrons

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Shigellosis is an enteric infectious disease in which antibiotic treatment is effective, shortening the duration of symptoms and reducing the excretion of the pathogen into the environment. *Shigella* spp., the etiologic agent, are considered emerging pathogens with a high public health impact due to the increase and global spread of multidrug-resistant (MDR) strains. Since *Shigella* resistance phenotype varies worldwide, we present an overview of the resistance phenotypes and associated genetic determinants present in 349 Chilean *S. sonnei* strains isolated during the periods 1995–1997, 2002–2004, 2008–2009, and 2010–2013. We detected a great variability in antibiotic susceptibility patterns, finding 300 (86%) MDR strains. Mobile genetic elements (MGE), such as plasmids, integrons, and genomic islands, have been associated with the MDR phenotypes. The *Shigella* resistance locus pathogenicity island (SRL PAI), which encodes for ampicillin, streptomycin, chloramphenicol, and tetracycline resistance genes, was detected by PCR in 100% of the strains isolated in 2008–2009 but was less frequent in isolates from other periods. The presence or absence of SRL PAI was also differentiated by pulsed-field gel electrophoresis. An atypical class 1 integron which harbors the bla_{OXA-1}-*aadA1-IS1* organization was detected as part of SRL PAI. The *dfrA14* gene conferring trimethoprim resistance was present in 98.8% of the 2008–2009 isolates, distinguishing them from the SRL-positive strains isolated before that. Thus, it seems an SRL-*dfrA14 S. sonnei* clone spread during the 2008–2009 period and declined thereafter. Besides these, SRL-negative strains harboring class 2 integrons with or without resistance to nalidixic acid were detected from 2011 onward, suggesting the circulation of another clone. Whole-genome sequencing of selected strains confirmed

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the results obtained by PCR and phenotypic analysis. It is highlighted that 70.8% of the MDR strains harbored one or more of the MGE evaluated, while 15.2% lacked both SRL PAI and integrons. These results underscore the temporal dynamics of antimicrobial resistance in *S. sonnei* strains circulating in Chile, mainly determined by the spread of MGE conferring MDR phenotypes. Since shigellosis is endemic in Chile, constant surveillance of antimicrobial resistance phenotypes and their genetic basis is a priority to contribute to public health policies.

Keywords: *Shigella sonnei*, antibiotic resistance, mobile genetic elements (MGE), integrons, SRL pathogenicity island, multidrug-resistant (MDR) bacteria, *dfrA14*, class 2 integron

INTRODUCTION

Shigellosis is one of the most common diarrheal diseases in children under 5 years old, especially in developing countries, although it occurs in people of all ages worldwide (Kotloff et al., 2018). *Shigella* spp., the etiologic agent, are considered emerging pathogens with a high public health impact due to the increase and global spread of multidrug-resistant (MDR) strains (Chu et al., 1998; Ashkenazi et al., 2003; Vrints et al., 2009; Shiferaw et al., 2012; Gu et al., 2017; Puzari et al., 2018). This situation is particularly worrying because shigellosis is one of the few enteric infections for which antibiotics are recommended as therapeutic management (Williams and Berkley, 2016, 2018). Antibiotics shorten the severity and duration of symptoms, reducing the excretion time of the pathogen and potential complications of the infection (Chu et al., 1998).

Shigella resistance phenotype varies worldwide. The emergence of *Shigella* spp. resistant to the most commonly used antibiotics for treating this disease is reported in Latin America, North America, Europe, and South Asia (Chu et al., 1998; Oh et al., 2003; Gu et al., 2012; Shiferaw et al., 2012; Marcoleta et al., 2013; Puzari et al., 2018; Chung The et al., 2019, 2021; Sati et al., 2019; Bardsley et al., 2020).

The genetic determinants of MDR phenotypes in *Shigella* spp. have been associated with the presence of different plasmids, integrons, and genomic islands (Muñoz et al., 2003; Turner et al., 2003; Toro et al., 2005; Barrantes and Achí, 2016; Miranda et al., 2016; Kang et al., 2019; Ranjbar and Farahani, 2019). The *Shigella* resistance locus pathogenicity island (SRL PAI) is a 66-kb genomic region inserted into the *serX* gene (which codifies for $tRNA^{Ser}$), conferring resistance to ampicillin, streptomycin, chloramphenicol, and tetracycline. A ferric dicitrate transport system and genes involved in D-aspartate metabolism are also encoded in SRL PAI, described firstly in *Shigella flexneri* 2a YSH6000 ($SRL_{YSH6000}$; Luck et al., 2001; Turner et al., 2003; Henríquez et al., 2020). Additionally, integrons have played an essential role in the spread of antimicrobial resistance genes since their discovery in 1989. Integrons are composed of three elements: the *intI* gene that encodes a tyrosine recombinase (integrase), the adjacent recombination site (*attI*) recognized by the integrase, and the promoter (P_c) necessary for the efficient transcription and expression of gene cassettes present in the integron. They have the ability to capture and express foreign genes, such as resistance gene cassettes, and facilitate their horizontal transfer to a wide range of pathogens (Stokes and Hall, 1989; Recchia and Hall, 1995). Class 1 and class 2 integrons have widely disseminated among species of the family *Enterobacteriaceae* and other Gram-negative bacteria. In *Shigella* spp., antimicrobial resistance is often due to both classes of integrons (Pan et al., 2006; Chang et al., 2011; Kaushik et al., 2018; Shariati et al., 2018; Kang et al., 2019).

The heterogeneous geographical distribution of antimicrobial-resistant strains and mobile genetic elements (MGE) underscores the importance of keeping the local antimicrobial resistance surveillance. Hence, the objective of this study was to characterize the phenotypic and genotypic diversity of Chilean *S. sonnei* that have circulated for almost two decades from 1995 to 2013, focusing on the MDR strains.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

A total of 349 *Shigella sonnei* strains were obtained from a bacterial collection belonging to the Programa de Microbiología y Micología, ICBM, Universidad de Chile, isolated from stool samples of patients suffering from acute diarrhea (n = 190; 1995–2004) and from the Instituto de Salud Pública de Chile [ISP], 2019 (Public Health Institute of Chile) (n = 159; 2010–2013). These strains were grouped into five time periods according to the isolation date: period A, between 1995 and 1997 (n = 60); period B, between 2002 and 2004 (n = 50); period C, between 2008 and 2009 (n = 80); period D, between 2010 and 2011 (n = 80); and period E, between 2012 and 2013 (n = 79) (**Figures 1B,C**). *S. sonnei* strains from periods A and B were isolated from patients living in Región Metropolitana. Period C is composed of strains mostly isolated from Región Metropolitana (61/80) and Antofagasta (15/80), a region located 1,000 km north of Región Metropolitana. Periods D and E include representative strains from Región Metropolitana (40 in each period) and northern and southern areas of the country (**Figures 1B,C**). The isolates were stored at −80°C in trypticase soy broth with 30% glycerol. The *Shigella* strains were identified by conventional and automated biochemical methods (VITEK-2, bioMérieux) and serotyped by agglutination with type-specific antisera (phase I and II, Denka Seiken, Tokyo, Japan). All strains were routinely cultured at 37°C on LB broth or agar.

Antimicrobial Susceptibility

The antibiotic susceptibility of *S. sonnei* strains was determined by disk diffusion assay tests, performed according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2018). The antibiotics included the following: ampicillin (AMP), 10 μg; cefotaxime (CTX), 30 μg; chloramphenicol (CHL), 30 μg; tetracycline (TET), 30 μg; streptomycin (STR), 10 μg; sulfamethoxazole/trimethoprim (SXT), 23.75/1.25 μg; trimethoprim (TMP), 30 μg; nalidixic acid (NAL), 30 μg; and ciprofloxacin (CIP), 5 μg. *Escherichia coli* ATCC 25922 was included as control. For purposes of the current analysis, both intermediate and resistant isolates were considered resistant.

Detection of Integrons and *Shigella* Resistance Locus Pathogenicity Island

The presence of the MGE associated with antibiotic resistance was detected by conventional PCR. DNA templates were obtained from bacterial lysates of individual colonies or genomic DNA preparation (E.Z.N.A. Bacterial DNA kit, Omega, Bio-Tek, Atlanta, United States). Specific primers for class 1 and class 2 integrases were designed (**Supplementary Table 1**). Two microliters of the sample were added to 20 μl of the reaction mix containing 3 mM $MgCl_2$, 400 μM (each) dNTPs (Invitrogen, United States), and primers (25 nM) together with 1 U of *Paq* polymerase (Agilent Technologies, United States). PCR reactions were performed as follows: an initial 2-min denaturation cycle at 95°C, followed by 30 cycles at 95°C for 1 min, 56°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1% agarose gels and stained with ethidium bromide.

Primers for the detection of SRL PAI were designed using the sequences of the flanking regions of the *serX* gene and the SRL island of *S. flexneri* YSH6000 (GenBank accession number: AF326777.3; Luck et al., 2001). The presence of SRL PAI was defined by three PCR reactions; the presence of SRL PAI (SRL-positive) was defined by the following pattern: SRL I (−), SRL II (+), and SRL III (+). When the PCR pattern was SRL I (+), SRL II (−), and SRL III (−), strains were classified as SRL-negative. Any other amplification pattern was considered also as SRL-negative. The PCR reactions were performed with an initial 2-min denaturation cycle at 95°C, followed by 30 cycles at 95°C for 1 min, 50°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1.5% agarose gels and stained with ethidium bromide.

Detection of Antimicrobial Resistance Genetic Markers

DNA templates were obtained as mentioned before. Two microliters of the sample was added to 20 μl of the reaction mix containing 3 mM $MgCl_2$, 400 μM (each) dNTPs (Invitrogen, United States), and primers together with 1 U of *Paq* polymerase (Agilent Technologies, United States). Specific primers for trimethoprim resistance genes (*dfrA1*, *dfrA8*, and *dfrA14*), bla_{OXA-1} for ampicillin, *cat* for chloramphenicol, and *qacEΔ1* for quaternary ammonium salts resistance were previously described (**Supplementary Table 1**; Di Conza et al., 2002; Toro et al., 2005; Miranda et al., 2016). The PCR reactions were performed as follows: an initial 2-min denaturation cycle at 95°C, followed by 30 cycles at 95°C for 1 min, 56°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

Pulsed-Field Gel Electrophoresis

According to PulseNet protocol (CDC)[1], bacterial suspensions of 276 *S. sonnei* strains were embedded in agarose plugs, lysed, and then digested with endonuclease *Xba*I (Thermo Fischer Scientific, United States) at 37°C for 2 h. The macrorestriction of genomic DNA fragments was separated by pulsed-field gel electrophoresis on a CHEF-DRIII Chiller system (Bio-Rad Laboratories, Richmond, CA, United States) in 1% agarose gel using 0.5X TBE buffer at 6 V/cm and 14°C, with ramped pulse times of 2.2–54.2 s for 21 h. *Salmonella enterica* serovar Braenderup strain H9812 was included as molecular size standard three times on each gel to normalize the images and to compare the fingerprints among several gels. The DNA band profiles were analyzed with GelCompar software (version 3.0; Applied Maths, Sint-Martens-Latem, Belgium). A similarity dendrogram was constructed using the unweighted-pair group method with arithmetic mean (UPGMA) with the Dice similarity coefficient and a band tolerance of 1.5%. Pulsegroups were defined by sharing 73% similarity and more than 93.5% for pulsetypes.

Genome Sequencing and Data Processing

Illumina sequencing was performed on 29 selected Chilean *S. sonnei* strains by Microbes NG (Birmingham, United Kingdom) using the Nextera XT library protocol. Sequencing was done on the Illumina MiSeq platform (Illumina, San Diego, CA, United States). Reads were processed with Trimmomatic 0.30 to remove adapters and for quality trimming. For *de novo* genome assembly, reads were processed using Shovill[2] with Spades 3.14.0 (Nurk et al., 2013)[3], with a Kmer range from 31 to 127 (31, 55, 79, 103, and 127). For SNP identification, the trimmed reads were mapped to the reference genome of *S. sonnei* SS046 using Snippy with default parameters[4]. The core SNPs were identified from the alignment, and a final phylogenetic tree was generated using FastTree (Price et al., 2010). Resfinder was used to predict the presence of known acquired resistance genes and chromosomal mutations associated with resistance to quinolones (Camacho et al., 2009; Bortolaia et al., 2020; Zankari et al., 2020)[5]. MLST typing using sequences was done by https://cge.cbs.dtu.dk/services/MLST/ (Larsen et al., 2012). All the assembled genomes from this study are available on NCBI under the BioProject accession PRJNA602693.

[1]https://www.cdc.gov/pulsenet/pathogens/protocols.html

[2]https://github.com/tseemann/shovill

[3]https://link.springer.com/chapter/10.1007%2F978-3-642-37195-0_13

[4]https://github.com/tseemann/snippy

[5]https://cge.cbs.dtu.dk/services/ResFinder/

Comparative Genomic Analysis

Draft genome sequences were managed using the Geneious software (v11.0.5; Biomatters, Ltd., New Zealand). The assembled contig sequence was mapped against the reference sequence of the SRL PAI carried by *S. flexneri* 2a str YSH6000 using BlastN in Geneious. Comparisons of the genetic structure of SRL PAIs were performed using EasyFig v2.1 (Sullivan et al., 2011).

Statistical Analysis

Evaluation of pairwise association between the timeframe of isolation of the strains and the specific genes that they carry was performed using contingency tables by odds ratios. The statistical significance of these associations was determined by Pearson's chi-square test or Fisher's exact test (when frequencies were less than 5) (R Core Team, 2014; Csardi, 2015; Wei et al., 2017). When any of the cell values of the contingency table was zero, Haldane correction (Haldane, 1940) was used by adding 0.5 to all cells.

RESULTS

Antibiotic Resistance Phenotypes of Chilean *S. sonnei* Strains

To characterize the molecular and phenotypic resistance profile of Chilean *S. sonnei* strains, we analyzed 349 available strains isolated between the years 1995 and 2013. To better understand the temporal dynamics of antibiotic resistance of these strains, they were grouped according to the date of isolation in periods A–E. This collection included a total of 80 strains isolated in 2008–2009, which corresponds to period C, when an increase of *S. sonnei* frequency was registered (**Figure 1A**). The geographical origin of the strains is shown in **Figures 1B,C**.

Antimicrobial susceptibility test (AST) for all these 349 strains showed that 100% were sensitive to ciprofloxacin, while 3 (0.9%) and 33 (9.5%) isolates were resistant to cefotaxime and nalidixic acid, respectively. In contrast, these strains displayed high rates of antibiotic resistance to streptomycin (305, 87.4%), trimethoprim (264, 75.6%), ampicillin (257, 73.6%), tetracycline (257, 73.6%), sulfamethoxazole/trimethoprim (256, 73.3%), and chloramphenicol (195, 55.9%). The resistant phenotype to the last five antibiotics in period C is particularly noteworthy (**Table 1**).

Isolates resistant to at least one antibiotic in three or more drug classes were considered MDR[6]. Consequently, 300 out of 349 strains (86%) presented an MDR phenotype. Furthermore, the most frequent MDR phenotype was AMP-CHL-TET-STR-SXT-TMP, displayed by 123 strains (35.2%), followed by AMP-CHL-TET-STR (36, 10.3%), AMP-STR-SXT-TMP (32, 9.2%), TET-STR-SXT-TMP (25, 7.2%), and AMP-CHL-TET-SXT-TMP (20, 6.7%) (**Supplementary Table 2**).

Figure 2 shows the temporal distribution of the resistance phenotypes by antibiotic (concentric circles) and MDR phenotypes simultaneously. It should be noted that all the strains of period C displayed resistance to six antibiotics (AMP-CHL-TET-STR-SXT-TMP). As mentioned above, this was the most frequent MDR profile in the present study. Interestingly, this MDR phenotype was observed with a lower frequency not only before period C (5/349, 1.4%) but also after this (38/349, 10.9%). On the contrary, in the other periods, heterogeneous MDR phenotypes were observed. Analyzing each period, the most frequent MDR phenotypes in period A were AMP-CHL-TET-STR (20/60, 33%) and AMP-STR-SXT-TMP (19/60, 32%). In period B, AMP-STR-SXT-TMP (12/50, 24%) and AMP-TET-STR-SXT-TMP (10/50, 20%) were detected. Meanwhile, these were AMP-CHL-TET-STR-SXT-TMP (19/80, 24%) and AMP-CHL-TET-SXT-TMP (15/80, 19%) in period D and TET-STR-SXT-TMP-NAL (25/79, 32%) and AMP-CHL-TET-STR-SXT-TMP (19/79, 24%) in period E. Notably, 32 out of 33 NAL^R strains were isolated in period E, and 25 of them displayed the TET-STR-SXT-TMP-NAL MDR phenotype, suggesting the emergence of a new clone of *S. sonnei* at that time.

Distribution of Genetic Elements Associated to Multidrug-Resistant in Chilean *S. sonnei* Strains

The simultaneously resistant phenotype to ampicillin, streptomycin, chloramphenicol, and tetracycline in some *S. sonnei* strains suggested the presence of the SRL PAI. To determine whether these strains harbor this genetic element, three PCR assays were designed. A combination of SRL I, SRL II, and SRL III PCR amplification patterns allowed us to detect the insertion of SRL PAI within the *serX* gene. The SRL I (−), SRL II (+), and SRL III (+) pattern was expected for SRL-positive strains. Conversely, the SRL I (+), SRL II (−), and SRL III (−) pattern was expected for no insertion at *serX*, indicating the absence of SRL PAI.

Of the 349 strains tested, 192 (55%) were classified as SRL-positive and 157 (45%) as SRL-negative. The 192 SRL-positive strains were distributed as follows: 24/60 (40%) from period A, 2/50 (4%) from period B, 80/80 (100%) from period C, 52/80 (65%) from period D, and 34/79 (43%) from period E (**Figure 3A**). The detailed SRL PAI distribution in the strains is described in **Supplementary Table 2**. The group of SRL-positive strains includes five strains that should have been classified as SRL-negative due to the PCR pattern. Nonetheless, because their antibiotic profile corresponded to the SRL-positive phenotype, we further evaluated the presence of pathogenicity island integrase and *orf58* genes as well as antibiotic resistance determinant genes in these strains (**Supplementary Table 1**). The results allowed us to confirm the presence of SRL PAI probably at another insertion site (SRL + out) (data not shown). Any other PCR pattern was considered negative for the presence of SRL, which was obtained for 14 strains (**Supplementary Table 2**).

In addition, class 1 and class 2 integrons were searched by PCR, using specific primers for integrases (**Supplementary Table 1**). Class 1 integron was detected in 194/349 (55.6%) strains, including 100% of the SRL-positive strains (**Figure 3B**). This result is consistent with the presence of an atypical class 1 integron within SRL PAI, which harbors the bla_{OXA-1}-*aadA1-IS1* gene organization. Indeed, we assessed and detected the adjacent

[6]https://www.cdc.gov/narms/resources/glossary.html

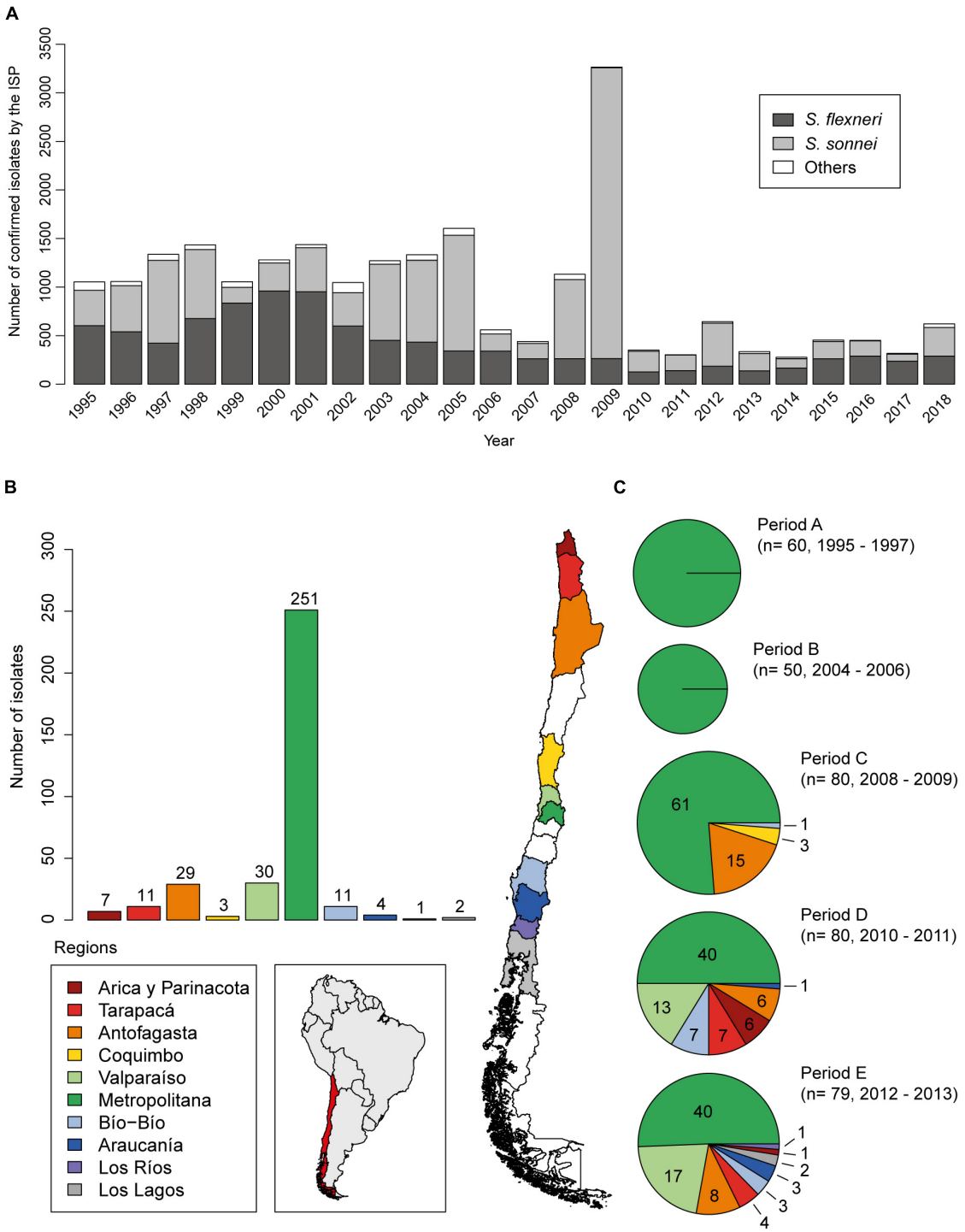

FIGURE 1 | Epidemiological distribution of *Shigella sonnei* in Chile. **(A)** Nationwide detection of *Shigella* spp. strains confirmed by Instituto de Salud Pública de Chile, ISP from 1995 to 2018. **(B)** Geographical distribution of 349 *Shigella sonnei* strains analyzed in this work. **(C)** Characterization of groups defined by time of isolation and geographical distribution. *S. sonnei* strains from periods A and B were isolated from patients living in Region Metropolitana. Period C is composed by strains mostly isolated from the Region Metropolitana (61/80) and from Antofagasta (15/80), a region 1,000 km north from Santiago (Región Metropolitana, Chile). Periods D and E include representative strains from Región Metropolitana (40 in each period) and from the northern and southern regions of the country, which were kindly provided by the ISP **(B,C)**.

TABLE 1 | Antimicrobial resistance of Chilean *Shigella sonnei* strains from 1995 to 2013.

Antimicrobial agent	Periods					
	A (1995–1997)	B (2002–2004)	C (2008–2009)	D (2010–2011)	E (2012–2013)	Total (1995–2013)
Streptomycin	60 (100)	50 (100)	80 (100)	51 (63.8)	64 (81)	305 (87.4)
Trimethoprim	28 (46.7)	27 (54)	80 (100)	61 (76.3)	68 (86.1)	264 (75.6)
Ampicillin	52 (86.7)	31 (62)	80 (100)	53 (66.3)	41 (51.9)	257 (73.6)
Tetracycline	27 (45)	17 (34)	80 (100)	64 (80)	69 (87.3)	257 (73.6)
Sulfamethoxazole/trimethoprim	28 (46.7)	25 (50)	80 (100)	57 (71.3)	66 (83.5)	256 (73.4)
Chloramphenicol	25 (41.7)	1 (2)	80 (100)	53 (66.3)	36 (45.6)	195 (55.9)
Nalidixic acid	0 (0)	0 (0)	0 (0)	1 (1.3)	32 (40.5)	33 (9.5)
Cefotaxime	0 (0)	0 (0)	0 (0)	1 (1.3)	1 (1.3)	3 (0.9)
Ciprofloxacin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

The number of strains analyzed by period are described as follows: A = 60, B = 50, C = 80; D = 80, and E = 79.
The number of strains and percentage in parenthesis are shown for each cell.

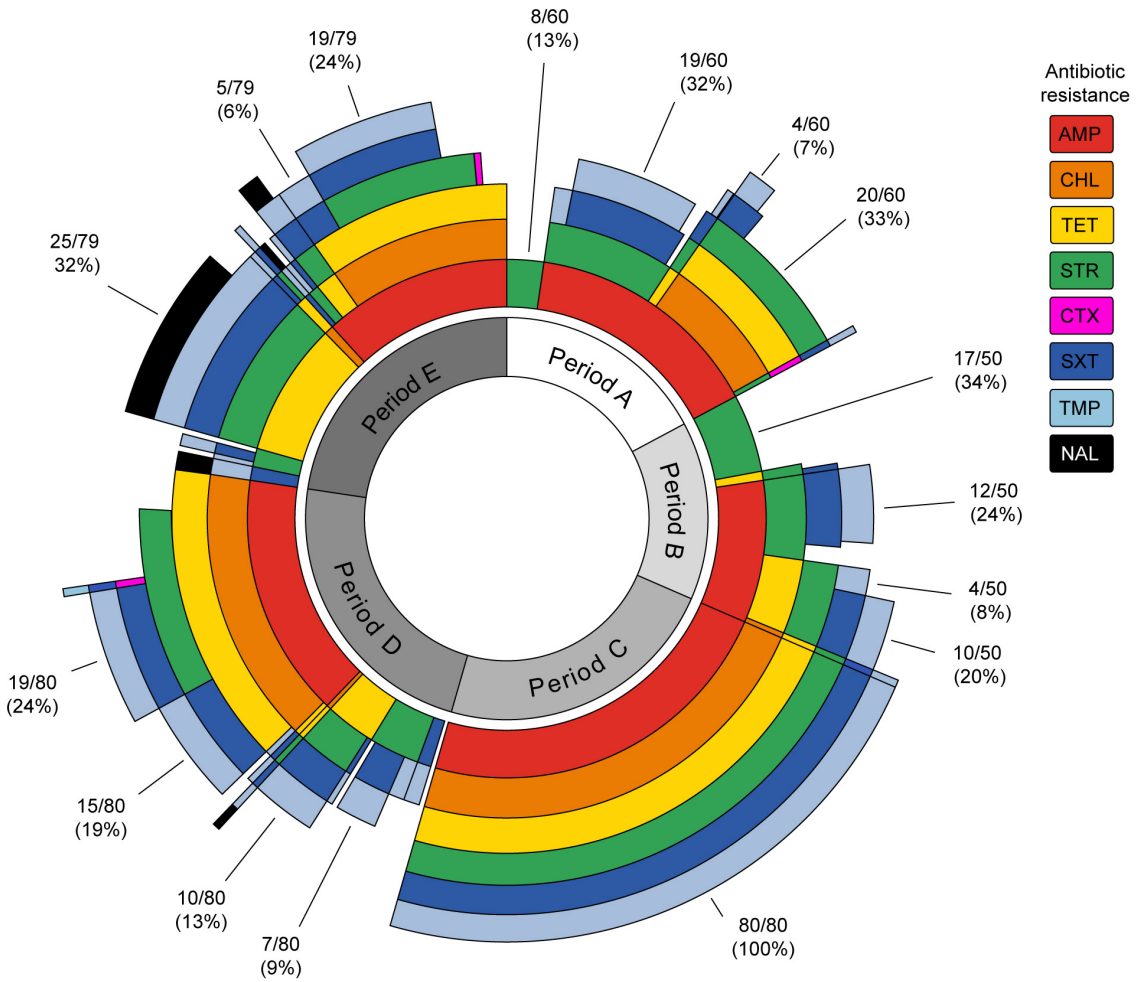

FIGURE 2 | Sunburst plot showing the antibiotic resistance and multi-drug resistance phenotypes of *Shigella sonnei* strains isolated in different time periods. Each antibiotic is represented by a concentric circle of a different color. The sum of the concentric circle represents the resistance phenotype pattern for each strain. The percentages of most frequent profiles for each period are shown. AMP, ampicillin; CHL, chloramphenicol; TET, tetracycline; STR, streptomycin; CTX, cefotaxime; SXT, sulfamethoxazole/trimethoprim; TMP, trimethoprim; NAL, nalidixic acid. The figure was prepared using the sunburstR (Bostock et al., 2019) package in R (R Core Team, 2014).

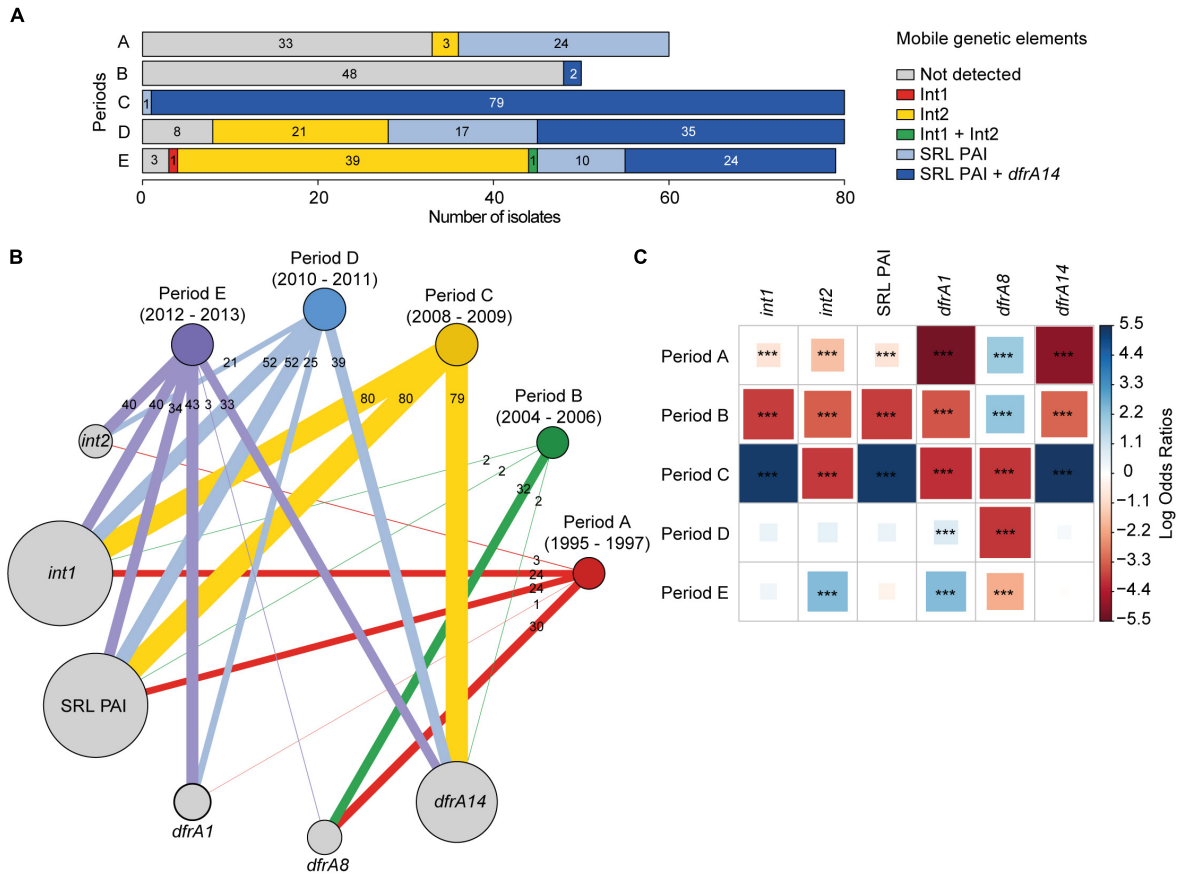

FIGURE 3 | Patterns of association between the *Shigella sonnei* isolation date (periods A–E) and genetic elements related to antimicrobial resistance. **(A)** Distribution of class 1, class 2 integrons, SRL PAI, and *dfrA14* gene. **(B)** Graph of modules showing the interactions between periods of isolation and specific genetic determinants. Modules are weighted by the number of strains. Besides this, links are weighted by both the number of strains linked between modules and the number of strains within modules. The figure was prepared using the igraph (Csardi, 2015) package in R (R Core Team, 2014). **(C)** Pairwise association plot for periods of isolation and specific genetic elements. Red and blue squares represent negative and positive associations, respectively. The color scale represents the magnitude of the association determined by odds ratios. ***$p < 0.001$ determined by Pearson's chi-square test or Fisher's exact test. The figure was prepared using the package corrplot (Wei et al., 2017) in R (R Core Team, 2014).

bla_{OXA-1} and *aadA1* genes by PCR for at least 70 SRL-positive strains (data not shown).

Of the 157 SRL-negative strains, only one SRL-negative strain from period E harbored a typical class 1 integron (0.3%) (**Figure 3A**), defined by the *dfrA1-aadA1-qacEΔ1-sul1* gene cluster and further confirmed by PCR and whole-genome sequencing (WGS) of the strain.

On the other hand, class 2 integron was detected in 64/349 (18.3%) strains; all of them were SRL-negative isolates. They belong to the following periods: 3 to period A, 21 to period D, and 40 to period E, with one of them harboring class 1 and class 2 integrons simultaneously (**Figure 3A**). It should be noted that class 2 integron was neither detected among strains from period B nor C (**Figure 3B**). In addition, 92 isolates (26.4%) were negative for MGE, SRL PAI, and integrons. Of this group, 53 isolates (15.2%) were MDR.

It is interesting to remark that 144 out of 192 (75%) SRL-positive strains displayed an MDR phenotype to AMP-CHL-TET-STR and also to SXT and TMP. Therefore, we searched for the presence of previously detected *dfrA1*, *dfrA8*, and *dfrA14* genes, which confer resistance to TMP (TMP^R) (Miranda et al., 2016). Overall, *dfrA1*, *dfrA8*, and *dfrA14* genes were detected in 69 (19.7%), 65 (18.6%), and 153 (43.8%) strains, respectively (**Figure 3B**). Focusing on the 264 TMP^R strains, we detected *dfrA1* in 54 strains (20.5%), *dfrA8* in 51 (19.3%) strains, and *dfrA14* in 142 (53.8%) strains as sole TMP^R genetic marker. The combinations *dfrA1-dfrA8*, *dfrA1-dfrA14*, and *dfrA8-dfrA14* were scarcely present in 1.1% (3), 3.8% (10), and 0.4% (1), respectively. Three TMP^R strains did not carry any of these TMP gene markers, while two TMP-sensitive strains harbored *dfrA1* cassette, and 10 carried *dfrA8* (**Supplementary Table 2**).

Notably, 140 out of 144 (97.2%) SRL-positive TMP^R strains harbored the *dfrA14* gene, distributed as follows: 2 strains in period B, 79 in C, 35 in D, and 24 in E. From the four remaining strains, two of them harbored *dfrA8*, and two lacked the tested TMP^R genes. On the other hand, there were 120 SRL-negative TMP^R strains that harbored a unique TMP^R gene, distributed as follows: 54 with *dfrA1*, 49 with *dfrA8*, and 4 with *dfrA14*. The

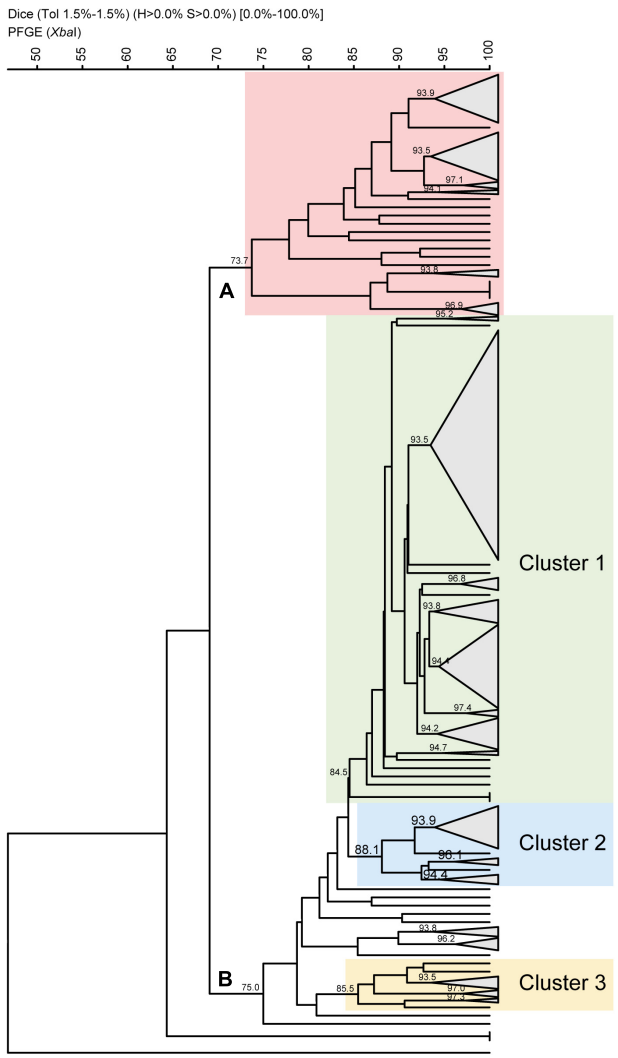

FIGURE 4 | Dendrogram based on Dice coefficients of similarity for 276 Chilean *Shigella sonnei* strains by pulsed-field gel electrophoresis patterns. The dendrogram was constructed by UPGMA (GelCompar II). Shown are two pulsegroups **(A,B)** with a similarity of 69.1%. Twenty-two pulsetypes were defined with a similarity of >93.5%. Within pulsegroup A, all the strains belong to periods A and B, and most of them harbored *dfrA8* as TMP resistance marker. Pulsegroup B comprises 214 strains divided in three clusters. Within cluster 1, there are 144 SRL and SRL/*dfrA14* strains. Cluster 2 contains 25 strains, of which 21 are NAL-resistant strains harboring class 2 integron. Cluster 3 is composed of 13 strains, of which 11 carry class 2 integron, but they are NAL-sensitive strains.

presence of *dfrA1-dfrA8* or *dfrA1-dfrA14* pairs was detected in 3 and 9 strains, respectively, and one strain was negative for these markers. In addition, the presence of *dfrA1* gene was detected among 62 out of 64 class 2 integron-positive strains.

All these associations are illustrated in **Figure 3B**, where it can be seen that the distribution of these genetic determinants depends on the isolation periods. In this context, strains from periods A and B were significantly associated with the presence of the *dfrA8* gene; the presence of the SRL PAI, the atypical class 1 integron, and the *dfrA14* gene was associated with strains from period C, while the presence of the class 2 integron and the *dfrA1* gene was associated with strains from period E (**Figure 3C**).

In summary, we detected 300 (86%) MDR strains out of 349. They were distributed in 247 (70.8%) containing one or more of the MGE evaluated in this work. In contrast, only 53 (15.2%) were negative for SRL PAI and class 1 and 2 integrons.

Pulsed-Field Gel Electrophoresis Analysis of Chilean *S. sonnei* Strains

To assess the clonality of the strains in this study, we performed a pulsed-field gel electrophoresis analysis of 276 *S. sonnei* isolates using *Xba*I digestion. The samples covering all the periods were randomly selected, including 56 from period A, 36 from period B, 71 from period C, 48 from period D, and 65 from period E. The obtained tree was split into two major pulsegroups with 69.1% of similarity (**Figure 4**). Pulsegroup A comprises 62 strains with similarities greater than 73.7% and whose common feature was the absence of SRL PAI. In addition, 55 isolates out of 62 belonged to periods A and B, i.e., strains isolated between 1995 and 2004 (88.7%), and 42 of them (67.7%) carried the genetic marker *dfrA8*.

Pulsegroup B includes 214 strains sharing 75% similarity, and it represents a more heterogeneous group with 3 subgroups or clusters (**Figure 4**). The largest subgroup, named cluster 1, includes 158 strains sharing 84.5% of similarity. Here 144 out of the 163 tested SRL-positive strains were grouped, with 108 of them carrying SRL/*dfrA14* and 36 just SRL. Cluster 1, the largest pulsetype defined at 93.5% similarity, included 84 strains: 70 isolates carried SRL/*dfrA14*, 10 strains harbored only SRL PAI, and 4 strains were without the island, regardless of the collection period. A subgroup composed of 25 strains (cluster 2) includes 23 isolates harboring the class 2 integron, 21 of which are simultaneously resistant to nalidixic acid. Finally, there is a subgroup composed of 13 strains (cluster 3), from period E, 11 of which have a class 2 integron. Hence, both clusters B2 and B3 include 75.6% of class 2 integron-positive strains and 84% of nalidixic acid-resistant strains of this analysis.

Whole-Genome Sequence Analysis From Chilean *S. sonnei* Strains

A total of 29 isolates representing different patterns of resistant phenotypes were selected for sequencing. Strains from all periods, positive and negative for SRL PAI and class 1 or 2 integrons, were included. Genetic determinants of resistance were obtained by Resfinder (Bortolaia et al., 2020) and were concordant with phenotypical AST and PCR results (**Table 2**). Seventeen out of 29 sequenced strains harbored SRL PAI: 11 were SRL/*dfrA14*-positive, and the remaining 6 strains carried only SRL PAI. The presence of the bla_{OXA-1}, *aadA1*, *cat*, and *tetB* genes in those strains was confirmed. In those SRL/*dfrA14*-positive strains, this gene was harbored in the pABC-3 plasmid. In all cases, disruption of the *strA* gene by the insertion of *dfrA14*, included in the *sul2*/*strA*::*dfrA14*/*strB* genes cluster, was identified. In addition, the *dfrA15* gene was detected as another determinant of TMP^R through sequencing of one of the three TMP^R strains negative for the other alleles (**Table 2**).

TABLE 2 | Phenotypic and genotypic characterization of 29 WGS Chilean *Shigella sonnei* strains.

						Antibiotic susceptibility profiles									PCR detection						WGS		WGS predicted antibiotic resistance genes[a]																
Period	Strain	Refseq assembly accession	Origin region	Isolation year	PFGE	AMP	CTX	CHL	TET	STR	SXT	TMP	NAL	CIP	Class 1 *int1*	Class 2 *int2*	SRL PAI	*dfrA1*	*dfrA8*	*dfrA14*	WGS-predicted MLST[b]	Lineage classification	*blaOXA*	*blaTEM*	*cat*	*cmlA1*	*tetB*	*tetA*	*aadA1*	*strA*	*strB*	*sul1*	*sul2*	*dfrA1*	*dfrA8*	*dfrA14*	*dfrA15*
A	a0044	GCF_019732475.1	Metropolitana	1995	+	R	S	S	S	R	S	R	S	S	−	+	−	−	+	−	ST-152	IIa	−	+	−	−	−	−	−	+	+	−	+	−	+	−	−
A	a0047	GCF_019732435.1	Metropolitana	1995	+	R	S	R	R	R	R	R	S	S	+	−	+	−	−	−	ST-152	IIIa	+	−	+	+	+	−	+	+	+	+	+	−	−	−	+
A	a0148	GCF_019732455.1	Metropolitana	1996	+	R	S	R	R	R	S	S	S	S	+	−	+	−	−	−	ST-152	IIIa	+	−	+	−	+	−	+	+	+	−	+	−	−	−	−
A	a0157	GCF_019732395.1	Metropolitana	1996	+	R	S	S	R	R	R	R	S	S	−	+	−	+	+	−	ST-152	IIIa	−	+	−	−	−	+	−	+	+	−	+	−	+	−	−
A	a0189	GCF_019732375.1	Metropolitana	1997	+	R	S	R	R	R	S	S	S	S	+	−	+	−	−	−	ST-152	IIIa	+	−	+	−	+	−	+	+	+	−	+	−	−	−	−
A	a0195	GCF_019732415.1	Metropolitana	1997	+	R	S	S	S	R	R	R	S	S	−	−	−	−	+	−	ST-152	IIb	+	+	−	−	−	−	−	+	+	−	+	−	−	−	−
A	a0214	GCF_019732315.1	Metropolitana	1997	+	R	S	R	R	R	R	R	S	S	+	−	+	−	+	−	ST-152	IIIa	+	+	+	−	+	−	+	+	+	−	+	−	+	−	−
B	b0061	GCF_019732335.1	Metropolitana	2005	+	R	S	S	R	R	R	R	S	S	+	−	+	−	+	+	ST-152	IIIa	+	+	+	−	+	+	+	+[c]	+	−	+	−	+	+	−
B	b0074	GCF_019732345.1	Metropolitana	2004	+	S	S	S	S	R	S	S	S	S	−	−	−	−	−	−	ST-152	V	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−
B	b0200	GCF_019732295.1	Metropolitana	2006	+	R	S	R	R	I	R	R	S	S	+	−	+	−	−	+	ST-152	IIIa	+	−	+	−	+	−	+	+[c]	+	−	+	−	−	+	−
B	b0566	GCF_019732275.1	Metropolitana	2005	+	R	S	S	R	R	R	R	S	S	−	−	−	−	+	−	ST-152	IIIa	−	+	−	−	−	−	−	+	+	−	+	−	+	−	−
C	c0725	GCF_019732245.1	Antofagasta	2008	+	R	S	R	R	I	R	R	S	S	+	−	+	−	−	+	ST-152	IIIa	+	+	+	−	+	+	+	+[c]	+	−	+	−	+	+	−
C	c0736	GCF_019732235.1	Antofagasta	2008	+	R	S	R	R	I	R	R	S	S	+	−	+	−	−	+	ST-152	IIIa	+	−	+	−	+	−	+	+[c]	+	−	+	−	−	+	−
C	c8225	GCF_019732175.1	Metropolitana	2009	+	R	S	R	R	R	R	R	S	S	+	−	+	−	−	+	ST-152	IIIa	+	−	+	−	+	−	+	+[c]	+	−	+	−	−	+	−
C	c8763	GCF_019732185.1	Metropolitana	2009	+	R	S	R	R	R	R	R	S	S	+	−	+	−	−	+	ST-152	IIIa	−	−	+	−	+	−	+	+[c]	+	−	+	−	−	+	−
C	c8852	GCF_013821725.1	Metropolitana	2009	+	R	S	R	R	R	R	R	S	S	+	−	+	−	−	+	ST-152	IIIa	+	−	+	−	+	−	+	+[c]	+	−	+	−	−	+	−
C	c8870	GCF_019732195.1	Metropolitana	2009	+	R	S	R	R	I	R	R	S	S	+	−	+	−	−	+	ST-152	IIIa	+	−	+	−	+	−	+	+[c]	+	−	+	−	−	+	−
D	d0150	GCF_019732155.1	Bío Bío	2011	+	S	S	S	S	S	R	R	S	S	−	−	−	−	−	+	ST-152	IIIa	−	−	−	−	−	−	−	+[c]	+	−	+	−	−	+	−
D	d0237	GCF_019732135.1	Metropolitana	2010	+	R	S	R	R	R	R	R	S	S	+	−	+	−	−	+	ST-152	IIIa	+	−	+	−	+	−	+	+[c]	+	−	+	−	−	+	−
D	d0930	GCF_019732105.1	Valparaíso	2010	−	R	S	S	R	S	R	R	S	S	−	+	−	+	−	−	ST-152	IIIb	−	−	−	−	−	−	+	+	+	−	+	+	−	−	−
D	d1397	GCF_019732095.1	Arica y Parinacota	2011	+	R	S	R	R	S	R	R	S	S	+	−	+	−	−	+	ST-152	IIIa	+	−	+	−	+	−	+	+[c]	+	−	+	−	−	+	−
D	d1533	GCF_019732055.1	Metropolitana	2011	+	R	S	R	S	S	S	R	S	S	+	−	+	−	−	+	ST-152	IIIa	+	−	+	−	+	−	+	+[c]	+	−	+	−	−	+	−
D	d2078	GCF_019732025.1	Valparaíso	2011	+	S	S	S	S	R	S	R	S	S	−	+	−	+	−	−	ST-152	IIIb	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−
D	d4511	GCF_019732015.1	Metropolitana	2010	−	S	S	S	R	R	R	R	S	S	−	+	−	+	−	−	ST-152	IIIb	−	−	−	−	−	−	+	+	+	−	+	+	−	−	−
E	e0300	GCF_019732075.1	Los Lagos	2012	+	S	S	S	S	S	I	R	R	S	−	+	−	+	−	−	ST-152	Global	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
E	e1006	GCF_019731995.1	Metropolitana	2012	+	I	S	S	R	R	R	R	R	S	−	+	−	+	−	−	ST-152	Global	−	−	−	−	−	+	−	+	+	−	+	+	−	−	−
E	e1087	GCF_019731955.1	Valparaíso	2013	+	R	S	R	R	S	S	S	S	S	+	−	+	−	−	−	ST-152	IIIa	+	−	+	−	+	−	+	−	−	−	−	−	−	−	−
E	e1409	GCF_013821745.1	Antofagasta	2012	−	R	I	R	R	S	S	S	S	S	+	−	+	−	−	−	ST-152	IIIa	+	−	+	−	+	−	+		−	−	−	−	−	−	−
E	e1660	GCF_019731975.1	Antofagasta	2013	+	R	S	S	R	R	R	R	S	S	+	−	−	+	−	−	ST-1504	Global	−	+	−	−	−	+	+	+	+	+	+	+	−	−	−

[a] *Predicted by ResFinder 4.1 tool (https://cge.cbs.dtu.dk/services/ResFinder/).*

[b] *Predicted by MLST 2.0 tool (https://cge.cbs.dtu.dk/services/MLST/).*

[c] *strA gene is interrupted by the insertion of dfrA14s.*

The lineage of the strains was determined according to the location of the genome in the full-core SNP phylogeny, comparing 470 publicly available *S. sonnei* sequences (**Figure 5**). This analysis allowed categorizing 20 out of 29 Chilean *S. sonnei* strains as sublineage IIIa. In fact, all SRL-positive strains sequenced in this study were sublineage IIIa. The seven sequenced strains harboring the class 2 integron were more heterogeneous, being part of sublineages IIa, IIIa, and IIIb and global. Three class 2 integron-positive strains were classified in sublineage IIIb, sharing this classification with most of the Argentinian strains. Two NAL^R-sequenced strains, displaying a D87Y mutation in *gyrA* and class 2 integron, belonged to the global lineage (**Table 2** and **Figure 5**).

Using the MLST 2.0 tool, we found that all but one of the sequenced strains had sequence type ST-152 (**Table 2**). The exception was a strain isolated in period E, whose sequence type was ST-1504. This strain belonged to the global sublineage and was confirmed to be the only one harboring the typical class 1 integron mentioned above.

Genomic Organization of *Shigella* Resistance Locus Pathogenicity Island in *Shigella*

Since the SRL PAI was widely distributed among Chilean *S. sonnei* strains isolated over a period of 18 years, we investigated the genetic conservation of this PAI. For this, we performed alignments between the prototypical SRL PAI harbored by the *S. flexneri* 2a str. YSH6000 ($SRL_{YSH6000}$) and five draft genomes of *S. sonnei* representative strains of each period (see section "Materials and Methods"). As a result, we found several contigs containing DNA regions with >90% identity with $SRL_{YSH6000}$. *In silico* analyses allowed us to map these homologous sequences against the genetic structure of $SRL_{YSH6000}$, assembling the complete genetic structure of this PAI in those five analyzed genomes (**Figure 6**). The SRL PAIs harbored by the *S. sonnei* strains comprise approximately 75 kb. As expected, in all five draft genomes, there is a high degree of conservation compared with the one in the *S. flexneri* YSH6000 strain. Among the genes identified in these PAIs, we found the antibiotic resistance genes (bla_{OXA-1}, *aadA1*, *cat*, and *tet*), a ferric dicitrate transport system, and the recently described *orfs* 8 and 9 that participate in the metabolism of D-aspartate (Henríquez et al., 2020). However, a significant difference observed in the SRL PAI identified in the *S. sonnei* strains corresponds to a deletion of a gene that encodes an autotransporter protein of the Ag43 allele family. One possible explanation is that this deletion originates from the insertion of a DNA fragment of about 10 kb, containing genes encoding for integrases and hypothetical proteins.

DISCUSSION

According to data from ISP, the epidemiology of shigellosis in Chile registered around 1,000 *Shigella* isolates per year between 1995 and 2008, from which 47% corresponded to *S. flexneri* and 47% were *S. sonnei*. In contrast, in 2009, there was an increase of up to 3,000 isolates, when a *S. sonnei* peak was observed rising up to about 90%. Furthermore, a sharp decline to less than 500 isolates during the years 2010–2018 was registered. Nevertheless, *S. sonnei* and *S. flexneri* frequency remained near to 50% (ISP, Boletín Vigilancia de Laboratorio, *Shigella* 2014–2018; **Figure 1A**).

The ISP reports also showed the antibiotic resistance profiles. However, the genetic determinants of resistance in our country have barely been studied. In this context, we described SRL PAI as one of the MGE present in Chilean *S. sonnei* strains. This genetic element was first described in *S. flexneri* 2a YH6000 (Luck et al., 2001). The 66-kb SRL PAI includes an SRL locus encoding resistance determinants to streptomycin (*aadA1*), ampicillin (bla_{OXA-1}), chloramphenicol (*cat*), and tetracycline (*tetB*). The SRL PAI is likely acquired due to horizontal gene transfer underscoring the role of this mechanism to disseminate *Shigella* MDR phenotype (Turner et al., 2003, 2004). It has also been described in *S. dysenterieae* serotype 1 and *S. flexneri* serotypes 3a and 3b (Baker et al., 2015; Njamkepo et al., 2016; Parajuli et al., 2019). On the other hand, we described the presence of the *dfrA14* gene in 79 out of 80 strains isolated in 2008–2009, differentiating from strains isolated before that period. This marker is coded in the pABC-3 plasmid in the *sul2/strA*::*dfrA14/strB* gene cluster (Miranda et al., 2016).

Based on the results of this study, we considered CHL resistance in MDR strains as a predictive phenotypic marker of the presence of SRL PAI, which pointed us to look at this marker more closely. The 2000–2010 report from Foodnet at CDC highlighted a high level of MDR *S. sonnei* strains in United States (Shiferaw et al., 2012). It is noteworthy that, from the 954 *S. sonnei* strains analyzed in that period, only 10 isolates (1%) included the CHL resistance phenotype, suggesting a low frequency of SRL-positive strains.

The *S. sonnei* strains circulating in Europe have diverse MDR patterns. Surprisingly, in the 18 years of surveillance in Belgium (Vrints et al., 2009), non-CHL resistance was detected in almost 5,000 isolates, suggesting the absence of SRL-positive strains. On the other hand, a study conducted in England and Wales characterized 341 *S. sonnei* isolated during 2015 by WGS (Sadouki et al., 2017). From that collection, only 8 strains (2.3%) appeared to carry both elements, the SRL PAI and the pABC-3 plasmid, based on the presence of sequence-predicted resistance genes. These strains could have been imported by travelers (Kotloff et al., 2018). Besides these, studies conducted by Baker et al. (2018) also demonstrated the presence of SRL and plasmid pABC-3 not only in *S. sonnei* but also in *S. flexneri* 2a strains.

In China, it is challenging to realize the presence of the SRL PAI in *S. sonnei* strains since CHL is not included in the AST. However, in a 10-year surveillance in Jiangsu (2002–2011), atypical class 1 integron was detected in 168 of 340 *S. sonnei* isolates (49.4%) harboring bla_{OXA-1}-*aadA1* gene cassettes (Gu et al., 2017; Kang et al., 2019). As mentioned, this integron is part of the SRL locus, suggesting the presence of SRL PAI in strains isolated from this region. Indeed Zhang et al. (2014) described the presence of SRL in an epidemic clone of *S. flexneri* MDR strain, carrying genes for resistance against streptomycin, ampicillin, chloramphenicol, and tetracycline.

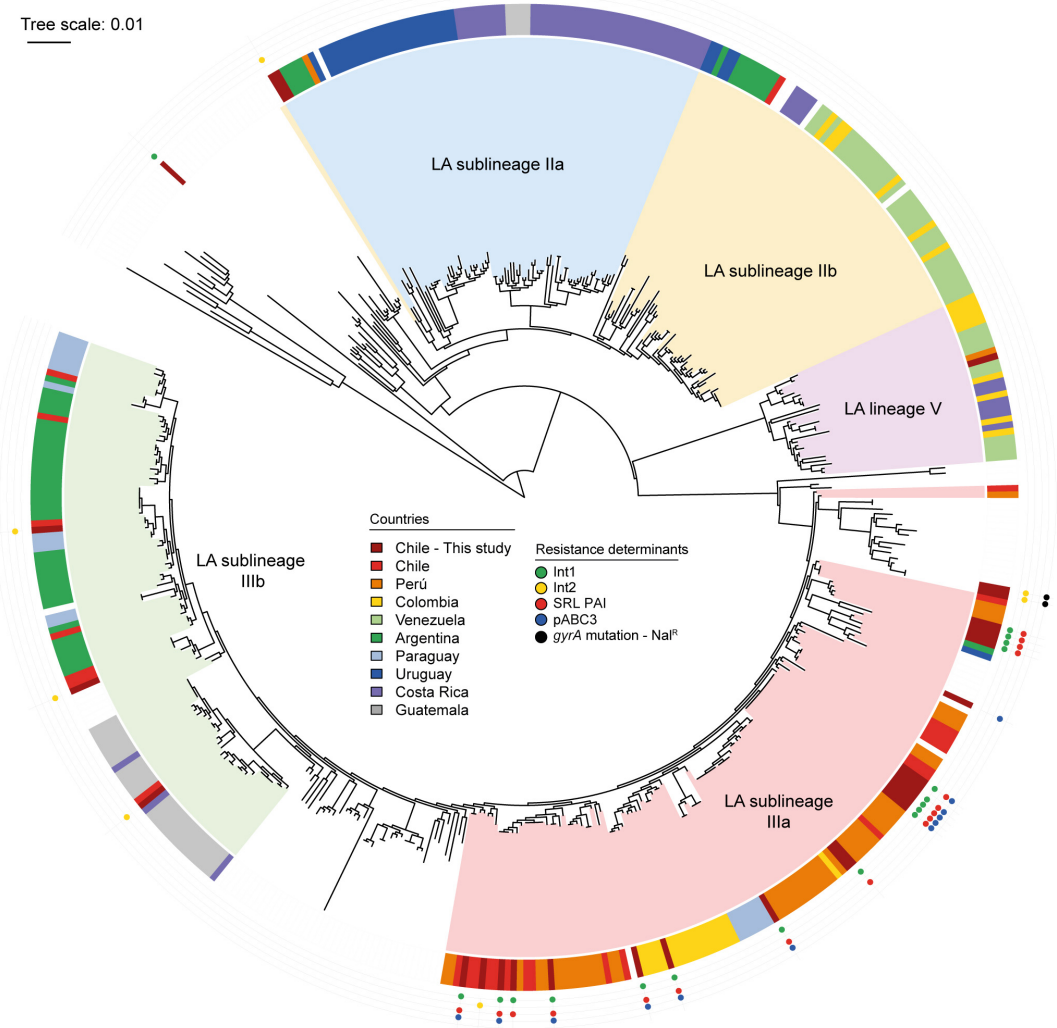

FIGURE 5 | Phylogenetic analysis of *Shigella sonnei* genomes sequenced in this study and publicly available *S. sonnei* genomes. Maximum likelihood phylogenetic tree (midpoint rooted) based on core genome SNPs showing phylogenetic relationships among 470 *S. sonnei* strains. The Latin-American (LA) lineages are based in the study by Baker et al. (2017). The country of origin of LA strains is shown in the colored ring, according to the legend. The global reference strains in the tree do not have country labels. The presence of antimicrobial resistance determinants of Chilean *S. sonnei* strains sequenced in this study is shown in the outer tracks, according to the legend. The tree was edited using iTOL (Letunic and Bork, 2016).

A completely different picture was observed with the Chilean *S. sonnei* strains. Since 1998, a high resistance to CHL has been reported (Fullá et al., 2005; Toro et al., 2005; Marcoleta et al., 2013). The present study detected 55.9% (195/349) of CHL-resistant strains among isolates from the 1995–2013 period, 191 of which possess the SRL PAI, thus correlating it as the most critical genetic element associated with CHL resistance.

The landscape of *S. sonnei* in Latin America was recently described by Baker et al. (2017). From WGS studies, it is possible to infer the circulation of SRL PAI in this region. Based on supplementary data analysis, we confirmed that 79 out of the 323 strains characterized by the PulseNet Latin American group at least harbored the SRL locus. The distribution of predicted SRL-positive strains was 46/48 Peruvian strains, 16/31 Colombian strains, and 17/27 Chilean *S. sonnei* isolates. All these strains belonged to sublineage IIIa. The sequence analysis of Peruvian *S. sonnei* strains showed that 40 out of the 48 strains harbor the SRL locus and the *dfrA14* gene probably as part of pABC-3 plasmid (Miranda et al., 2016), while 6 out of the 48 seem to carry only the SRL locus. In that dataset, the oldest Peruvian isolates were obtained in 1999, indicating the simultaneous presence of SRL PAI and the pABC-3 plasmid in those strains. In Colombia, 2 and 14 strains were predicted to harbor SRL PAI and SRL/pABC-3 genetic elements, respectively. For the Chilean strains analyzed in that work, the authors had access to strains isolated in the period 2010–2011 from different areas of the country, and the presence of SRL and SRL/pABC-3 was predicted in 3 and 14 of them, respectively

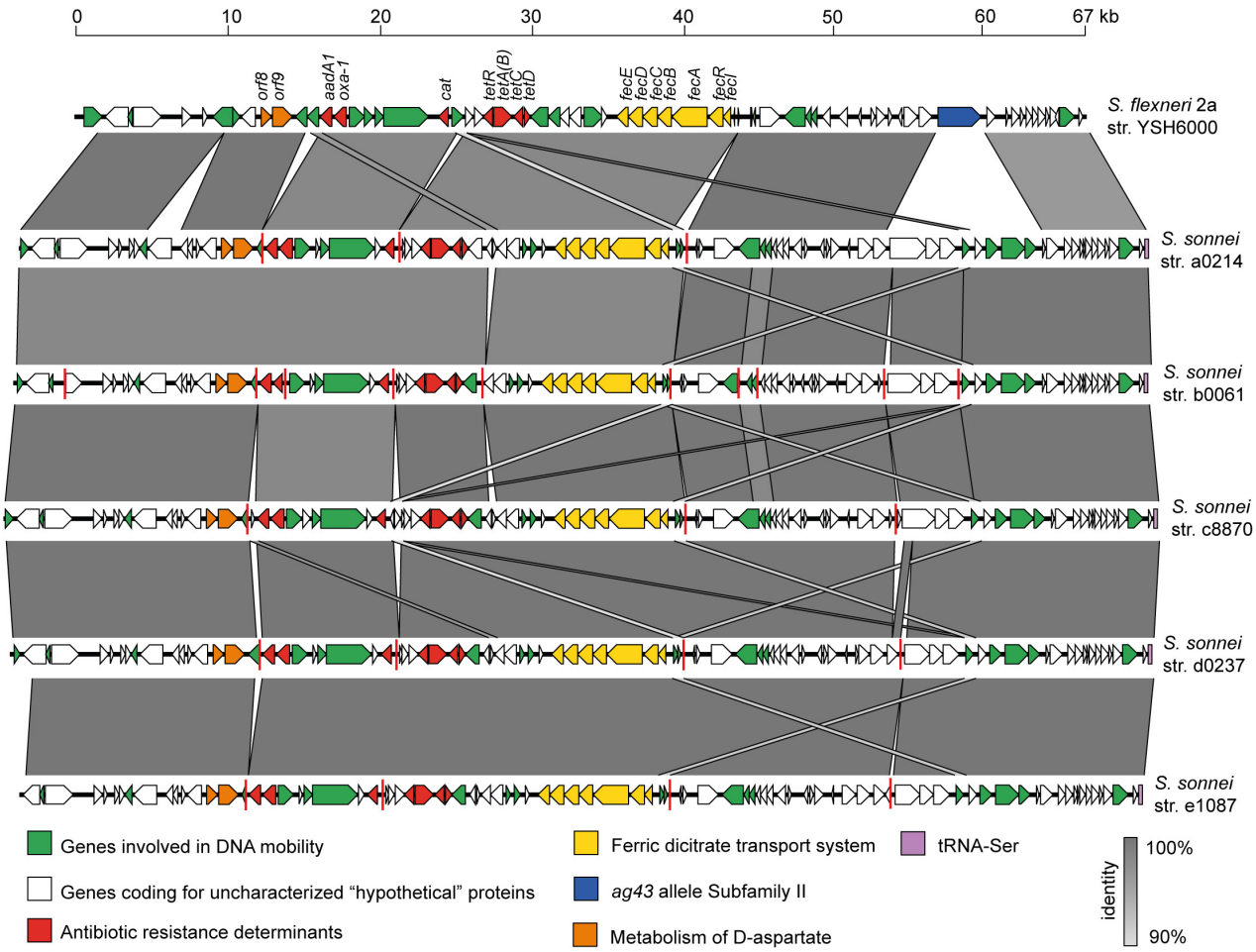

FIGURE 6 | Comparison of the genetic structure of SRL pathogenicity islands carried by the *Shigella flexneri* 2a YSH6000 strain and Chilean *S. sonnei* strains. The predicted genes and the direction of transcription are represented as block arrows. The genes are color-coded according to gene function, as indicated in the legends at the bottom. The names of some genes are indicated. The conserved regions are shaded in gray, and the intensity of the color indicates the nucleotide identity levels, from 88 to 100%. Contig boundaries are shown as red lines. The GenBank accession numbers are indicated in **Table 2**. The figure was prepared using EasyFig (Sullivan et al., 2011).

(Baker et al., 2017). It is very likely that more than one of these strains coincide with those included here in period D, where we described 21% SRL-positive strains and 43.8% of SRL/pABC-3 strains. Our results show the close phylogenetic relationship in sublineage IIIa of all the Chilean strains harboring the SRL PAI to be nearly related with the Peruvian and Colombian isolates, suggesting the interchange of strains among these countries (**Figure 5**).

The results of the current study show that SRL-positive strains have been circulating in Chile from 1995 onward, and *dfrA14* was not present until 2006 when a SRL/pABC-3-positive strain was isolated for the first time. After that, all the strains included in period C (2008–2009 period) harbored SRL/pABC-3 genetic elements. It should be noted that this group is formed by 61 strains from different areas of Región Metropolitana, and 15 were isolated in Antofagasta, showing the dissemination to a much wider geographical area of the country. It is not known why these strains spread massively in our country in those years. Even the diminished prevalence of *Shigella* after 2009 and the co-circulation of different clones later have not been resolved (**Figure 1A**).

Interestingly, the *S. sonnei* strains circulating in Brazil appear to be different clones. A study of 72 Brazilian strains isolated over 31 years from 1983 to 2014 suggests two prevalent subtypes that differ little; however, neither of them displays the MDR associated with the presence of SRL PAI (Seribelli et al., 2016). Although a more recent study showed that most *S. sonnei* strains isolated in northeastern Brazil were associated with azithromycin resistance, it seems that the SRL element is circulating as 20% of them were resistant to CHL (Medeiros et al., 2018).

Surveillance for MDR *S. sonnei* infections acquired from domestic and international sources allowed the isolation of two *S. sonnei* strains in Pennsylvania from patients infected during a international travel to Peru (Abelman et al., 2019), showing the spread of SRL-positive strains to North America. The predicted antimicrobial gene resistance markers harbored by those strains

strongly suggest the presence of the same Latin American clone, including SRL PAI (bla_{OXA-1}, *aadA1*, *tetB*, and *cat1*) and the plasmid pABC-3 (*strA*, *strB*, *sul2*, and *dfrA14*). The key marker is again CHL resistance; only these strains had the appropriate phenotype and the genetic markers for the presence of SRL PAI.

The increase in *Shigella* spp. MDR strains has been a cause for concern worldwide over the past two decades. The spread of class 1 and class 2 integrons has also been documented in Latin America (Barrantes and Achí, 2016). The emergence and successful spread of a particular MDR strain of *S. sonnei* biotype g carrying a class 2 integron, resistant to STR, SXT, and TET, was reported in several countries, including Australia, Senegal, Taiwan, Japan, Korea, and Iran (McIver et al., 2002; Oh et al., 2003; Gassama-Sowa et al., 2006; Seol et al., 2006; Ranjbar et al., 2007; Izumiya et al., 2009; Chang et al., 2011). Among the Chilean *S. sonnei* strains, we could detect the presence of class 2 integron to be circulating steadily just since 2011.

The World Health Organization has recommended fluoroquinolones and cephalosporins as the preferred drugs for the treatment of *Shigella* infections (Williams and Berkley, 2016, 2018). However, resistance to these antibiotics has been described. During 1998–2009, the nalidixic acid resistance rates in *S. sonnei* isolated from Asia and Africa have increased to 44%, whereas in Europe and America they were less than 3%. Consequently, the spread of ciprofloxacin-resistant *S. sonnei* has been increasing globally, apparently from South Asia (Gu et al., 2012; Chung The et al., 2016, 2019, 2021). In Chile, ciprofloxacin resistance was not detected during the period 1995–2013, but the presence of 9.5% of NAL^R strains among these 349 isolates, concentrated in a short time between 2011 to 2013, is of great concern. Two of these Chilean NAL^R strains were sequenced, thus finding the D87Y mutation in *gyrA*. Moreover, the latest report of ISP showed the emergence of CIP^R in Chilean *S. sonnei* strains to be at 2–3% (ISP, Boletín Vigilancia de Laboratorio, *Shigella* 2014–2018).

Our results regarding the emergence of NAL^R strains agree with reports from Latin American (Sati et al., 2019). Such surveillance provides evidence of the increasing prevalence of NAL^R *S. sonnei* in this region since 2011. This information supports the presence of class 2 integron and the MDR phenotype to TET, STR, SXT, TMP, and NAL, suggesting that the entry of NAL^R strains of *S. sonnei* is more likely due to global world trade and human travel (Chung The et al., 2016, 2021).

In summary, this work shows an overview of the resistance phenotypes associated with genetic determinants, emphasizing the MDR molecular mechanisms that have circulated in Chile during the period 1995–2013. We were able to demonstrate the presence of a clone or variant of *S. sonnei* SRL-*dfrA14*, harboring the pABC-3 plasmid, which spread during the 2008–2009 period. After that, different clones harboring class 2 integron have emerged. Therefore, our results underscore the role of the SRL PAI and integrons as genetic elements associated with the MDR phenotype. On the other hand, the high similarity of the SRL sequences from representative strains of different periods would rule out that this element has affected by itself the spread of *S. sonnei* isolated during period C, 2008–2009. Even considering the limitations of the current study, the data presented in this work revealed the temporal dynamics of antimicrobial resistance in *S. sonnei* strains circulating in Chile, mainly determined by the widespread dissemination of MGE conferring MDR phenotypes. Since shigellosis is endemic in Chile, constant surveillance of antimicrobial resistance phenotypes and their genetic basis is a priority in order to contribute to the orientation of public health policies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

CT and JS contributed to study design, data analysis, data interpretation, manuscript writing, and revision of the manuscript. DM contributed to data analysis, data interpretation, manuscript writing, figures design, and revision of the manuscript. JU contributed to data analysis, data interpretation, and revision of the manuscript. JD contributed to data analysis and revision of the manuscript. LC and RC contributed to data acquisition and data analysis. TH and CG contributed to data acquisition, data analysis, and data interpretation. GH and MU contributed to study design, data analysis, data interpretation, and revision of the manuscript. CT was the principal investigator at the FONDECYT grant that funded this work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.794470/full#supplementary-material

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Enrofloxacin Promotes Plasmid-Mediated Conjugation Transfer of Fluoroquinolone-Resistance Gene *qnrS*

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This study aimed to determine the effect of enrofloxacin (ENR) on the transfer of the plasmid-mediated quinolone resistance (PMQR) gene *qnrS* from opportunistic pathogen *Escherichia coli* (E2) to *Salmonella* Enteritidis (SE211) and to analyze the resistance characteristics of SE211-*qnrS* isolates. The plasmid carrying *qnrS* gene of E2 was sequenced by Oxford Nanopore technology. The plasmid carrying *qnrS* gene belonged to incompatibility group IncY. *In vitro*, the transfer experiment of IncY plasmid was performed by the liquid medium conjugation method. The conjugation transfer frequency of the IncY plasmid was 0.008 ± 0.0006 in the absence of ENR, 0.012 ± 0.003 in 1/32 MIC_{ENR}, 0.01 ± 0.008 in 1/8 MIC_{ENR}, and 0.03 ± 0.015 (Mean ± SD) in 1/2 MIC_{ENR}, respectively. After inoculation of *E. coli* E2 and SE211, chickens were treated with different doses of ENR (3.03, 10, and 50 mg/kg b.w.) for 7 days consecutively. To screen the SE211-*qnrS* strains from intestinal tract of chickens, the resistance genes and susceptibility of isolates were identified. The amount of *E. coli* E2 and the copy number of *qnrS* gene in the chicken intestinal tract were determined by colony counting and qPCR, respectively. *In vivo*, more SE211-*qnrS* strains were isolated from the treated group compared with the untreated group. SE211-*qnrS* strains not only obtained IncY plasmid, but also showed similar resistance phenotype as E2. In conclusion, ENR treatment can promote the spread of a IncY-resistance plasmid carrying the *qnrS* fluoroquinolone-resistance gene in *Escherichia coli* and the development of drug-resistant bacteria.

Keywords: *qnrS*, *Escherichia coli* E2, *Salmonella* enterica serovar enteritidis SE211, enrofloxacin, resistance transfer

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INTRODUCTION

Escherichia coli (abbreviated as *E. coli*) is a common opportunistic pathogen in the gastrointestinal tract. An estimated 44 million ETEC-related diarrheal diseases occur annually, resulting in 113,000 deaths in 2015 (Roussel et al., 2020), and the mortality is 3–5% in *E. coli* infection.[1] Non-typhoidal *Salmonellae*, one of the leading causes of bacterial diarrhea worldwide, are estimated to cause approximately 153 million cases of gastroenteritis and 57,000 deaths globally each year.[2] Fluoroquinolones (FQs) are commonly used in the treatment of colibacillosis and salmonellosis and can induce resistance of intestinal bacteria (Li J. et al., 2019). *QnrS* gene, a plasmid-mediated FQ-resistance gene, was the most prevalent quinolone gene in *E. coli* strains isolated from poultry feces (Gosling et al., 2012). Antibiotic resistance genes (ARGs) can be the agent of an outbreak by transferring resistance to multiple unrelated pathogens (Lerminiaux and Cameron, 2019). In the case of antibiotic abuse, whether opportunistic pathogens transfer ARGs to pathogens has attracted widespread attention. *E. coli* was used as a vector to transmit the *qnrS* gene, which could make pathogenic bacteria obtain drug-resistance genes and reach the level of clinical drug resistance (Gosling et al., 2012). Furthermore, the plasmid carrying *qnrS* gene in *E. coli* also contained other ARGs (Veldman et al., 2014; Slettemeas et al., 2019; Koyama et al., 2020), which could cotransfer with the *qnrS* gene. *Salmonella* Enteritidis 211 (abbreviated as SE211) is highly pathogenic (Cui et al., 2020, 2021), and it is sensitive to enrofloxacin. *E. coli* E2 carrying *qnrS* gene is a multidrug resistant (MDR) strain (Li et al., 2017). Previous research results show that the *qnrS* gene promotes the transfer efficiency of ARGs from *E. coli* E2 to *E. coli* strain EC600 *in vitro*, suggesting the intraspecies transfer capacity of resistance genes (opportunistic pathogen *E. coli* E2 to model recipient *E. coli* EC600). However, the interspecies transfer (opportunistic pathogen *E. coli* to pathogenic *Salmonella*) capacity of the *qnrS*-carrying *E. coli* under the antibiotic selection pressure in broiler chicken was unexplored. ENR can favor the broiler gastrointestinal (GI) tract acting as a niche for selection of MDR commensal coliforms (Li et al., 2017). Furthermore, the intestinal tract of chicken provides a habitat for different bacteria. The multifactorial and complex relationships of the microbiota ecosystem of the gut contribute to the spread of these bacteria between animals and humans (Tewari et al., 2019). Therefore, we analyzed the effect of enrofloxacin on the transfer of *qnrS* gene from *E. coli* E2 to SE211 both *in vitro* and *in vivo* and the resistance characteristic of SE211 carrying *qnrS* gene (SE211-*qnrS*). The transfer of *qnrS* gene from *E. coli* E2 to SE211 in the absence of ENR and in the presence of subminimal inhibitory concentration (sub-MIC) of ENR was performed by the liquid medium conjugation method. The chickens were gavaged with a bacteria suspension of *E. coli* E2 and SE211 before ENR administration. SE211-*qnrS* isolates were screened on plates supplemented with ENR. The colonization levels of *E. coli* E2 and SE211-*qnrS*, copy number of *qnrS*, were determined by traditional culture methods and molecular biology identification techniques.

MATERIALS AND METHODS

Ethics

The study *in vivo* was carried out in accordance with the guidelines established by the China Regulations for the Administration of Affairs Concerning Experimental Animals (1988) and Regulations for the Administration of Affairs Concerning Experimental Animals in Hubei province (2005) (Project No.2017YFC1600100 and Animal Welfare Assurance No. HZAUCH-2020-0005). All work was made to treat the experimental animals ethically and to minimize suffering in this study.

Experimental Strains and Reagents

Donor and Recipient Bacteria

E. coli E2 containing the *qnrS* gene was used as a donor strain (MIC_{ENR} = 128 mg/L). *Salmonella* SE211 was used as a recipient strain (MIC_{ENR} = 0.25 mg/L). They were preserved by the Cooperative Innovation Center for Sustainable Pig Production (HZAU), Huazhong Agricultural University.

Reagents

The standards of enrofloxacin, chloramphenicol, ampicillin, trimethoprim, and sulfamethoxazole were purchased from Dr. Ehrenstorfer (Germany). Ciprofloxacin standard was obtained from MedChemExpress (New Jersey, United States). Tetracycline standard was from China Institute of Veterinary Drug Control (Beijing, China), and 2 × EasyTaq PCR SuperMix and Phanta super-fidelity DNA Polymerase were purchased from Vazyme (Nanjing, China). The pUCm-T vector, IPTG, and X-Gal were from Beyotime Biotechnology (Nantong, China). The primers of this study were all synthesized by Genscript (Nanjing, China).

Plasmid Sequencing and Analysis

E. coli E2 was grown in LB broth, and the culture was centrifuged and quickly frozen in liquid nitrogen. Relying on Wuhan Bena Technology Service Limited Company, the genomic DNA was extracted by sodium dodecyl sulfate (SDS) and purified with a 13323 kit. The plasmid sequencing was performed by the Oxford Nanopore Technologies DNA sequencing platform. Annotation of plasmid type and resistance genes were carried out on the website.[3] According to the plasmid sequencing result, NCBI was used to design the primers for plasmid replicon and resistance genes. Comparison of IncY plasmids was created by BRIG tools.[4] The comparison of IncY plasmids was performed in the following order (inner to outer circles): pTET-GZEC065 (GenBank accession no. CP048027), pTetA_020022 (CP032890), pE2 (CP086663).

[1] http://www.who.int/news-room/fact-sheets/detail/e-coli

[2] https://wwwnc.cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/salmonellosis-nontyphoidal

[3] http://www.genomicepidemiology.org/index.html

[4] https://sourceforge.net/projects/brig/

Conjugation Transfer and Construction of Animal Model

Transfer of *qnrS in vitro*

The conjugation experiment was conducted using the liquid mating procedure according to previous report (Lambrecht et al., 2018). *E. coli* E2 and SE211 with 4:1 volume ratio were cocultivated for 4 h in LB broth supplemented with 0, 1/2 MIC_{ENR} (0.0125 mg/L), 1/8 MIC_{ENR} (0.003 mg/L), and 1/32 MIC_{ENR} (0.0007 mg/L). The coculture was diluted 10^4 times by LB broth and spread on the chromogenic *Salmonella* agar (second generation) (Hopebio, Qingdao, China). The plates were supplemented with 0.25 mg/L of ENR to screen the SE211-*qnrS* strains. The conjugation transfer efficiency = $\frac{Number\ of\ transconjugants}{Number\ of\ recipients}$.

Chickens and Housing

The animal trial was performed in the animal room at Ke Qian of HuaZhong Agricultural University. Twenty specific-pathogen-free (SPF) male chickens (1-day-old) were purchased from Bejing Boehringer Ingelheim Vital Biotechnology Limited Company and kept in four individual PQ3 type stainless steel poultry isolators (Suzhou Suhang Technology Equipment Co., Ltd., China), which can prevent pollution from external environmental factors. They were fed with sterile water and SPF feed (Beijing Ke Ao Xie Li Feed Limited Company, China).

Model Construction and Enrofloxacin Treatment

On day 7, all chickens were inoculated with 0.5 mL of the donor strain ($\sim 10^9$ CFU/mL). On day 10, all chickens were inoculated with 0.5 mL of the recipient strain ($\sim 10^9$ CFU/mL). The chickens were gavaged with bacterial suspension. After successful colonization of the intestine with the donor and recipient bacteria on day 12, the 20 chickens were equally divided into four groups. Then, they were treated with different dosages of ENR (10% enrofloxacin oral solution, Bayer, Germany). The first group (group 3.03) was given a prophylactic dose (3.03 mg/kg b.w.) (Li et al., 2017). The second group (group 10) was given the ENR (10 mg/kg b.w.) as a clinical recommended dose. The third group (group 50) was given a high dose (50 mg/kg b.w.), which could effectively inhibit pathogenic bacteria. The fourth group did not receive treatment and served as a non-treated control group (group NTC). The different dosages of ENR were given for 7 days consecutively (**Figure 1**).

Sample Collection and Strain Isolation

Collection of Fecal and Cloacal Samples

Prior to the inoculation with *E. coli* E2 and SE211, cloacal swabs were taken from each chicken, then cultivated on chromogenic *Salmonella* agar (second generation) plates and in LB broth (Hopebio, Qingdao, China). *QnrS* and *repA* genes were identified by PCR. None of the chickens was found to be positive for *Salmonella*, resistance gene *qnrS*, and IncY plasmid. No contamination of *Salmonella*, IncY plasmid, and *qnrS* gene were observed in the drinking water and SPF feed. Cloacal swabs and fecal samples were collected on day 12 (1 day before ENR treatment); days 13–19 (2, 4, 6, 8 days of the treatment of ENR); and days 21, 23, 25, 27, 33, and 40 (1 and 2 weeks after stopping ENR treatment), respectively (**Figure 1**).

Bacterial Isolation

Two swabs were taken from each chicken. One swab was emulsified in 2 mL of selenite cystine broth (SC) (Hopebio, Qingdao, China) and then further inoculated on chromogenic *Salmonella* agar (second generation) plates supplemented with 0.25 mg/L of ENR. *Salmonella* represented a typical purple single colony on the chromogenic *Salmonella* agar (second generation). The colonies were randomly screened for further amplification of *qnrS* and *repA* genes to identify SE211-*qnrS* strains. In the meantime, all the *Salmonella* and putative SE211-*qnrS* isolates were stored at −20°C. Another swab was weighed, which was emulsified in 1 mL of sterile 0.9% NaCl and then further diluted 10^5 times. An aliquot (50 μL) of the appropriate dilution was spread onto eosin-methylene blue agar (Hopebio, Qingdao, China) plates supplemented with ENR (32 mg/L). These plates were incubated at 37°C overnight to detect the colonization level of *E. coli* E2.

Quantitation PCR

Standard Curve

Snapgene software (Version 3.1.1) was used to design primers (5′–3′) for amplification of the full length (657 bp) of *qnrS* gene (F-5′-ATGGAAACCTACAATCATACATATCGG-3′ and R-5′-TTAGTCAGGATAAACAACAATACCC-3′). PCR product of *qnrS* gene was obtained by recycling from 1% agarose gels. Then, the recombinant DNA of pUCm-T-*qnrS* was obtained by ligation and transformation (DH5α). The plasmid pUCm-T-*qnrS* was extracted with E.Z.N.A.® Plasmid Mini Kit II (Wuhan Tianyuan Huida Biotechnology Limited Company, China). The copy number of *qnrS* gene was calculated by the equation: Copy number (copies/μL) = $6.02 \times 10^{23} \times$ cDNA (g/mL)/MW, molecular weight (MW) = (plasmid vector length + insertion fragment length) × 660 dalton/bp. Then, pUCm-T-*qnrS* of 1.17×10^8, 1.17×10^7, 1.17×10^6, 1.17×10^5, and 1.17×10^4 copies were used as the standards to determine the corresponding CT values. CT = $-KlgC_0+b$ was used as the standard curve formula, and C_0 represented the copy number of *qnrS* gene.

DNA Isolation

Total DNA was extracted from 210 mg of fecal samples collected from chickens using by commercial extraction kits (Rapid extraction kit for fecal genomic DNA, Aidlab, Beijing, China) according to the manufacturer's instruction and our modification. We increased the number of samples through the AC adsorption column and the proteinase K.

Analysis of the Quantities of the *qnrS* Gene

Quantitative PCR (qPCR) was performed by SYBR Green (Vazyme, Nanjing, China) detection in triplicate using a QuantStudio 3 real-time PCR detection system (Thermo Fisher Scientific, Germany) by the following procedure: 1 cycle at 95°C for 30 s, 40 cycles at 95°C for 10 s, 57°C for 10 s, 72°C for 30 s. The specificity of the PCR products was confirmed by melting curve. The copy number was calculated by equation of standard curve.

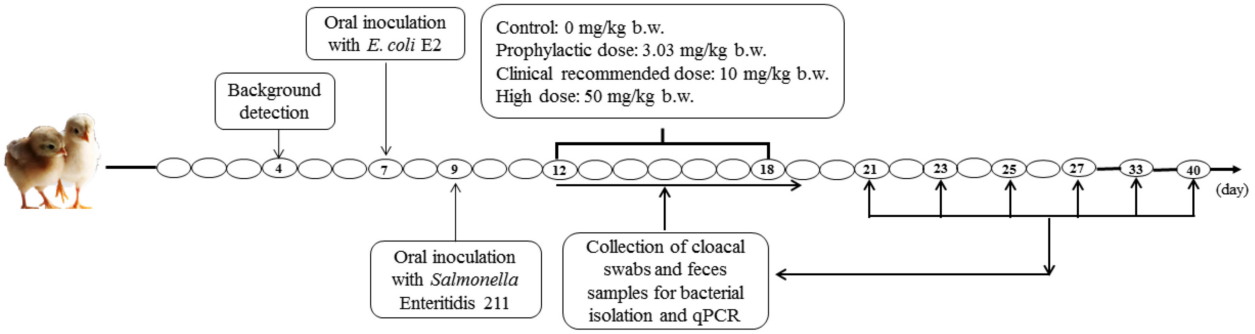

FIGURE 1 | Experimental design. The 12–18 days represent ENR treatment for 7 days. The 21, 23, 25, 27, 33, 40 days represent 2, 4, 6, 8, 14, 21 days after termination of ENR treatment, respectively.

Verification of SE211-*qnrS*

All of the isolates were confirmed to be SE211 strains and then tested the IncY plasmid by the PCR method using the primer pairs (5′–3′) for *qnrS* (F-5′-CATACATATCGGCACCACAAC-3′ and R-5′-CAGGATAAACAACAATACCCAGT-3′), *repA* (F-5′-AATTCAAACAACACTGTGCAGCCTG-3′ and R-5′-GCGAGAATGGACGATTACAAAACTTT-3′). All of the SE211-*qnrS* strains were tested for the resistance genes located in the IncY plasmid by PCR method using the primers (5′–3′) for $bla_{\mathrm{TEM-135}}$ (F-5′-TTGATCGTTGGGAACCGGAG-3′ R-5′-AATAAACCAGCCAGCCGGAA-3′), *tet*(A) (F-5′-CATTTCGCTTGCCGCATTTG-3′ and R-5′-TCATTCCGAGCATGAGTGCC-3′), *floR* (F-5′-CGGATTCAGCTTTGCCTTCG-3′ R-5′-GCCAATGTCCCGACGATACT-3′), and *dfrA*-14 (F-5′-CAACGATGTTACGCAGCAGG-3′ R-5′-CAATCGCGGAAAAGGCGTAG-3′).

Susceptibility Tests

MICs of four antibiotics (enrofloxacin, ampicillin, chloramphenicol, tetracycline) for all SE211-*qnrS* strains were determined by the broth microdilution method, and their antibiotic resistance level was interpreted by the Clinical and Laboratory Standards Institute guidelines (CLSI) (CLSI, 2020). In particular, the resistant breakpoint of ampicillin, chloramphenicol, and tetracycline for *Salmonella* strains was interpreted by the CLSI criteria (ampicillin $\geq$ 32 mg/L, chloramphenicol $\geq$ 32 mg/L, tetracycline $\geq$ 16 mg/L), whereas no resistant breakpoint of ENR was interpreted. Ciprofloxacin was interpreted based on the CLSI breakpoint (R $\geq$ 1 mg/L). *E. coli* ATCC 25,922 served as a quality control strain.

Stability of Plasmid

In this study, the plasmid stability experiment was different from the other report (Wein et al., 2019), which was evaluated by serial passages. In this study, we aimed to obtain colonies of SE211-*qnrS* from the glycerol bacteria. Method 1: The SE211-*qnrS* glycerol bacteria were cultured in LB broth supplemented with ENR (0.25 mg/L) at 37°C for 12–16 h, and then 20 μL culture was spread on chromogenic *Salmonella* agar (second generation) plates supplemented with ENR (1 mg/L) and incubated at 37°C for 18–24 h. Then, colonies were randomly selected on the resistant plates as a template for PCR identification. Method 2: The glycerol bacteria of SE211-*qnrS* was supplemented with ENR (0.25 mg/L) and stored in −20°C for 48 h. Cells of SE211-*qnrS* from the glycerol stock were supplemented with ENR (0.25 mg/L) and stored at −20°C for 48 h. The cells were cultured in LB broth supplemented with ENR (0.25 mg/L) at 37°C for 12 h, and 50 μL of the culture was spread on LB agar plates supplemented with (0.25 mg/L) and incubated again at 37°C for 12 h. Cells were harvested from these plates and resuspended in LB broth supplemented with (0.25 mg/L) and used as a template for PCR. *QnrS* and *repA* genes were amplified from resuscitative and eluotropic bacteria solutions to identify the SE211-*qnrS* strains.

Statistical Analysis

Using Graphpad Prism 7.0 statistical software, the value was expressed by mean ± SD, and the differences among different time points were analyzed by Student's *t*-test. $^{*}P \leq 0.05$ was set as the significance level, and $^{**}P \leq 0.01$ was set as the extremely significance difference.

RESULTS

Genetic Characteristics of IncY Plasmids

The plasmid (pE2) harbored in *E. coli* E2 had a size of 94, 190 bp with an average G + C content of 49.59%. The replicon gene *repA* showed 100% identity with the replicon of IncY plasmids in the PlasmidFinder database. The plasmid distribution system *parA* and *virB* genes were associated with the self-transmitting of IncY plasmids. An *oriT* sequence was found between the positions 89,095 and 89,379 by *oriTfinder*. Multiple mobile elements were distributed on pE2, such as insertion sequences (IS) IS421, IS1, ISKpn19, IS26, IS5, IS91; integron functional element IntI1; and transposon Tn3. Additional resistance genes were found on the plasmid, such as *tet*(A), *dfrA*-14, $bla_{\mathrm{TEM-135}}$, and *floR*. The sequence of plasmid pE2 was highly similar to that of pTET-GZEC065 and pTetA_020022 (85% query coverage and 100% sequence identity). These plasmids all contained the *floR* and *tet*(A) resistance genes, including insertion elements (**Figure 2**).

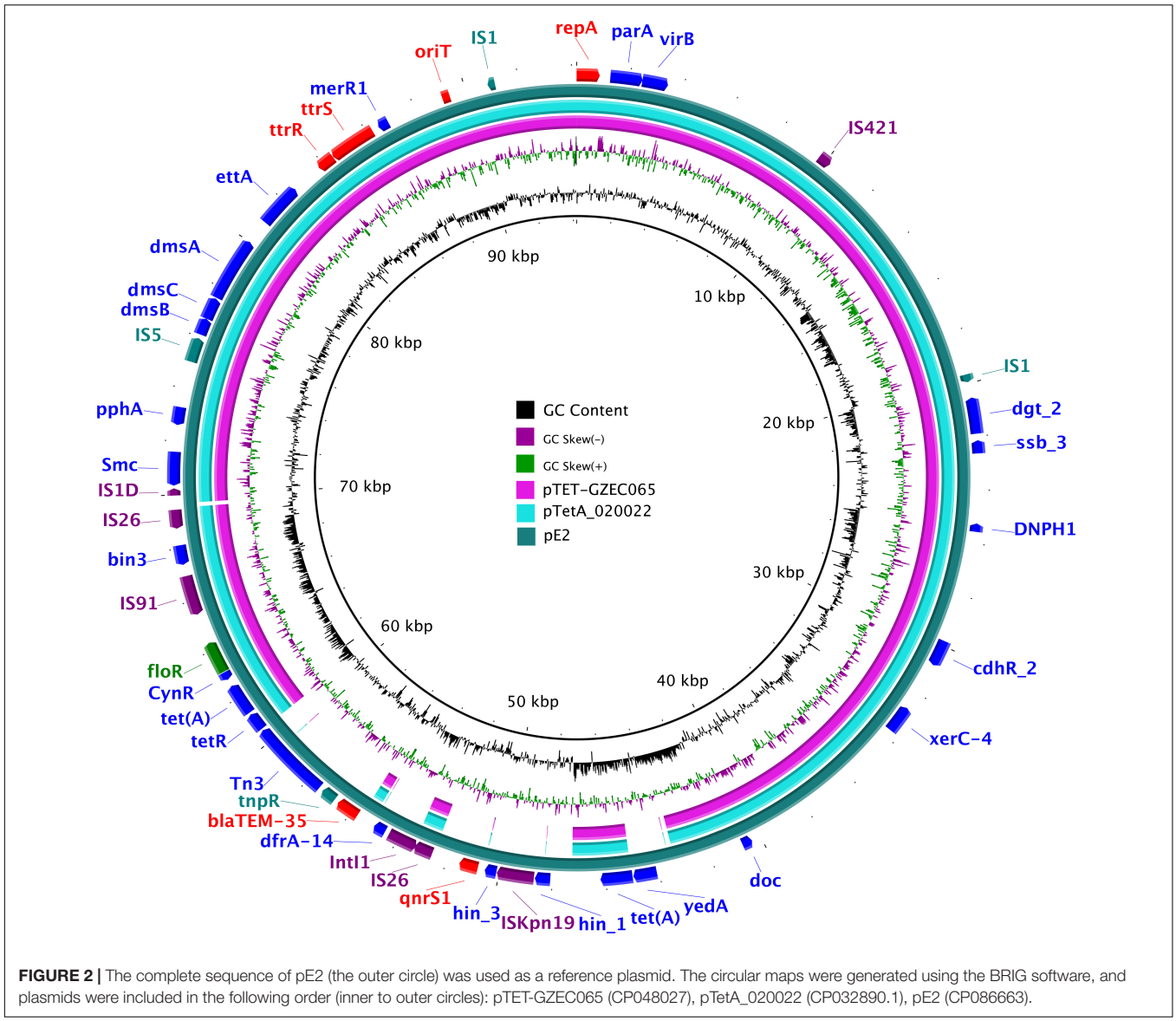

FIGURE 2 | The complete sequence of pE2 (the outer circle) was used as a reference plasmid. The circular maps were generated using the BRIG software, and plasmids were included in the following order (inner to outer circles): pTET-GZEC065 (CP048027), pTetA_020022 (CP032890.1), pE2 (CP086663).

The Effect of Subminimal Inhibitory Concentration of Enrofloxacin on the Transfer Frequency of *qnrS* Gene *in vitro*

No significant changes in the transfer frequency of *qnrS* gene from resistant *E. coli* E2 to susceptible SE211 was observed under 0, 1/2 MIC_{ENR} (0.125 mg/L), 1/8 MIC_{ENR} (0.03 mg/L), and 1/32 MIC_{ENR} (0.007 mg/L) conditions. However, the transfer frequency increased with increasing concentration of ENR (**Table 1**).

The Effect of Enrofloxacin on the Persistence and the Transfer of *qnrS* Gene *in vivo*

Prior to inoculation, cloacal swabs of the chickens showed no *E. coli* carrying *qnrS* gene and *Salmonella* as judged by the EMB agar plates supplemented with ENR (32 mg/L) and the chromogenic *Salmonella* agar (second generation) plates. Before ENR treatment, *E. coli* E2 and SE211 strains had colonized the GI tracts of chickens. *E. coli* E2 reached levels of $10^5 \sim 10^7$ CFU per g of feces (**Figure 3**). SE211 reached levels of $10^3 \sim 10^5$ CFU per mL of feces. The chickens infected with the SE211 strain showed a somnolent state and cold sensitivity and also passed out green and white loose feces. The *E. coli* E2 strain was still detected in the ENR treatment period and within 3 weeks after termination of ENR treatment. In the NTC group, however, *E. coli* E2 was not detected on day 21 after termination of ENR treatment (**Figure 3**). This result suggests that the resistant *E. coli* E2 strain could persist in the intestinal tract of chickens under the selection pressure of ENR. In this study, although *E. coli* E2 was isolated from the GI tracts of chickens (Li J. et al., 2019), it was easily excreted from the chicken intestine without the selective pressure of antibiotics. Then, the copy number of the *qnrS* gene was determined to evaluate the prevalence of the

TABLE 1 | Transfer frequency of *qnrS* gene under sub-MIC of ENR.

Concentration of ENR	Transfer frequency
0	0.008 ± 0.0006
1/32 MIC_{ENR}	0.012 ± 0.003
1/8 MIC_{ENR}	0.01 ± 0.008
1/2 MIC_{ENR}	0.03 ± 0.015

Three replicates in each group, the transfer frequency was analyzed by Student's t-test.

resistance gene in the chicken intestinal tract. The standard curve is $C_T = -3.237\ lgC_0 + 37.172$, $R^2 = 0.998$, Efficiency = 103.693%. This standard curve is credible. In the NTC group, the copy number of *qnrS* gene was higher than the initial detection level on days 1 (1 day after ENR treatment), 3, 7, S.4 (4 days after termination of ENR treatment), S.6, S.14, and lower than the initial detection level on days 5, S.2, S.8, S.21, indicating that the *qnrS* gene could persist in feces (**Figure 4**). In the prophylactic dose group, the copy number of the *qnrS* gene in the intestinal tracts of chickens showed a downward trend within 3 days of ENR treatment. From days 3 to S.4, the copy number of *qnrS* gene tended to a stable state. From days S.4 to S.21, the copy number of *qnrS* gene increased slightly, but it was still lower than the level before ENR treatment. In the clinically recommended dose group, the copy number of *qnrS* gene was lower than that time point before ENR treatment except on days 7 and S.2. In the high-dose group, the copy number of *qnrS* gene was higher than that time point before ENR treatment from the duration of ENR treatment to day S.4. Within 5 days of ENR treatment, the prophylactic and therapeutic doses of ENR reduced the copy number of *qnrS* resistance gene in chicken intestinal microflora. The high dose of ENR increased the copy number of the *qnrS* gene. Similarly, in the clinically recommended dose group, the copy number of the *qnrS* gene had an increasing trend during 5–7 days of ENR treatment. Therefore, high-dose (50 mg/kg b.w.) and long-term clinically recommended dose (> 5 days) ENR treatment increases the risk of *qnrS* gene transmission. All of the *Salmonella* and putative SE211-*qnrS* strains were screened from selective agar plates supplemented with ENR (0.25 mg/L). In ENR treatment duration, no SE211strain was isolated from the high-dosage group. However, the SE211 and putative SE211-*qnrS* strains were isolated from the other groups. The number of putative SE211-*qnrS* strains were changeful. Prior to ENR treatment, no SE211-*qnrS* was detected on ENR-supplemented plates. After 1 day of ENR treatment, one SE211-*qnrS* strain was acquired in the prophylactic dose group. After 2 days of ENR treatment, three SE211-*qnrS* strains were obtained in the clinically recommended dose group. One SE211-*qnrS* strain was isolated from the non-treated control group. After termination of ENR treatment, four SE211-*qnrS* strains were obtained from the clinically recommended dose group (**Table 2**).

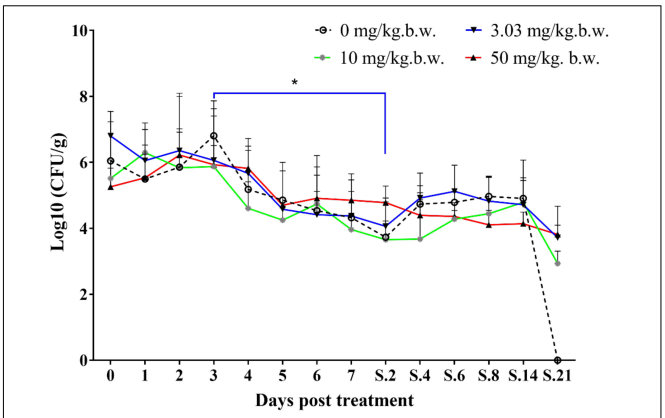

FIGURE 3 | Colonization level of drug-resistant *E. coli* E2 in chicken intestine. The 0–7 represent days for ENR treatment. S represents days after termination of ENR treatment. S.2 represents 2 days after termination of ENR treatment. The $^*p \leq 0.05$ that was set as significance level.

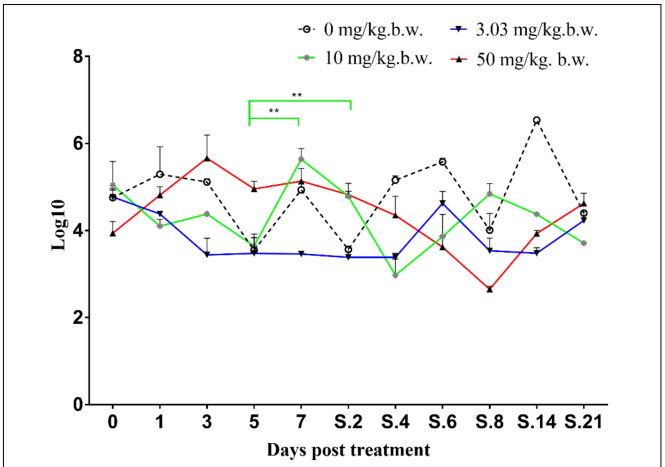

FIGURE 4 | Copy number of *qnrS* gene in fecal genome. The 0–7 represent days for ENR treatment. S represents days after termination of ENR treatment. S.2 represents 2 days after termination of ENR treatment. The $^{**}p \leq 0.01$ that was set as extremely significance difference.

TABLE 2 | The isolates of putative SE211-*qnrS* obtained.

Sampling day	Group [n/(Total)]			
	NTC	3.03 mg/kg b. w.	10 mg/kg b. w.	50 mg/kg b. w.
1	0 (15)	1 (15)	0 (12)	–
2	0 (12)	0 (9)	3 (15)	–
3	1 (9)	0 (15)	0 (12)	–
S.2	0 (9)	0 (15)	3 (9)	–
S.21	–	–	1 (3)	–

S, presents discontinue medication; n, number of SE211-qnrS strains; Total, total number of Salmonella isolates; –, no Salmonella isolates; NTC, presents non-treated control group.

Sensitivity of SE211-*qnrS* to Four Antibiotics

The resistance phenotypes of SE211-*qnrS* strains were similar to that of the donor strain *E. coli* E2, which exhibited the MDR phenotype as *E. coli* E2 (**Table 3**). 9 SE211-*qnrS* strains also obtained the bla_{TEM-35}, *dfrA*-14, *floR*, and *tet*(A) genes located on the IncY plasmid.

TABLE 3 | MIC of four antibiotics to E2, SE211, and SE211-*qnrS*.

The source of strains	The name of the strains	MIC (mg/L)			
		ENR	AMP	TET	CHL
Donor	E2	128	>256	128	128
Recipient	SE211	0.25	0.5	1	2
NTC	3-9-2	64	>256	128	128
Group 3.03	1–14	64	>256	64	128
	2-29-1	64	> 256	128	128
	2-29-2	128	> 256	128	128
	2-29-3	64	> 256	128	128
Group 10	S.2-23-1	128	> 256	128	128
	S.2-23-2	64	> 256	128	256
	S.2-23-3	128	> 256	128	256
	S.21–24	64	> 256	128	128

ENR, enrofloxacin; AMP, ampicillin; TET, tetracycline; CHL, chloramphenicol; NTC, non-treated control group; Group 3.03, prophylactic dose (3.03 mg/kg b. w.) of ENR; Group 10, clinical recommended dose of ENR (10 mg/kg b. w.).

Instability of Plasmid in *Salmonella*

The SE211-*qnrS* single colony was not recovered from the glycerol bacteria of 26 SE211-*qnrS* (17 *in vitro* and 9 *in vivo*). Although we identified two SE211-*qnrS* strains with positive *repA* and *dfrA*-14 genes in the screening process, they were still sensitive to trimethoprim/sulfamethoxazole when they were tested for the sensitivity to trimethoprim/sulfamethoxazole by glycerol bacteria resuscitation again. It suggested that the two strains also lost the IncY plasmid.

DISCUSSION

Subinhibitory Concentration of Enrofloxacin Promotes the Transfer of *qnrS* Gene

PMQR genes can be transmitted among bacteria through horizontal gene transfer (HGT). In this study, transfer of *qnrS* gene from *E. coli* E2 to SE211 was observed both *in vitro* and *in vivo*. Under the laboratory condition, the transfer frequency slightly increased with the increasing concentration of ENR, which might be correlated with the upregulation of conjugation-associated gene expression (Shun-Mei et al., 2018). In this experimental model of intestinal colonization in chickens with MDR *E. coli* E2 and sensitive SE211strains, more SE211-*qnrS* strains were isolated from the ENR-treated group than that from the non-treated control group. This suggests that the resistance gene *qnrS* can be easily transferred to *Salmonella* strains *in vivo* under selective pressure of antibiotics. However, one SE211-*qnrS* strain was also obtained from the NTC group. This indicates that transfer of *qnrS* gene in the intestinal tract of chicken might occur among different bacteria under natural conditions (Gosling et al., 2012), and this kind of horizontal transfer behavior was independent with antibiotic pressure (Le Devendec et al., 2011).

Enrofloxacin Accelerates the Emergence of Resistant Bacteria

The abuse of antibiotics in poultry and the residues of antibiotics in animals and the environment play a considerable role in the development of resistance among zoonotic food-borne microorganisms (Racewicz et al., 2020). The half-life of ENR is 59.1 days/115.0 days, 88.9 days, and 190.8 days in the dark/light, aerobic, and anaerobic conditions, respectively (Slana and Sollner-Dolenc, 2016). Thus, ENR can exist in chicken feces for a long time. The use of FQs can lead to the increase of resistant strains (Marshall and Levy, 2011). The horizontal transfer of resistance plasmids was the main reason for increasing the resistance level. The treatment of ENR promotes the transfer of the *qnrS* gene to the SE211strain in the intestinal environment of chickens. The isolates of SE211-*qnrS* from the ENR treatment groups were more than that from the NTC group, which confirms the hypothesis that ENR promotes *qnrS* gene transfer. In addition, antibiotics can promote the transfer of resistance genes in chickens (Chen et al., 2016). Similarly, horizontal transfer of plasmid-encoded resistance determinants is reported in the animal intestinal tract under both the presence and absence of antibiotic selective pressure, and the usage of florfenicol and ENR can facilitate the transmission of resistance gene *oqxAB* (Chen et al., 2016). Trimethoprim significantly increased both the HGT and vertical gene transfer frequencies (Li B. et al., 2019). Furthermore, the persistence of antibiotics may lead to the maintenance of resistant bacteria and resistance genes. The colonization results of *E. coli* E2 indicates that ENR can maintain the persistence of *E. coli* E2 carrying *qnrS* gene. It also indicates that treatment with ENR can increase the number of resistant *E. coli* in chicken gut (Roth et al., 2017). Therefore, it is necessary to continuously strengthen the monitoring of resistance genes in clinical strains and take effective measures to eliminate resistance genes and plasmids so as to prevent the spread of resistance genes from aggravating clinical drug resistance.

QnrS Cotransferred With Other Resistance Genes

The other resistance genes coexisting with the *qnrS* gene on the IncY plasmid were generally β-lactam-resistance genes. In the reported IncY plasmid carrying *qnrS* gene, it usually came from *E. coli* in the food chain (Roschanski et al., 2017) and healthy people (Mshana et al., 2016). Similar to this study, the IncY plasmid carried MDR genes to mediate MDR. IncY plasmid as a repository of MDR genes, *E. coli* can pose a

threat to human health through the food chain. Here, we found that the *qnrS* gene was cotransferred with the other resistance genes (bla_{TEM-35}, *tetA*, *floR,* and *dfrA*-14) on the IncY plasmid. In *E. coli*, cotransfer of the *qnrS* gene with β-lactam-resistance genes is reported (Wu et al., 2008; Jiang et al., 2012, 2014). pTET-GZEC065, pTetA_020022, pE2 plasmids had high similarities, carrying different resistance genes, which might be Tn3-family members that confer resistance to antibiotics (Nicolas et al., 2015). Recently, there are many identified Tn3 family members with different combinations of antibiotic-resistance determinants (Stokes and Gillings, 2011; Nordmann et al., 2012). Antimicrobial susceptibility tests show that the resistance spectrum of the SE211-*qnrS* strain was similar to that of donor strain *E. coli* E2 (**Table 3**). The resistance phenotypes were consistent with the resistance genotypes. In addition, we also obtained a drug-resistant *Salmonella* strain (MIC_{ENR} = 4 mg/L) from the intestinal tract of chicken without the IncY plasmid and *qnrS* gene. We speculate that the resistance mechanism may be attributed to mutations in the *parE* gene. The emergence and increase of MDR bacteria pose a great threat to public health.

IncY Plasmid Instability in Conjugant

The level of conjugated plasmid-mediated drug resistance in *Salmonella* was found to be slightly higher than that in *E. coli* (Chen et al., 2018). Our research also confirms this finding. In this study, MIC_{ENR} of SE211-*qnrS* strains were 64 or 128 mg/L, whereas MIC_{ENR} of EC600-*qnrS* strain was 8 mg/L (Li J. et al., 2019). The plasmids generally impose a fitness cost on their hosts (Carroll and Wong, 2018). The replication of plasmids in the host bacteria caused metabolic load, and the expression of plasmid-encoded genes was one of the important reasons resulting in the metabolic load of plasmids in the host bacteria (Silva et al., 2012). The expression of plasmid-encoded resistance genes led to the stress response of the host bacteria, resulting in the loss of plasmids (Rozkov et al., 2004). In this study, IncY plasmid carried *qnrS*1, *tet*(A), *floR*, *dfrA*-14, and $bla_{TEM-135}$ resistance genes and mediated the resistance of ENR, TET, CHL, TMP, and AMP. Compared with the reported IncY plasmid carrying *qnrS*, the pE2 resistance spectrum was larger. The expression of MDR genes in IncY plasmid undoubtedly increased the metabolic burden of the host bacteria. In addition, the plasmid also had several insertion elements IS421, IS1, ISkpn19, IS26, IS5, and IS91; integron functional elements IntI1; and transposon Tn3, the expression of IS elements and transposons caused the instability of plasmid DNA structure (Haddadin and Harcum, 2005). The copy number of plasmids was also one of the factors determining the metabolic load of plasmids, and the selective pressure of antibiotics was an important condition for maintaining the stability of resistance plasmids. Plasmids with high copy numbers, especially those containing the β-lactam-resistance gene, were unstable and lost quickly in *Salmonella* without antibiotic selection pressure both *in vivo* and *in vitro* (Zhang et al., 2005). In this study, we did not obtain SE211-*qnrS* colonies on plates supplemented with ENR. Although the *repA* and *dfrA*-14 genes of two SE211-*qnrS* strains were positive in the screening process, they were still sensitive to trimethoprim/sulfamethoxazole following glycerol bacteria resuscitation (data not shown). We speculate that antibiotic-free glycerol preserved SE211-*qnrS* strains might easily lose the IncY plasmid. In this study, loss of the IncY plasmid in SE211 might be caused by the absence of antibiotic selective pressure. Thus, antibiotics should be appropriately added to the glycerol bacteria of *Salmonella* transconjugants.

CONCLUSION

The *QnrS* gene located on IncY plasmid can transfer from *E. coli* E2 to *Salmonella* SE211 both under laboratory conditions and in the chicken intestinal environment. Sub-MIC of ENR and the clinically recommended dose of ENR can promote conjugation transfer of the *qnrS* gene. The selective pressure exerted by ENR on the intestinal environment of chickens contributed to the persistence of resistance *E. coli* and *qnrS* genes, thus increasing the risk of resistance gene transmission as well as the resistance gene reservoir.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

ETHICS STATEMENT

The animal study was reviewed and approved by the study *in vivo* was carried out in accordance with the guidelines established by the China Regulations for the Administration of Affairs Concerning Experimental Animals (1988) and Regulations for the Administration of Affairs Concerning Experimental Animals in Hubei province (2005) (Project No. 2017YFC1600100 and Animal Welfare Assurance No. HZAUCH- 2020-0005). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MD conceived and designed the study, wrote, reviewed, and edited the manuscript. YZ, ZC, LC, TH, KG, and FZ performed the experiments. YZ and ZC wrote the draft manuscript. All authors participated in the interpretation of the results and read and approved the manuscript.

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Molecular Genetic Characteristics of Plasmid-Borne *mcr-9* in *Salmonella enterica* Serotype Typhimurium and Thompson in Zhejiang, China

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Salmonella enterica is a zoonotic food-borne pathogen threatening public health around the world. As is the case with many other pathogens, the spread of mobilized colistin resistance (*mcr*) alleles is of grave concern. In this study, totally 689 clinical *Salmonella* isolates were collected from a local hospital in Hangzhou, Zhejiang Province, China between 2009 and 2018. Resistance genes were screen by PCR. Two *mcr-9*-positive *Salmonella* strains S15 and S639 were identified which belong to serotype Typhimurium and Thompson, respectively. We observed that both *mcr-9* genes were located on conjugative IncHI2 plasmids which encoded numerous resistance genes, likely facilitating the dissemination of *mcr-9* by co-resistance mechanisms. The *mcr-9* cassettes encoded on the two plasmids were not identical: downstream of the *mcr-9* genes, we found IS*1* on one plasmid (pS15), while the other had a *WbuC*-IS*26* (pS639). Despite the presence of *mcr-9* cassettes, the strains were not rendered colistin resistant. Yet, it is of epidemiological importance to implement surveillance to be able to observe and possibly control the spread of *mcr-9* due to its potential to mediate resistance to the last-resort antibiotic colistin.

Keywords: *mcr-9*, colistin, IncHI2 plasmid, *Salmonella* Typhimurium, *Salmonella* Thompson

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INTRODUCTION

Colistin is an effective antibiotic for the treatment of infections caused by multidrug-resistant Gram-negative bacteria as one of the last-resort therapeutic options (Nation and Li, 2009). Since the plasmid-encoding colistin-mediated resistance gene *mcr-1* was reported in *Escherichia coli* of animal origin in China (Liu et al., 2016), plasmid-borne *mcr* alleles have gained increasing attention and have been extensively researched. Successively, *mcr-2* to *mcr-10* have been identified, most from animals (Carroll et al., 2019; Lima et al., 2019; Wang et al., 2020). According to the current

TABLE 1 | The primers used in this study.

Primer	Sequence of primer (from 5′ to 3′)	Usage
mcr-2-F	CAAGTGTGTTGGTCGCAGTT	Screening for *mcr* alleles
mcr-2-R	TCTAGCCCGACAAGCATACC	
mcr-3-F	TTGGCACTGTATTTTGCATTT	
mcr-3-R	TTAACGAAATTGGCTGGAACA	
mcr-4-F	ATTGGGATAGTCGCCTTTTT	
mcr-4-R	TTACAGCCAGAATCATTATCA	
mcr-5-F	ATGCGGTTGTCTGCATTTATC	
mcr-5-R	TCATTGTGGTTGTCCTTTTCTG	
mcr-6-F	AGCTATGTCAATCCCGTGAT	
mcr-6-R	ATTGGCTAGGTTGTCAATC	
mcr-7-F	GCCCTTCTTTTCGTTGTT	
mcr-7-R	GGTTGGTCTCTTTCTCGT	
mcr-8-F	TCAACAATTCTACAAAGCGTG	
mcr-8-R	AATGCTGCGCGAATGAAG	
mcr-9-F	TTCCCTTTGTTCTGGTTG	
mcr-9-R	GCAGGTAATAAGTCGGTC	
mcr-10-F	GGACCGACCTATTACCAGCG	
mcr-10-R	GGCATTATGCTGCAGACACG	
XH104-F	AAAGTCATCATCCCCTAATGCTTTTG	Verification of transconjugants
XH104-R	TGACAGTATTAGGATTTGCGGTTG	
S15-mcr9.1-F	TGTATGAATCCCGCCTGAAGGGA	
S15-mcr9.1-R	TGCAGCGAATAAGGCAATCATAA	

data, *mcr-1* and *mcr-9* are the most common colistin resistance cassettes with *mcr-9* prevalent in *Salmonella enterica* (Ling et al., 2020). *Salmonella enterica* is an important zoonotic pathogen, which can disseminate between animals and people through contaminated food (Lima et al., 2019). Nontyphoidal *Salmonella* usually causes self-limited enterocolitis with diarrhea. Occasionally an infection with the pathogen can result in more severe diseases including bloodstream infections especially in young children, the elderly, and immunocompromised people (Crump et al., 2015). Thus, the increasing antimicrobial resistance in *Salmonella* species needs to be monitored (Lozano-Leon et al., 2019).

In a previous study, we focused on the prevalence of the *mcr-1* gene in 689 clinical *Salmonella* isolates in a local hospital and six *mcr-1* positive strains were identified (Fan et al., 2020). Five strains belonged to *S.* Typhimurium and one belonged to *S.* Indiana. In this work, we have screened the *Salmonella* isolates for other *mcr* alleles (*mcr*-2 to *mcr*-10). Here, we identified two plasmid-borne *mcr-9* in *Salmonella* Typhimurium and *Salmonella* Thompson. To our knowledge, this is the first detailed description of *mcr-9* plasmid of *Salmonella* Thompson. In this work, we characterized the composition of the *mcr-9* carrying plasmids and the genetic environment surrounding the *mcr-9* cassettes, which differed in the two plasmids.

MATERIALS AND METHODS

Clinical Isolates and Identification

Salmonella clinical isolates were isolated from patients' specimens such as blood, feces, synovial fluid and pus from abdominal and skin and soft tissue infections in the First People's Hospital of Hangzhou, Zhejiang Province, China, between 2009 and 2018. Bacterial species were identified by the automated Vitek 2 system (BioMérieux, Marcy-l'Étoile, France) and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) (Bruker, Bremen, Germany). *Salmonella* serotyping was identified by slide agglutination with specific antisera (Tianrun Bio-Pharmaceutical Co., Ltd., Ningbo, China) according to the White-Kauffmann-Le Minor scheme (9th edition).

mcr Alleles Screened by PCR and Sequencing

All *Salmonella* isolates were screened for *mcr-2* to *mcr-10* by using PCR with corresponding pairs of primers (**Table 1**). The amplification products were subsequently sequenced by Sanger sequencing for confirmation.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by broth microdilution, including colistin, ampicillin, amoxicillin, piperacillin-tazobactam, cefazolin, cefoxitin, ceftriaxone, cefepime, ceftazidime, aztreonam, ertapenem, imipenem, meropenem, amikacin, gentamicin, kanamycin, ciprofloxacin, levofloxacin, tigecycline, tetracycline, trimethoprim-sulfamethoxazole, and fosfomycin. The minimum inhibitory concentration (MIC) of nitrofurantoin was performed using E-test method. Antimicrobial susceptibility testing of the transconjugants was performed by broth microdilution, including colistin, ampicillin, amoxicillin, piperacillin-tazobactam, amikacin, gentamicin, kanamycin, and tetracycline.

The results of antimicrobial susceptibility testing were interpreted by Clinical and Laboratory Standards Institute guidelines (CLSI) (M100, 30th ed.; CLSI, 2020), except that colistin and tigecycline were used the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints v8.1. The quality control strain was *E. coli* ATCC 25922.

Genome Sequencing and Analysis

Genomic DNA of two *mcr-9*-positive strains S15 and S639 were sequenced by HiSeq (Illumina, San Diego, CA, United States) and MinION sequencer (Oxford Nanopore Technologies, Oxford, United Kingdom). The short read and long read sequence data were hybrid *de novo* assembled by Unicycler v0.4.8 (Wick et al., 2017). The gene sequences were annotated by Prokka (Seemann, 2014) and NCBI Blast (Camacho et al., 2009). Resistance genes and insertion sequence (IS) were identified by BacAnt (Hua et al., 2021). Multi-locus sequence typing (MLST) was identified by using mlst.[1] The gene sequences were compared and visualized by Easyfig 2.2.5 (Sullivan et al., 2011) and BRIG-0.95 (Alikhan et al., 2011).

Conjugation Experiments

Conjugation assays were conducted by using rifampicin-resistant *Salmonella* strain XH1984 and *E. coli* strain EC600 as the recipient strain and strain S15 as the donor. The Mueller-Hinton agar plates containing rifampicin (100 μg/ml) and ampicillin (4 μg/ml for S15 and XH1984; 32 μg/ml for S15 and EC600) were used

[1]https://github.com/tseemann/mlst

for selection. The successful transconjugants of S15 and XH1984 were verified by PCR using two pairs of primers: XH104-F and XH104-R; S15-mcr9.1-F and S15-mcr9.1-R (**Table 1**). The former pair of primers was used to identify XH1984 and the latter was used to identify pS15. The transconjugants of S15 and EC600 were verified by S15-mcr9.1 primers and MALDI-TOF MS. The conjugation frequency of pS15 was determined.

Phylogenetic Trees of *mcr-9*-Carrying *Salmonella*

The assembled *mcr-9* carrying *S.* Thompson and *S.* Typhimurium genomes were downloaded from NCBI and annotated using prokka 1.13 (Seemann, 2014). The maximum likelihood phylogenetic tree was constructed with IQTree 2.1.2 (Nguyen et al., 2015) from a multiple alignment of the core genomes generated by Roary 3.7.0 (Page et al., 2015). The trees were visualized with ggtree (Yu, 2020) and ggtreeExtra (Xu et al., 2021) in R.

RESULTS

Screening for *mcr-2* to *mcr-10*

We previously screened 689 clinical *Salmonella* isolates from hospital patient specimens for the presence of the colistin resistance gene *mcr-1* (Fan et al., 2020). This follow-up study, we have screened all isolates for other types of *mcr* genes, including *mcr-2* to *mcr-10*. While none of the strains contained any *mcr-2* to *mcr-8* or *mcr-10* genes, we found two (0.29%) *mcr-9*-positive *Salmonella* spp. strains, S15 and S639. The strain S15 was isolated from the stool of a 53-year-old female patient in 2011, while S639 was a stool sample isolate from a 24-year-old woman obtained in 2018. Both patients came to the outpatient service with symptoms of diarrhea.

Results of Antimicrobial Susceptibility Testing

The antimicrobial susceptibility results are displayed in **Table 2**. Two *mcr-9*-positive strains were both resistant to ampicillin, amoxicillin, and tetracycline. S639 was additionally resistant to cefazolin, cefoxitin, ceftriaxone, ceftazidime, aztreonam, amikacin, kanamycin, ciprofloxacin, levofloxacin, and trimethoprim-sulfamethoxazole. Both strains were sensitive to colistin, cefepime, ertapenem, imipenem, meropenem, gentamicin, tigecycline, and nitrofurantoin.

Whole-Genome Sequencing Analysis

The serotype of S15 was *Salmonella* Typhimurium (O4:Hi), belonging to ST 34. The strain contained a single plasmid only which we called pS15. The plasmid encoded the *mcr-9* gene which showed a 100% identity and coverage to the previously reported *mcr-9* in *Enterobacterales* (WP_044704969.1). The genome size of IncHI2 plasmid pS15 was 266,098 bp and the GC content was 46%. This strain contains 12 resistance genes on the plasmid and 20 resistance genes on the chromosome (**Table 3**).

The serotype of S639 was *Salmonella* Thompson (O7:Hk:H1,5), belonging to ST 26. Again, this strain contained only a single plasmid which we called pS639. Here, the *mcr-9* exhibited 100% identity and coverage to another colistin resistance gene in the

TABLE 2 | Summary of antimicrobial susceptibility testing.

Antibiotics (μg/ml)	Strains					
	S15	**S639**	**XH1984**	**XH1984-pS15**	**EC600**	**EC600-pS15**
Colistin	0.5	0.5	1	1	0.06	0.06
Ampicillin	512	>2,048	1	>64	8	>128
Amoxicillin	32	64	1	>64	16	>128
Piperacillin-tazobactam	4/4	32/4	2/4	4/4	4/4	8/4
Cefazolin	2	>1,024				
Cefoxitin	4	128				
Ceftriaxone	0.5	8				
Cefepime	0.25	0.5				
Ceftazidime	0.5	>64				
Aztreonam	0.06	32				
Ertapenem	0.032	0.25				
Imipenem	0.5	1				
Meropenem	0.06	0.125				
Amikacin	4	32	2	2	4	4
Gentamicin	1	0.5	0.25	0.25	0.25	0.25
Kanamycin	2	32	2	2	16	16
Ciprofloxacin	0.032	0.5				
Levofloxacin	0.125	1				
Tigecycline	0.25	0.5				
Tetracycline	32	256	1	32	4	>32
Nitrofurantoin	24	16				
Trimethoprim-sulfamethoxazole	2/38	>32/608				
Fosfomycin	4	128				

TABLE 3 | Resistance genes in two strains.

Strains	ST	Plasmid type	Resistance genes in plasmid	Resistance genes in chromosome
S15	ST34	IncHI2, IncHI2A	*terW,terZ,merD,merB,merR_Ps,* bla_{TEM-1}*,tet(A),dfrA16,aadA2, mcr-9,pcoS,pcoE*	*sinH,golS,golT,mdsB,pcoE, pcoS,pcoD,pcoC,pcoB,pcoA,silP,silB,silF, silC,silS,silE,arsC_gluta,arsB_ pKW301,arsA,arsR_pKW301*
S639	ST26	IncHI2, IncHI2A	*terW,terZ,aadA2,sul1,* bla_{TEM-1}*, merT,merA,merE,mph(A),sul1,* bla_{OXA-10}*,aacA34,arr-3,aph(6)-ld,aph(3″)-lb,sul2,catA2,tet(D), pcoE,pcoS,mcr-9.1,aph(6)-ld, aph(3″)-lb,dfrA19,sul1,* bla_{DHA-1}*, qnrB4*	*sinH,golS,golT,mdsB*

Enterobacterales, WP_001572373, *mcr-9.1*. This sequence was missing a single codon (for tryptophan) right before the STOP codon compared to *mcr-9* in S15. The genome size of IncHI2 plasmid pS639 was 308,491 bp and the GC content was 48%. This strain contains 27 resistance genes on the plasmid while only four resistance genes are found on the chromosome (**Table 3**).

Most of the chromosome-encoded resistance genes in S15 and S639 are related to metal resistance aside from the multidrug efflux RND transporter gene *mdsB*. The resistance genes in two plasmids differ greatly from each other: for example, pS15 contains genes associated with resistance to tellurium (*terW* and *terZ*), mercury (*mer* gene cluster), β-lactams (bla_{TEM-1}), tetracycline [*tet(A)*], trimethoprim (*dfrA16*), streptomycin (*aadA2*), colistin (*mcr-9*), and copper (*pcoS* and *pcoE*). Plasmid pS639 encodes genes mediating resistance to all substances which are facilitating resistance in pS15, some of which were different alleles like *dfrA19* and *tet(D)*. Additionally, pS639 encodes genes associated with resistance to sulfonamide (*sul1* and *sul2*), macrolide [*mph(A)*], β-lactams (bla_{OXA-10}), aminoglycoside (*aacA34*), rifamycin (*arr-3*), streptomycin [*aph(6)-ld* and *aph(3″)-lb*], chloramphenicol (*catA2*), cephalosporin (bla_{DHA-1}), and quinolone (*qnrB4*). This abundance of additional ARGs might explain why S639 exhibited higher MIC values and a wider resistance to more antibiotics than S15.

Comparison of the Plasmid Sequences

The two plasmids we found are similar to other IncHI2 plasmids. **Table 4** lists plasmids for comparison, some of which share high query coverage and identity with pS15 and pS639 from different species. The backbone structures of pS15, pEcl10-1 (CP048704), sLN794248 (LN794248), and pC45-VIM4 (LT991958) are closely related (**Figure 1**). The main differences between them are the resistance gene cluster regions where most insertion elements (ISs) were located. Although pEcl10-1 and sLN794248 were more similar to pS15 in their sequence, they do not contain *mcr-9.1* and *dfrA16*. Plasmid pC45-VIM4 encodes a *mcr-9.1* gene but shows more differences in the 90–140 kbp region of pS15 compared to other plasmids. When comparing pS15 to three other plasmids, pS15 contains a gene encoding group II intron reverse transcriptase/maturase (around 210 kb). Two resistance gene clusters are present in pS639, found in two sections, from ~100 to 180 kb and from ~240 to 270 kb, respectively (**Figure 2**). The first section shares some similarities but also substantial differences with two other plasmid sequences, p48212_MCR (CP059413) and pMOL665_IncHI2 (OU015720). pS639 additionally encodes *mph(A)*, bla_{OXA-10}, *aacA34*, *aar-3*, and *catA2*, genes which are able to facilitate the resistance to macrolide, β-lactam, aminoglycoside, rifamycin, and chloramphenicol. In the other resistance gene cluster which includes *mcr-9.1*, the main difference is an insertion of the two resistance genes bla_{DHA-1} and *qnrB4* while a gene cluster encoding phage shock protein is also present.

Characterization of the Genetic Context Surrounding *mcr-9* Genes

Our genetic analyses regarding the sequences surrounding the mcr-9 genes revealed two types (**Figure 3**). The mcr-9 surrounding structure of pS639 was *pcoE-pcoS*-IS*903B-mcr-9-WbuC*-IS*26* similar to p48212_MCR and pMOL665_IncHI2. However, in our case, the genetic context that embedded the *mcr-9* gene was *pcoE-pcoS*-IS*903B-mcr-9*-IS*1* which is present in both, the *Salmonella* plasmid pS15 and pC45-VIM4, a plasmid found in a bacterium of the Enterobacter cloacae complex. There was an insertion of IS*1* and *catA* in the position of *mcr-9* and IS*903B*.

Transferability of *mcr-9*-Carrying Plasmids

We also tested the ability of the plasmids to be transferred to other strains and their ability to convey antibiotic resistance. We first tested the plasmid pS15 which was successfully transferred to the rifamycin-resistant *Salmonella* strain XH1984 and *E. coli* strain EC600. When determining the MICs for ampicillin, amoxicillin, piperacillin-tazobactam, and tetracycline, we found increased resistance likely conferred by the presence of the plasmid-encoded bla_{TEM-1} and *tet(A)* genes (**Table 2**). However, *mcr-9* in pS15 could not confer colistin resistance in neither *Salmonella* nor *E. coli* strains. The conjugation efficiency was calculated in pS15 plasmid conjugation assays, which was 1.9×10^{-6} transconjugants per

donor when pS15 was transferred to XH1984 and 2.1×10^{-8} transferred to EC600 (**Table 5**).

Our attempt in transferring the plasmid pS639 was unsuccessful. The reason for this is that we did not have a suitable recipient strain available which would allow the use of an antibiotic selection marker, as our strains exhibited resistance to the antibiotics encoded on the plasmid. Also, the possibility that the recipient strains were genetically not suitable to receive this specific plasmid cannot be excluded.

Genetic Homology of *mcr-9* Carrying *Salmonella*

A total of 175 *S.* Typhimurium strains and 21 *S.* Thompson strains carrying *mcr-9* have been deposited in NCBI till today (February 2022). The phylogenetic trees of two serotypes were displayed in **Figures 4, 5**, respectively. *S.* Typhimurium strains were mostly isolated from clinical samples. Australia and the United Kingdom were the countries where the most assembled sequences were uploaded from, which does not

TABLE 4 | Comparison of two *mcr-9*-positive plasmids with similar plasmids.

Plasmid	Similar plasmid	Species	Query coverage (%)	Identity (%)
pS15 (*Salmonella* Typhimurium)	pEcl10-1 (CP048704)	*Enterobacter hormaechei*	98	99.98
	sLN794248 (LN794248)	*Salmonella* Typhimurium	98	99.98
	pC45-VIM4 (LT991958)	*Enterobacter cloacae* complex	96	99.96
pS639 (*Salmonella* Thompson)	p48212_MCR (CP059413)	*Enterobacter hormaechei*	94	99.29
	pMOL665_IncHI2 (OU015720)	*Salmonella* Typhimurium	92	99.29

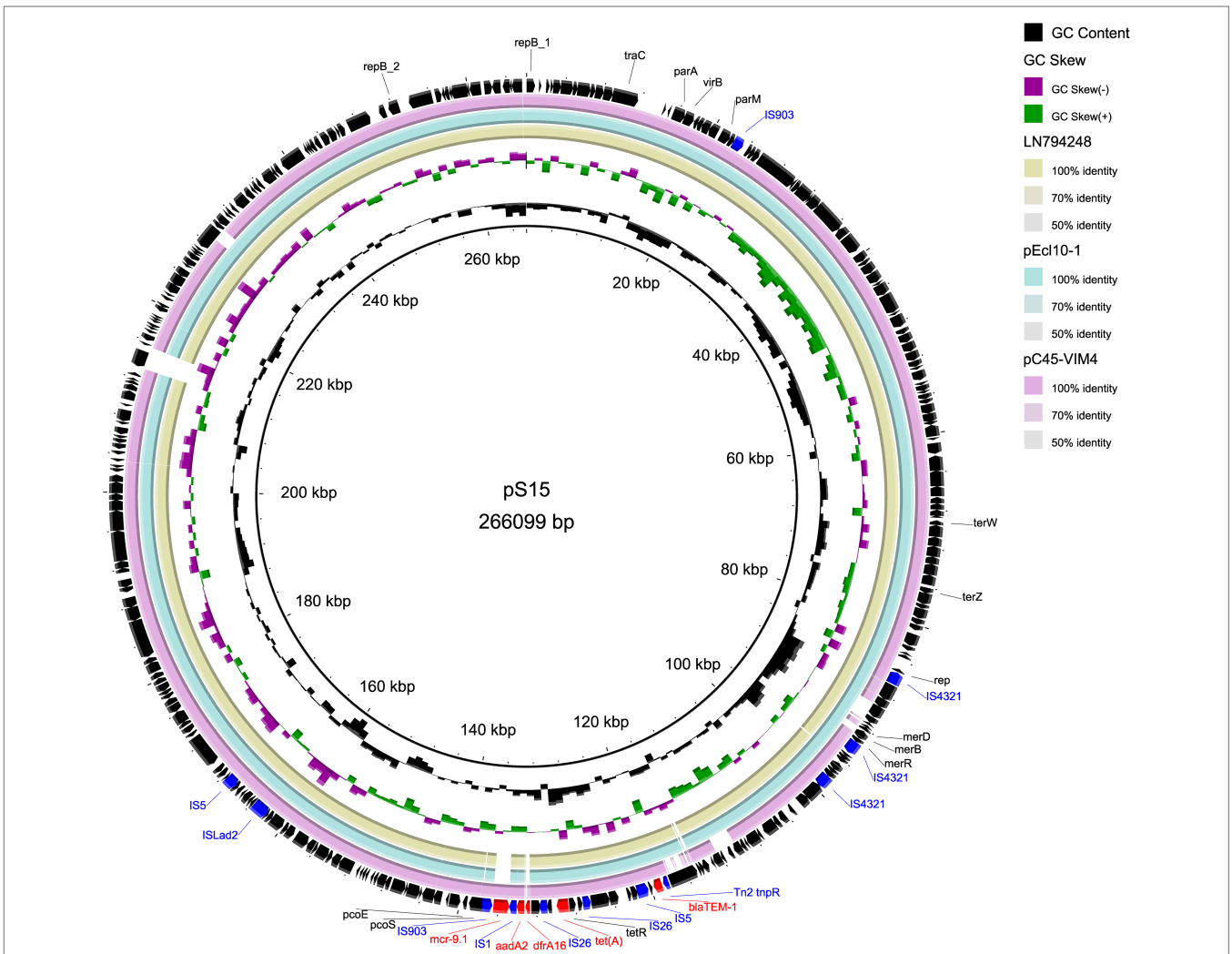

FIGURE 1 | Comparison of circular structures of pS15 and its similar plasmids. Arrows indicate the direction of predicted transcription of each gene. Red arrows indicate antibiotic resistance genes. Blue arrows indicate insertion sequences.

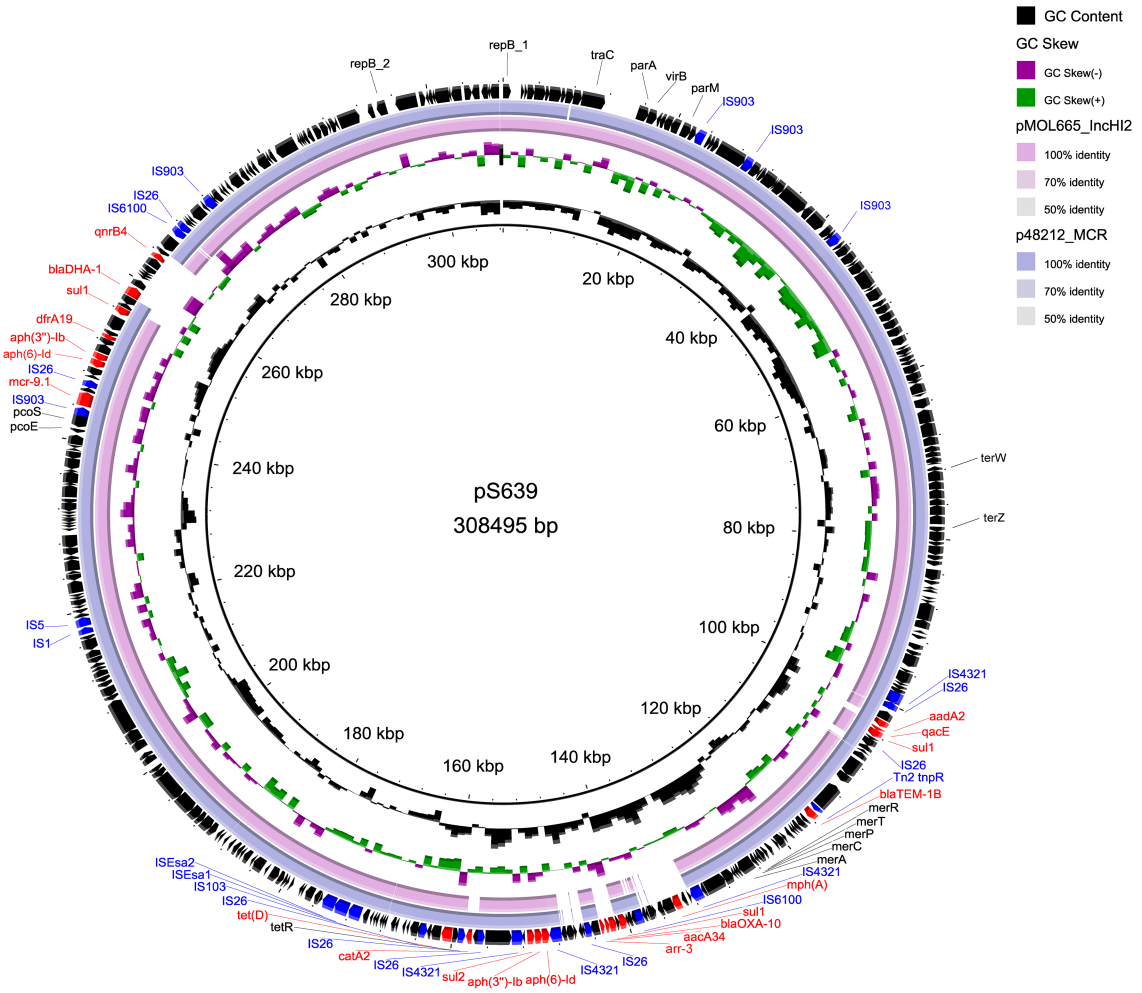

FIGURE 2 | Comparison of circular structures of pS639 and its similar plasmids. Arrows indicate the direction of predicted transcription of each gene. Red arrows indicate antibiotic resistance genes. Blue arrows indicate insertion sequences.

necessarily reflect the prevalence of the strains in these countries. *S.* Typhimurium S15 was most similar to FSIS32003798 isolated from pork in the United States. As for *S.* Thompson, only three of 21 strains were clinical origin including S639, which had the closest relationship with 813,389 isolated in the United Kingdom. Interestingly, both S15 and S639 were more closely related to strains isolated outside China, despite being isolated within the country.

DISCUSSION

With multidrug resistance continuously increasing, colistin now belongs to the last-resort antibiotics. Plasmid-bound *mcr* alleles that mediate resistance to the antimicrobial compound are of great concern in particular if plasmid encoded due to the risk of rapid spread (Smelikova et al., 2021). Several *mcr* genes have been found, with *mcr-9* first identified in *Salmonella* Typhimurium (Carroll et al., 2019). To date, *Salmonella* strains have been reported worldwide to carry various *mcr* alleles, with mcr-1 being the most common and Typhimurium being the most prevalent serotype (Lima et al., 2019; Paveenkittiporn et al., 2021; Portes et al., 2022). As a zoonotic food-borne pathogen, *mcr*-positive *S. enterica* strains were mostly isolated from livestock, including pork and poultry, due to the fact that colistin has and continues to be used in animal husbandry (Lima et al., 2019). Therefore, it is important to monitor the spread of *mcr* alleles in *S. enterica*.

In this study, we identified two *mcr-9*-positive *Salmonella enterica* from a total of 689 clinical *Salmonella* isolates. The positive rate of *mcr-9* (0.29%) might be lower than *mcr-1* (0.87%) as we previously reported (Fan et al., 2020) although due to the low numbers (of two and six strains, respectively), statistically sound conclusions are not possible. The two *mcr-9*-positive strains belong to a different serotype, Typhimurium and Thompson. *Salmonella* Typhimurium ST34 is most commonly prevalent in causing food-borne infections in China (Wong et al., 2013). While *Salmonella* Thompson is the main serotype

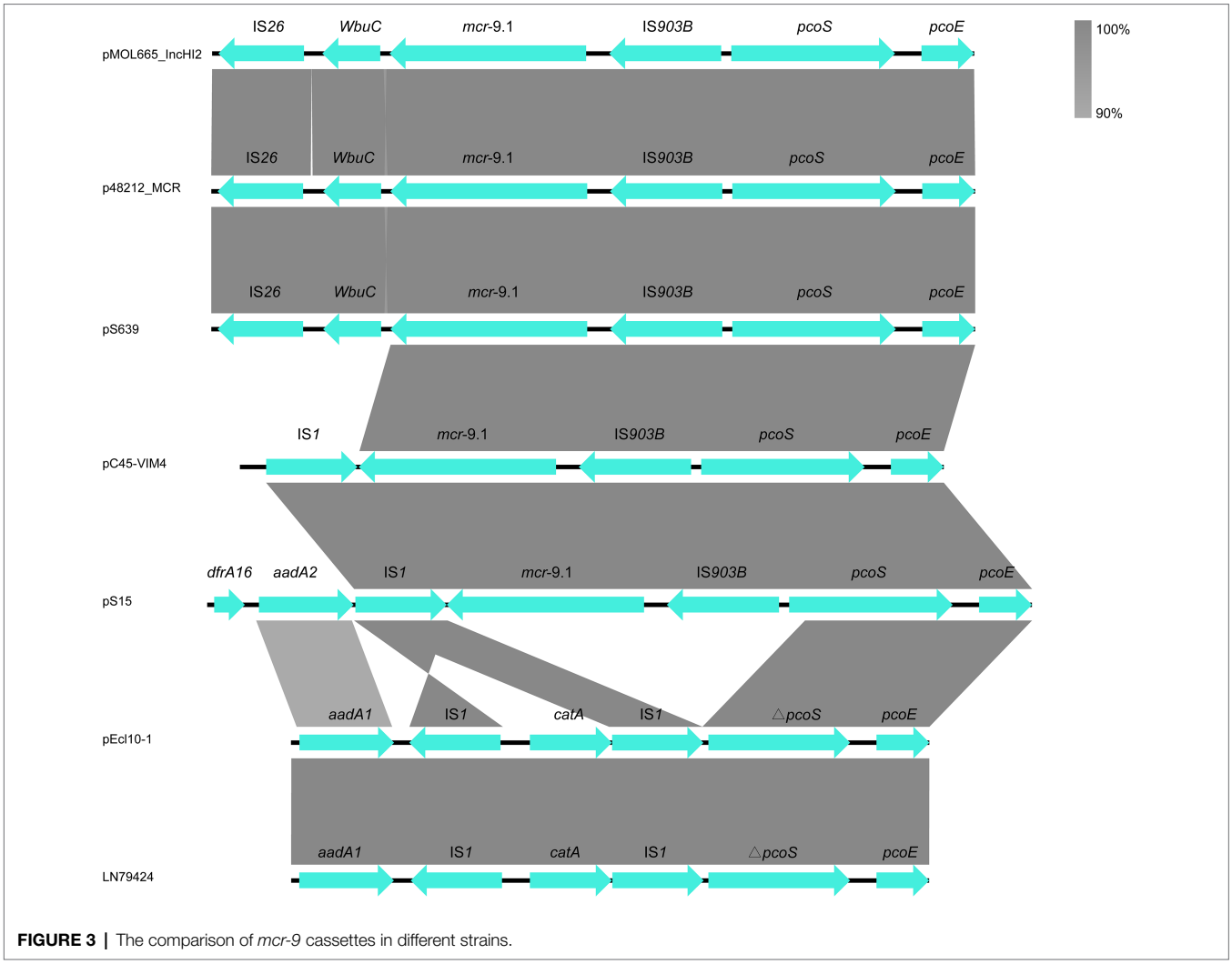

FIGURE 3 | The comparison of *mcr-9* cassettes in different strains.

TABLE 5 | Conjugation frequency of pS15 from S15 to XH1984 and EC600.

	S15-XH1984				S15-EC600			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
Donors (D)/ml	1.1×10^{11}	6.9×10^{11}	5.0×10^{10}		3.5×10^{11}	9.0×10^{10}	1.4×10^{11}	
Transconjugants (TC)/ml	3.4×10^{5}	5.7×10^{5}	9.0×10^{4}		8.6×10^{3}	1.8×10^{3}	2.5×10^{3}	
Conjugation frequency (TC/D)	3.1×10^{-6}	8.3×10^{-7}	1.8×10^{-6}	1.9×10^{-6}	2.5×10^{-8}	2.0×10^{-8}	1.8×10^{-8}	2.1×10^{-8}

isolated from poultry-based products (Yang et al., 2020; Elbediwi et al., 2021b). In this study, both strains were isolated from stools of patients with diarrhea caused by *Salmonella enterica* infections, likely to have been exposed to food contaminated with the pathogen. The surprising similarity between the strains we isolated in China and those found outside the country can be explained by the rapid development of international agricultural products trade.

IncHI2 plasmids were the predominant plasmid type carrying *mcr-9* (Li et al., 2020). The two plasmids that we characterized in our study, pS15 and pS639, also belonged to the IncHI2 type, both of them having IncHI2 and IncHI2A replicons, which indicates that they are hybrid plasmids. This type of plasmid is conjugative which can result in extensive spread of the *mcr-9* gene in recipient hosts (Ai et al., 2021; Elbediwi et al., 2021a; Khodor et al., 2021; Wang et al., 2021). Testing whether the plasmids we discovered can be transmitted to other strains, we found that pS15 was indeed conjugative to both, *Salmonella* and *E. coli*. The efficiency of conjugation to *Salmonella* was about 90 times

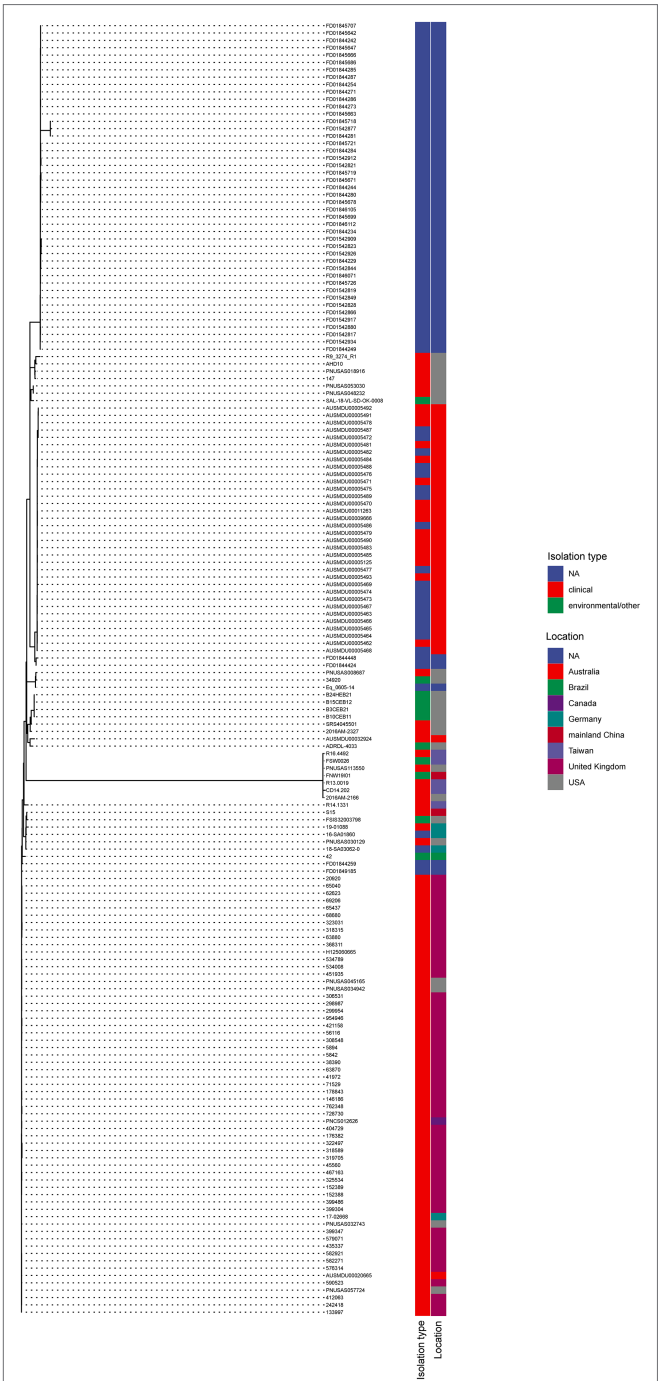

FIGURE 4 | The phylogenetic tree of *mcr-9* carrying *S.* Typhimurium strains.

higher than that to *E. coli*, indicating pS15 was easier to spread within species. The conjugation experiments using the plasmid pS639 could not be performed due to the fact that we did not have a suitable recipient strain to our disposal as we could not select for an antibiotic encoded on the plasmid. However, pS15 and pS639 were predicted to contain similar conjugative apparatus components by oriTfinder (Li et al., 2018). Thus, it is reasonable to conclude that pS639 is likely to be conjugative since the components of the two plasmids show strong similarities.

Compared to the low level colistin resistance mediated by *mcr-1*, most of the *mcr-9*-carrying strains do not present resistance to colistin (Luo et al., 2017; Wang et al., 2021). We also observed this to be the case with the strains S15 and S639, which we described in this study. However, the inducible expression of *mcr-9* could potentially lead to an increasing of colistin MIC after exposure to low concentrations of colistin, mediated by the *qseC* and *qseB* genes (Kieffer et al., 2019). This makes *mcr-9* a gene that should not be disregarded when addressing antimicrobial resistance. Apart from this, our study identified numerous resistance genes located in the two plasmids in addition to *mcr-9*, which were responsible for the drug resistance spectrum of two strains. A total of 12 resistance genes are found in pS15 and 27 in pS639. S15 and S639 were resistant to broad spectrum penicillin and tetracycline because they both had plasmid-encoded $bla_{\text{TEM-1}}$ and *tet*, which was verified by the transconjugants of pS15. In addition, pS639 encoded genes *sul*, $bla_{\text{OXA-10}}$, $bla_{\text{DHA-1}}$, *aacA34,* and *qnrB4*, accounting for the resistance to sulfonamides, cephalosporins, aminoglycosides, and quinolones. Since there are multiple resistance genes encoded on *mcr-9* plasmids, it is a matter of concern that co-resistance mechanism could facilitate the spread of *mcr-9*. The two types of *mcr-9* cassettes in our study, *pcoE-pcoS*-IS*903B-mcr-9-WbuC*-IS*26* and *pcoE-pcoS*-IS*903B-mcr-9*-IS*1*, did not include the *qseC-qseB* regulatory genes, indicating they might circulate silently. However, there might be other undetermined genes or molecules regulating *mcr-9* expression (Kananizadeh et al., 2020). Therefore, it is important to investigate the silent spread of *mcr-9* further and to monitor the dissemination of plasmids containing the colistin resistance gene.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The studies obtained ethical approval from the Ethics Committee of Hangzhou First People's Hospital (2020103-1).

AUTHOR CONTRIBUTIONS

JF, YF, DZ, and XH designed the study. JF, HC, YF, LZ, JH, and YYa performed the experiments. JF, YF, LZ, JH, and HC analyzed the bioinformatics data. JF, YF, and HC wrote the manuscript. QX, DZ, SL, YYu, and XH revised the manuscript. All authors contributed to the article and approved the submitted version.

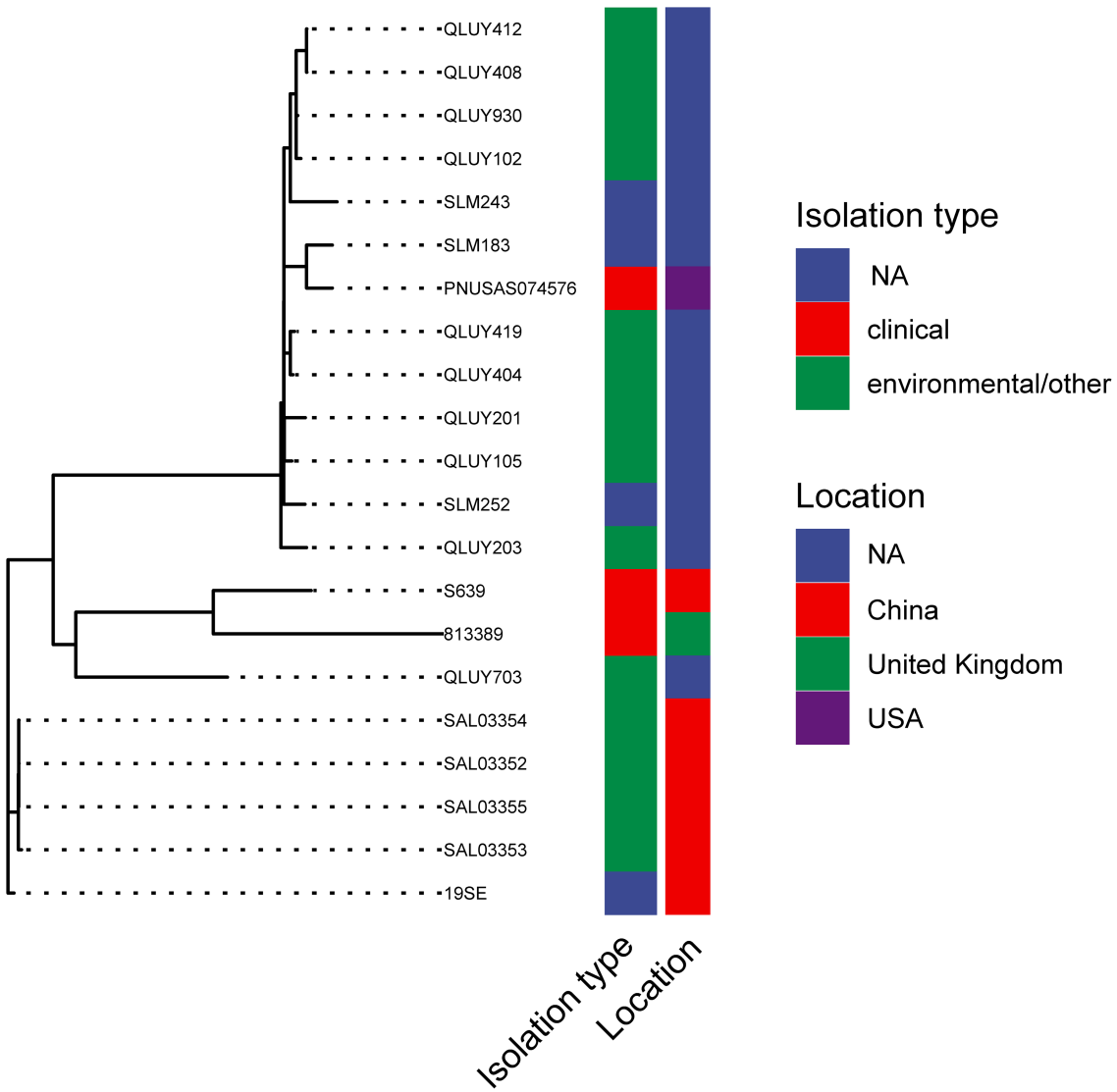

FIGURE 5 | The phylogenetic tree of *mcr-9* carrying *S.* Thompson strains.

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Increased Drug Resistance and Biofilm Formation Ability in ST34-Type *Salmonella* Typhimurium Exhibiting Multicellular Behavior in China

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Salmonella Typhimurium is an important food-borne pathogen. In this paper, multicellular behavior and associated characteristics of *S.* Typhimurium isolated from human and animal source food were studied. All the *S.* Typhimurium strains exhibiting multicellular behavior (100%) belonged to the ST34 type. In addition, most of the ST34-type multicellular behavior *S.* Typhimurium strains had a human origin (69.11%) and 98% of the ST34-type multicellular behavior strains exhibited strong biofilm formation capacity, which was much higher than that of non-multicellular behavior strains (7%, $P<0.01$). Antibiotic resistance in ST34-type multicellular behavior strains was significantly higher than in strains with non-multicellular behavior for most conventional drugs ($P<0.05$); notably, Polymyxin B (8%) and Imipenem (1%) resistances were also observed in the ST34-type strains. Furthermore, all the ST34-type multicellular behavior strains (100%) exhibited Multiple Drug Resistance (resistance to ≥3antibiotics), which was much higher than that of the non-multicellular behavior strains ($P<0.05$). Consistent with the drug-resistant phenotype, the carrying rates of most drug-resistant genes in ST34-type multicellular behavior strains were higher than that those in non-multicellular behavior strains ($P<0.05$). Therefore, this study revealed the emergence of a prevalent ST34-type multicellular behavior *S.* Typhimurium strains with increased biofilm formation ability and drug resistance rate, which poses a threat to public health safety, and highlights the need for comprehensive monitoring of the strains.

Keywords: *S.* Typhimurium, animal source food, ST34, multicellular behavior, biofilm, antibiotic resistance

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INTRODUCTION

Salmonella is an important zoonotic pathogen that causes food-borne diseases in many countries (Gomez et al., 1997). *Salmonella* has been reported to be the leading cause of death among food-borne bacterial pathogens in some countries (Casey et al., 2011) with 94 million infections annually and 155,000 deaths globally (Hendriksen et al., 2011). *S.* Typhimurium is one of the dominant serotypes among the >2,500 recognized *Salmonella* serotypes (Popoff et al., 2001; Liang et al., 2015; Yang et al., 2017). Furthermore, *S.* Typhimurium has numerous hosts and is widely distributed; it can not only infect animals but is also transmitted to humans through contaminated food (Adagbada et al., 2014) and the mortality caused by *S.* Typhimurium is 3-fold higher than the average mortality associated with *Salmonella* (Angulo and Kre, 2005).

S. Typhimurium has different sequence types (STs), which exhibit distinct biological characteristics including virulence (Singletary et al., 2016) and drug resistance (Li et al., 2017). An analysis of *S.* Typhimurium strains in the Enterobase database[1] revealed that from the end of the 19th century to the end of the 1980s, all the *S.* Typhimurium isolates belonged to ST19; however, since 1990, the proportion of ST34 has increased annually and has reportedly replaced ST19 in recent years as the prevalent ST (Wong et al., 2013; Li et al., 2017). Consequently, the prevalence trends of *S.* Typhimurium STs should be monitored constantly to facilitate the effective prevention and control of the associated epidemics.

Currently, the treatment of diseases related to *S.* Typhimurium infection mainly involves the administration of antibiotics. However, in the wake of widespread antibiotic use, the rapid emergence of Multi-Drug Resistance (MDR) is a new challenge for the global prevention and control of *S.* Typhimurium infection. *S.* Typhimurium can exhibit multicellular behavior under drug or environmental pressure (Römling, 2001) and the prevalence of *S.* Typhimurium exhibiting multicellular behavior has been reported sporadically in Ethiopia (Eguale et al., 2014) and Germany (Cimdins and Simm, 2017a), among other countries. Multicellular behavior of *S.* Typhimurium refers to the transition of strains from a single-cell planktonic state to a quiescent aggregated colony state and as a community (Römling, 2001), and studies have demonstrated that bacteria with multicellular behavior are mainly characterized by production of extracellular matrix in the forms of curli fibers and cellulose (Rmling et al., 1998; Zogaj et al., 2010), which can be combined with diazodye Congo Red so that they can show the Rdar (red, dry, and rough) phenotype on the Congo Red plate. On the contrary, those strains that are unable to produce multicellular behavior are usually characterized by saw (smooth and white) phenotypic, which do not express these matrix components (Cimdins and Simm, 2017b). Furthermore, in bacterial communities formed by multicellular behavioral strains, the matrix-coated bacteria are considered to have structural protection, which can enhance the strains' adaptability to adverse external environments and their stress tolerance (Eguale et al., 2014). Consequently, such structural protection poses a challenge to typical strategies of prevention and control of *S.* Typhimurium.

Although *S.* Typhimurium is one of the most prevalent *Salmonella* serotypes in China (Lu et al., 2011), systematic studies on the prevalence and associated roles of multicellular behavior in the increasingly prevalent ST34-type *S.* Typhimurium are still lacking. Monitoring the prevalence of multicellular behavior in *S.* Typhimurium and studying the associated biological characteristics could facilitate *Salmonella* infection prevention and control. Therefore, this study explored the prevalence and associated biological characteristics of multicellular behavior in *S.* Typhimurium isolated from human and animal source food in China. In addition, the authors investigated the factors that could facilitate the prevalence of the ST34-type *S.* Typhimurium and highlight the potential risks posed by multicellular behavior in *S.* Typhimurium to public health and safety.

MATERIALS AND METHODS

Bacterial Strains

Salmonella Typhimurium isolates were recovered from samples of clinically suspected patients and animal source food (such as chicken, pork, meat products, and breeding environment) in Fujian, Guangxi, Guangzhou, Hubei, Shandong, Shanxi, Shanghai, Sichuan, Xinjiang, and Chongqing from 2008 to 2017. Among them, 123 strains (61.50%) were of human origin, and 77 strains (38.50%) were of animal food origin. These strains were preserved at the Key Laboratory of Zoonoses Prevention and Control of Guangdong Province, Guangzhou, China.

Detection of the Multicellular Behavior Morphotype

The identification of multicellular behavioral phenotypes was carried out based on methods in previous reports, with slight modifications (Zhang et al., 2019). Briefly, 10 µl of the strains cultured overnight was dropped on a salt-free LB agar plate containing 40 mg/ml Congo Red and 20 mg/ml Coomassie Brilliant Blue G, and cultured at 27°C for 5 d. Subsequently, colony morphology on the plate was observed to determine whether the strain exhibited multicellular behavior, which was characterized based on the RDAR (red, dry, and rough) phenotype. On the contrary, those strains that were unable to produce multicellular behavior were characterized by saw (smooth and white) phenotypic.

Multi-Locus Sequence Typing Analysis

The genomic DNA of the bacteria was extracted by the boiling method as previously reported (Rmling et al., 1998); afterward, based on the primers designed by University of Cork (Achtman et al., 2017), seven pairs of housekeeping genes, including, *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and

[1]http://enterobase.warwick.ac.uk/species/index/senterica

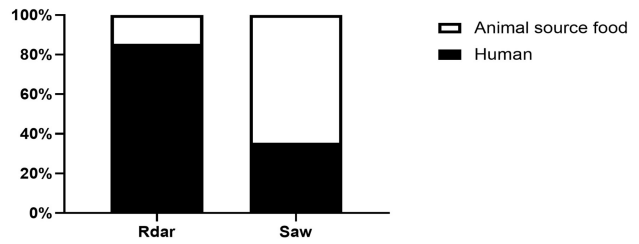

FIGURE 1 | Identification of multicellular behavior phenotypes of *Salmonella* Typhimurium from human- and animal source food.

thrA of *Salmonella*, were amplified by PCR, and the PCR products were sequenced and submitted to the Enterobase database[2] to obtain the corresponding allele numbers. Seven housekeeping genes together constitute the STs of all strains in this study.

Detection of Biofilm Formation Ability

Biofilm formation ability was assessed using a previously described protocol, with some modifications (Gomez-Baltazar et al., 2019). First, a single colony was added to Lucia-Bertani broth (LB) and cultured overnight at 37°C with shaking. On the following day, the bacterial solution was diluted into fresh salt-free LB broth (1,100), and the diluted bacterial solution (200 μl) from each sample was inoculated into each well of 96-well plates, and 200 μl of salt-free LB broth added to the well as a blank control, then incubated at 27°C for 48 h without shaking. Afterward, the bacterial suspension was discarded and the plates were washed three times with PBS solution. After drying, 200 μl of anhydrous methanol was added to each well and fixed for 15 min. The anhydrous methanol was discarded and the plates were dried; thereafter, 200 μl of 1% crystal violet was added to each well and dyed at 24–26°C for 15 min. Unbound dyes were removed gently and the plates were washed three times with PBS solution. After drying at 24–26°C, 200 μl of 33% acetic acid was added to the well to dissolve crystal violet. Finally, optical density was measured at 595 nm using an enzyme-labeling instrument (BIO-TEK). The strains were classified into four categories: strong biofilm forming ability, medium biofilm forming ability, weak biofilm forming ability, and none biofilm forming ability according to the criteria described in a previous study (Diez-Garda et al., 2012).

Antimicrobial Susceptibility Tests

Detection of *S.* Typhimurium susceptibility to antimicrobials was carried out using the Agar dilution method as described by the Clinical and Laboratory Standards Institute (CLSI; Cockerill et al., 2012). *Escherichia* coli ATCC25922 was used as the quality control strain. In the present study, 15 antimicrobial agents that accounted for 10 classes of antibiotics were used for the susceptibility analyses. The results of antibiotic sensitivity were assessed according to the CLSI criteria.

Detection of Antibiotic Resistance Genes

The PCR method was used to detect the resistance genes of isolates with the ACSuT (ampicillin, chloramphenicol, sulfamethoxazole, and tetracyclinen resistance) profile, and the resistance genes of isolates resistant to Polymyxin B and Imipenem. Therefore, PCR screening of 10 resistance genes, including *BlaCTXM*, *BlaTEM*, *Bloxa*, *BlaNDM-5*, *flo*, *sulI*, *sulII*, *TetA*, *TetB*, and *mcr-1*, was carried out (Ahmed et al., 2007; Az et al., 2019; Wang et al., 2019).

Statistical Analysis

The data were analyzed using GraphPad Prism v8.0 (GraphPad Software, San Diego, CA, United States). The Chi-square test was used to compare two test groups. $p < 0.05$ indicated significant difference.

RESULTS

Detection of the Multicellular Behavior Morphotype

S. Typhimurium with multicellular behavior detection was conducted by counting the strains displaying the Rdar phenotype on Congo red agar plate. Among 123 isolates from human sources, 85 (69.11%) and 38 (30.89%) exhibited the Rdar and saw phenotypes, respectively. Conversely, among the strains isolated from animal sources food, 15 (19.48%) and 62 (80.52%) strains exhibited the Rdar and saw phenotypes, respectively (**Figure 1**).

Multi-Locus Sequence Typing Analysis

A total of 81.00% of the strains detected belonged to the ST34 genotype, whereas only 19.00% of the strains belonged to the ST19 genotype. In addition, there were significant differences in ST typing between the Rdar and saw phenotypes. All the Rdar phenotype colonies (100%) belonged to the ST34 genotype, and all the remaining saw phenotypes (38%) belonged to the ST19 genotype (**Figure 2**). Between the two sources, 87.80% (108/123) of the strains of human origin belonged to the ST34 genotype, and 70.13% (54/77) of animal food origin belonged to the ST34 genotype (**Table 1**).

Biofilm Formation Ability of Colonies of Different Phenotypes

All the ST34-type multicellular behavior *S.* Typhimurium had the ability to form biofilms, and 98% exhibited strong biofilm formation ability. However, only 7% of the non-multicellular behavior *S.* Typhimurium exhibited strong biofilm formation ability. Among the non-multicellular behavior *S.* Typhimurium, most (57%) showed weak biofilm formation ability, and 16% of the non-multicellular behavior strains exhibited moderate biofilm formation ability. Furthermore, all strains with no biofilm formation ability were non-multicellular behavior

[2]http://enterobase.Warwick.ac.uk/species/index/senterica

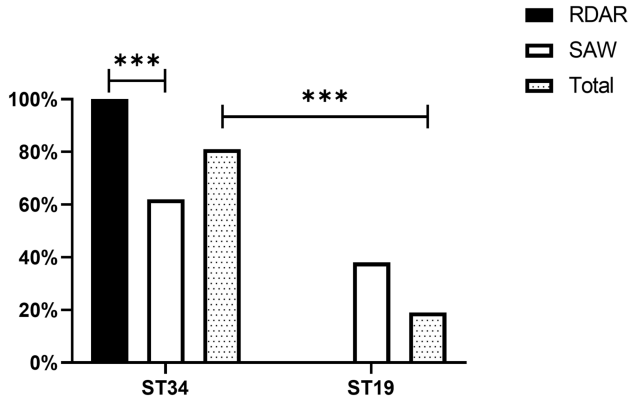

FIGURE 2 | Multi-locus sequence typing of colonies of different phenotypes (***$p < 0.001$).

TABLE 1 | Multi-locus sequence typing (MLST) of different of *Salmonella* Typhimurium isolates from human- and animal source food.

ST type	Human	Animal-based food
ST34	87.8%(108/123)	70.1%(54/77)
ST19	12.2%(15/123)	29.9%(23/77)

(**Figure 3**). Chi-square test results showed that strong biofilm formation ability was significantly more prevalent in Rdar phenotypes than in saw phenotypes ($P < 0.01$).

Antimicrobial Susceptibility

Among all the strains, resistance to Sulfisoxazole (84.50%) was the most frequently observed, followed by resistance to Tetracycline (80.50%), Florfenicol (66.50%), Ampicillin (64.00%), and Nalidixic acid (63.50%). However, resistance to Cefepime (2.00%) and Amikacin (0%) was low. Additionally, ST34-type multicellular behavior *S.* Typhimurium exhibited greater levels of resistance than non-multicellular behavior *S.* Typhimurium, with significant differences in antibiotic resistance, based on the Chi-square test ($p \leq 0.05$; **Figure 4**). Notably, eight out of the nine strains resistant to Polymyxin B were ST34-type multicellular behavior strains. In addition, one strain resistant to Imipenem was an ST34-type multicellular behavior strain.

Among all the strains, 85.50% developed Multi-Drug Resistance (MDR), and all the ST34-type multicellular behavior strains (100%) were phenotypically resistant to at least three classes of antimicrobial agents. A total of 32 (16.0%) isolates exhibited an ACSSuT resistance profile, and among the ST34-type multicellular behavior strains, 24% exhibited the ACSSuT profile, which was significantly higher than the rate observed for the non-multicellular behavior strains (8%; $P < 0.01$; **Figure 5**).

Antibiotic Resistance Genes

The ACSuT resistance genes included *BlaCTXM* (8.50%), *BlaTEM* (44.00%), *Blaoxa* (32.50%), *Flo* (44.00%), *Sul1* (27.00%), *Sul2* (66.00%), *TetA* (11.50%), and *TetB* (67.50%). In addition, one imipenem-resistant strain was found to harbor a *BlaNDM-5*-carrying IncX3 plasmid. The strain also had a IncHI2 plasmid that harbored nine resistant genes, including *aadA1*, *aadA3*, *aph(3′)-la*, *sul1*, *sul2*, *sul3*, *floR*, *cmlA*, and *dfrA12*. Moreover, all the Polymyxin B-resistant strains (100%) harbored the *mcr-1* resistance gene.

Analysis of the resistance genes of different phenotypes revealed that excluding *BlaTEM* and *TetA*, the detection rates of other antibiotic-resistant genes were higher in the ST34-type strains exhibiting multicellular behavior than in the non-multicellular strains (**Figure 6**). In addition, there were significant differences in the resistance genes between the Rdar and saw morphotypes ($P < 0.01$, **Figure 6**). Therefore, the resistance gene carrying rates of the ST34-type multicellular behavior strains were much higher.

DISCUSSION

S. Typhimurium is an important food-borne pathogen. It not only causes economic losses in the livestock and poultry breeding industry, but also poses serious risks to human health (Diez-Garda et al., 2012). The results of the present study revealed that ST34 is currently the main *S.* Typhimurium ST type from human and animal source food in China. Other studies have also reported that ST34 has replaced ST19 as the main ST type in some regions in China (Li et al., 2017) and in some other countries (Biswas et al., 2019). To explore the biological characteristics of the emerging ST34 epidemics, and the underlying factors involved in its prevalence and spread, we analyzed the capacities of different strains to exhibit multicellular behavior and their tolerance to antimicrobial factors. Generally, all the *S.* Typhimurium strains exhibiting multicellular behavior (100%) belonged to the ST34 type. Compared to other strains, the strains exhibiting multicellular behavior could have greater environmental adaptability and higher antibiotic resistance. Consequently, the multicellular behavior in the strains could be facilitating the gradual increase in the prevalence of the ST34-type strains.

Notably, according to the results of the present study, most of the ST34-type multicellular behavior strains were of human origin, and the finding is consistent with the results of Vestby et al. (Ahmed et al., 2007). In addition, Römling et al. (2003) reported that human-derived *S.* Typhimurium mainly exhibits multicellular behavior. Human-derived *S.* Typhimurium is more likely to exhibit multicellular behavior, which may be linked to the environments of the strains (Wang et al., 2019). Better or stronger antibiotics would be used to prevent and control *S.* Typhimurium in clinical practice, which puts human-derived *S.* Typhimurium under greater survival pressure, which could in turn facilitate the emergence of multicellular behavior and development of adaptive resistance (Biswas et al., 2019).

In the present study, some *S.* Typhimurium strains from different sources (human origin and animal source food origin) were observed to belong to the same ST type, and most human-derived *S.* Typhimurium strains exhibited multicellular behavior, which highlights the potential role of multicellular behavior in facilitating *S.* Typhimurium spread between animals and humans, and further, in epidemics. Generally, the emergence of multicellular behavior may be the reason for the current high prevalence of ST34, so

that further research on the adaptability of strains exhibiting multicellular behavior to unfavorable environments, based on biofilm formation and drug resistance characteristics, should be carried out, which could enhance the capacity of clinicians to manage the threats posed by such strains to public health.

In the present study, we observed that all ST34-type multicellular behavior *S.* Typhimurium isolates could form biofilms, and most of them (98%) displayed strong biofilm formation ability. The proportion observed was much higher than the proportion reported previously (24.14%; Manafi et al., 2020). On the contrary, most of the non-multicellular behavior strains in this study exhibited weak or no biofilm formation ability, indicating that ST34-type multicellular behavior *S.* Typhimurium had stronger biofilm formation ability. This may be due to the strains exhibiting multicellular behavior being characterized by the production of extracellular matrix, in the form of cellulose and curli, which can facilitate the establishment of hydrophobic networks in colonies and enhance biofilm formation (White et al., 2006). Therefore, the results of the present study reveal that the ST34-type multicellular behavior *S.* Typhimurium has a high capacity to form biofilms, which could enhance its adaptability to unfavorable environments and resistance to environmental stress, including antimicrobials (Farzan et al., 2008). The ability of *S.* Typhimurium to form biofilms increases the risk of outbreaks, because such strains can be released from the biofilms and cause food contamination during processing.

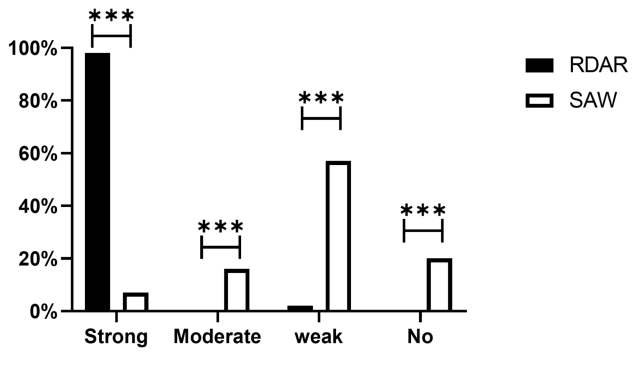

FIGURE 3 | Biofilm formation ability of different phenotype colonies. Strong, Strong biofilm formation ability; Moderate, Moderate biofilm formation ability; Weak, Weak biofilm formation ability; and No, none biofilm formation ability (***$p < 0.001$).

According to the results of the present study, the ST34-type multicellular behavior strains exhibited higher drug resistance than non-multicellular behavior strains. The resistance rates of ST34-type multicellular behavior strains to Nalidixic acid, Florfenicol, and Tetracycline were as high 100%, and the resistance rates to drugs such as Ampicillin exceeded 90%, which were higher than the previously reported resistance rates to Nalidixic acid (28.8%; Lina et al., 2018), Florfenicol (93%; Farzan et al., 2008), Tetracycline (68%; Li et al., 2017), and Ampicillin (72.2%; Tu et al., 2015). Notably, all of the ST34-type multicellular behavior strains (100%) in the present study displayed MDR, which is much higher than the previously reported MDR rate in *S.* Typhimurium (56.58%; Ke et al., 2014) and in the non-multicellular behavior *S.* Typhimurium in the present study. The ACSSuT profile is an important indicator for evaluating the drug resistance of non-typhoid *Salmonella*. In the present study, 24% of ST34-type multicellular behavior *S.* Typhimurium exhibited the ACSSuT resistance profile, which was a much higher rate than that of non-multicellular behavior *S.* Typhimurium, and higher than

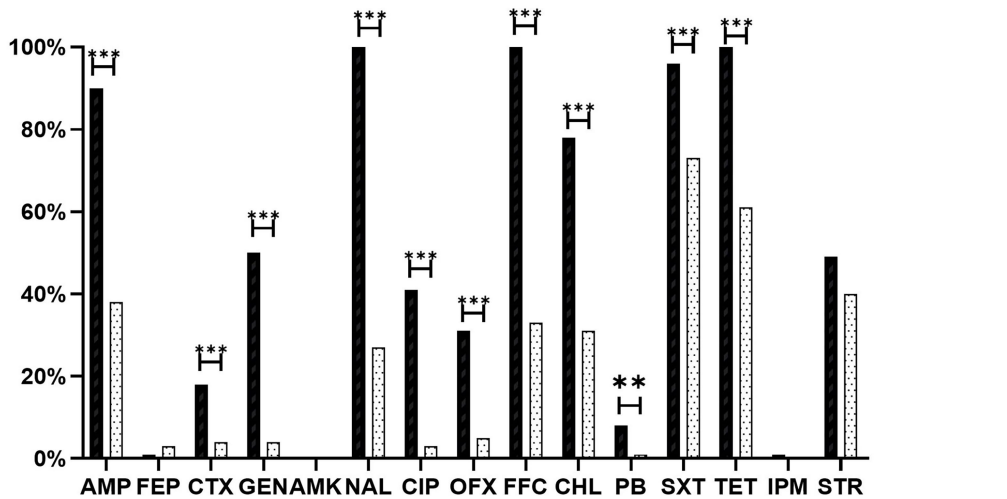

FIGURE 4 | Antimicrobial resistance of isolates and different phenotype colonies. AMP, Ampicillin; FEP, Cefepime; CTX, Cefotaxime; GEN, Gentamicin; AMK, Amikacin; NAL, Nalidixic acid; CIP, Ciprofloxacin; OFX, Ofloxacin; FFC, Florfenicol; CHL, Chloramphenicol; PB, Polymyxin B; SXT, Sulfaisoxazole; TET, Tetracycline; IPM, Imipenem; and STR, Streptomycin (**$p < 0.01$ and ***$p < 0.001$).

a 14.5% rate reported previously (Ma et al., 2018). The higher drug resistance of the ST34-type multicellular behavior strains could be attributed to excessive and improper use of antibiotics in clinical and animal breeding settings, which could lead to the adaptation of multicellular behavior in *S.* Typhimurium in response to stress. Furthermore, the extracellular matrix produced by strains with multicellular behavior forms a complex network structure that wrap the strains, and the ensuing protective mechanism could enhance resistance of the strains to antibiotics, so that the emergence of ST34-type multicellular behavior *S.* Typhimurium presents additional challenges for its prevention and control.

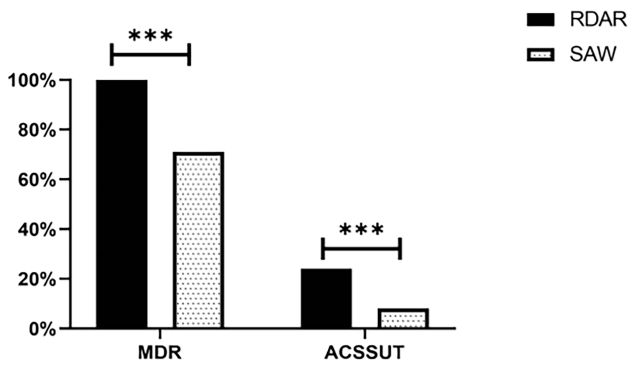

FIGURE 5 | Distribution of multi-drug resistance (MDR) in isolates and their ACSSuT profiles. MDR, Multiple antibiotic resistance; ACSSuT, resistance to Ampicillin, Chloramphenicol, Streptomycin, Sulfamethoxazole, and Tetracycline (***$p < 0.001$).

Considering the high drug resistance and MDR rates of the ST34-type multicellular behavior *S.* Typhimurium, the authors further studied the drug resistance characteristics of the ST34-type multicellular behavior *S.* Typhimurium at the molecular level. In general, the detection rates of drug-resistant genes in the ST34-type multicellular behavior *S.* Typhimurium were higher than those in the non-multicellular behavior strains. The trend could be attributed to the colonies formed by multicellular behavior strains offering opportunities for intra- and inter-species genetic exchange of antibiotic resistance genes (Diez-Garda et al., 2012; Eguale et al., 2014), which facilitates the emergence and spread of antibiotic resistance.

Contrary to expectations, although *blaTEM* is the main gene detected in β-lactamase resistance genes, its detection rates in non-multicellular behavioral phenotypic strains were much higher than in the ST34-type multicellular strains. Similarly, *TetA* had a higher detection rate in non-multicellular behavior strains for tetracycline antibiotic resistance genes. Overall, the results demonstrate why the multicellular behavior *S.* Typhimurium has high drug resistance, in addition to more drug resistance genes, multicellular behavior could facilitate the development of drug resistance, and further in-depth research and analyses are required on the underlying mechanisms of drug resistance. In addition, novel antimicrobial agents should be developed based on a multicellular behavior perspective.

The ST34-type multicellular behavior strains not only exhibited high drug resistance to conventional drugs but also to the last line of defense, Polymyxin B (8%) and Imipenem (1%). The rates were much higher than the resistance rates to Polymyxin B (2.0%) and Imipenem (0.5%) reported previously in China (Lina et al., 2018). The imipenem-resistant strains observed

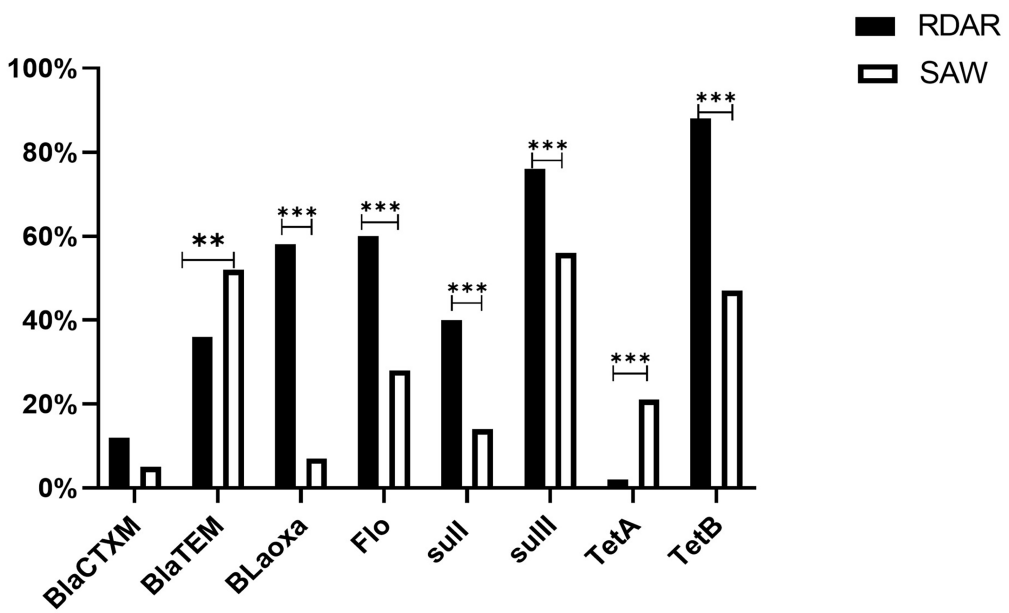

FIGURE 6 | Antimicrobial resistance genes of ACSuT, Ampicillin, Chloramphenicol, Sulfamethoxazole, and Tetracycline. Among them, *BlaCTXM, BlaTEM, and Bloxa* are β-lactam resistance genes, *Flo* is Phenicols resistance genes, *sulI* and *sulII* are Sulphonamide resistance genes, *TetA* and *TetB* are Tetracycline resistance genes, *mcr-1* is the Polymyxin B resistance gene, and *BlaNDM-5* is the Imipenem resistance gene (**$p < 0.01$ and ***$p < 0.001$).

in the present study were isolated from animal source food rather than human sources. Therefore, the multicellular behavior of the strains is a source of concern. Furthermore, in the present study, all (100%) strains resistant to Polymyxin B harbored the *mcr-1* resistance gene. Plasmid-mediated *mcr-1* can be transferred horizontally between different strains (Liu Liu et al., 2016) and is considered a novel "super bacteria" after *NDM-1*; the high detection rate of *mcr-1* in ST34-type multicellular behavior strains may be due to the presence of the gene in the strains with multicellular behavior that are coated with various substrates, which is conducive for the survival and transfer of host strains. In addition, in the present study, the Imipenem-resistant ST34-type multicellular strain harbored a *BlaNDM-5*-carrying IncX3 plasmid. In addition, the strain has been reported to harbor an IncHI2 plasmid that carries nine resistance genes, including *aadA1*, *aadA3*, *aph(3′)-la*, *sul1*, *sul2*, *sul3*, *floR*, *cmlA*, and *dfrA12* (Gao et al., 2020). Therefore, the emergence of an Imipenem-resistant ST34-type multicellular strain has implications for the emergence and spread of drug resistance. The findings also suggest that compared with non-multicellular behavioral strains, the ST34-type multicellular behavioral strains isolated in the present study have stronger drug resistance, with resistance against the last line of defense, which makes the prevention and control of *S.* Typhimurium more challenging.

In summary, multicellular behavior is potentially a major factor involved in the MDR of *S.* Typhimurium. Therefore, strategies for the prevention and control of *S.* Typhimurium should consider whether the strains exhibit a multicellular behavior phenotype.

CONCLUSION

In summary, the present study observed the emergence of ST34-type multicellular behavior *S.* Typhimurium isolated from different sources, including human and animal source food, in China. All the ST34-type multicellular behavior *S.* Typhimurium (100%) could form biofilms and 98% exhibited strong biofilm formation ability. In the drug resistance tests of the strains, the ST34-type multicellular behavior strains not only exhibited higher resistance than non-multicellular behavior strains under conventional antimicrobial drugs, but also was resistant to the last line of defense, Polymyxin B (8%) and Imipenem (1%). Furthermore, the ST34-type multicellular strains exhibited much higher MDR rates than the non-multicellular behavior strains and harbored more drug-resistant genes. The emergence of an ST34-type multicellular behavior *S.* Typhimurium with high biofilm formation ability and high drug resistance in China could facilitate the prevalence and spread of associated epidemics. Comprehensive and continuous research on the ST34-type *S.* Typhimurium with multicellular behavior is essential for its effective prevention, control, and for public health safety.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

In this study, all strains from human and animal sources food were preserved in the laboratory, which did not involve the isolation and identification of bacteria from samples of relevant sources. It did not include animal research and human research, and there were no ethical issues related to living animals and human.

AUTHOR CONTRIBUTIONS

KC: methodology, data curation, and writing (original draft preparation). YG: validation and investigation. LL: supervision and resources. WZ, JL, ZZ, HH, and ZC: investigation. ML: supervision and project administration. JZ: conceptualization and writing (reviewing and editing). All authors contributed to the article and approved the submitted version.

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The Current Landscape of Antibiotic Resistance of *Salmonella* Infantis in Italy: The Expansion of Extended-Spectrum Beta-Lactamase Producers on a Local Scale

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Salmonella enterica serovar Infantis is one of the five main causes of human salmonellosis in the European Union (EU) and in recent years, has been increasingly reported to carry multiple antimicrobial resistance determinants, including extended-spectrum beta-lactamase (ESBL) genes. In our study, we used WGS-based tools to characterize *S.* Infantis strains circulating in the Abruzzo and Molise regions of Italy between 2017 and 2020 and compared this local dataset to the *S.* Infantis population present in Italy over the last two decades. Phylogenetic analyses demonstrated that the majority of strains isolated from poultry and turkeys from Abruzzo and Molise were closely related and belonged to one of the two main genetic clusters present in Italy, which were grouped predominantly as ESBL-producing strains that harbored pESI-like plasmid. We showed that 60% of the local strains carried multiple antibiotic resistance genes, including ESBL gene $bla_{CTX-M-1}$ as well as *aad*A1, *dfr*A1, *dfr*A14, *sul1*, and *tet*(A) genes present on the pESI-like megaplasmid. The analysis of strains from Abruzzo and Molise and the publicly available Italian *S.* Infantis sequences revealed a dramatic increase in the number of identified AMR genes in the strains isolated after 2011. Moreover, the number of strains resistant to five or more antibiotic classes increased from 20–80% in the last decade likely due to the acquisition of the megaplasmid. The persistence of the ESBL-producing and the multidrug-resistant (MDR) clone of *S.* Infantis in poultry populations in Italy and in Europe requires rapid and efficient intervention strategies to prevent further expansion of the clone.

Keywords: *Salmonella* Infantis, ESBL–extended-spectrum beta-lactamase, multidrug resistance, genomics, WGS–whole-genome sequencing

INTRODUCTION

Non-typhoidal *Salmonella* is one of the main causes of acute enteric diseases world-wide and salmonellosis is the second most frequently notified zoonosis in the European Union (EU) (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2020, 2021). Among all serovars of *Salmonella*, *Salmonella enterica,* serovar Infantis is one of the five main serovars responsible for human infections in the EU and the primary cause of salmonellosis acquired by consumption or handling of contaminated poultry meat (Antunes et al., 2016). The rate of salmonellosis reported in Italy in 2019 was 5.4 cases per 100,000 population and 151 outbreaks have been registered (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2021). Although *S.* Infantis is frequently isolated from poultry meat, to date, only a few outbreaks caused by this serovar were reported in Italy (Chironna et al., 2014). In the EU between 2018 and 2019 *S.* Infantis was most frequently isolated from broiler chickens, both from animals and the related food sources, followed by turkey and turkey meat, and more than 50% of reported *S.* Infantis from broilers were isolated in Italy (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2020, 2021). Indeed, of all *Salmonella* serovars the proportion of *S.* Infantis isolated in Italy from poultry sources increased from 2.3% in 2008 to 22.7% in 2018 (IZSVenezie, 2008-2018). Importantly, this increase was also associated with a high proportion of *Salmonella* resistant to multiple antimicrobials, including ciprofloxacin and the 3rd-generation cephalosporins (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2021). Italy is one of the largest consumers of antimicrobials in the EU (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2021). Biomass-weighted consumption data reported for 2017 showed that the amount of antimicrobial agents used in food-producing animals in Italy was more than double of the average consumption in EU countries (European Medicines Agency [EMA], 2019; European Food Safety Authority [EFSA] and European Medicines Agency [EMA] and European Centre for Disease Prevention and Control [ECDC], and European Medicines Agency [EMA] et al., 2021). The most commonly purchased antimicrobials for veterinary use were penicillins, tetracyclines, and sulphonamides. Similarly, a high number of antimicrobials were acquired for human use making Italy the 6th largest consumer of antimicrobials per population number. Importantly, the use of 3rd and 4th level cephalosporins in humans was higher than in all EU countries except in Bulgaria (European Food Safety Authority [EFSA] and European Medicines Agency [EMA] and European Centre for Disease Prevention and Control [ECDC], and European Medicines Agency [EMA] et al., 2021).

Global dissemination of MDR strains of *Salmonella* and other Enterobacteriaceae and, in particular, the emergence of extended spectrum beta-lactamase (ESBL)-producing strains, has been increasingly observed in the last two decades. This has prompted the World Health Organization (WHO) to place the ESBL-producing Enterobacteriaceae on the list of "Critical Priority Pathogens" that pose a great risk to public health (World Health Organization [WHO], 2019). MDR strains of *S.* Infantis isolated in broilers have been now recorded in multiple countries, including Hungary (Szmolka et al., 2018), Germany (García-Soto et al., 2020), Italy (Alba et al., 2020; Proietti et al., 2020), the Netherlands (Mughini-Gras et al., 2021), Russia (Bogomazova et al., 2020), and United States (Tate et al., 2017). A recent study showed that global population of *S.* Infantis could be divided into three major lineages (Gymoese et al., 2019). The main lineage, which separated from the other branches approximately 75 years ago, contained the highest number of strains currently circulating worldwide. Several clusters of *S.* Infantis were reported in Europe. Most of these clusters were not geographically restricted and the same clones were isolated in multiple European countries likely due to the shared livestock suppliers (Alba et al., 2020).

An important factor for the increased prevalence of this serovar and the observed MDR has been the acquisition of the pESI-like conjugative megaplasmid, first described in an Israeli strain of *S.* Infantis (Aviv et al., 2014) and later identified worldwide (Franco et al., 2016; Alba et al., 2020; McMillian et al., 2020). This plasmid (∼300 kbp in size) was demonstrated to harbor virulence factor genes such as fimbriae (*ipf* and *fea*), yersiniabactin operon (*ybt*), toxin/antitoxin system, genes conferring resistance to heavy metals (*mer*A), disinfectants (*qac*EΔ), and several antimicrobial resistance genes, including $bla_{\mathrm{CTX-M}}$ genes responsible for production of ESBL enzymes (Aviv et al., 2014; Alba et al., 2021).

Following the global trend, an increased number of *S.* Infantis, isolated primarily from poultry and turkeys and resistant to several classes of antibiotics, including the 3rd generation cephalosporins, has been observed in the Abruzzo and Molise regions of Italy over the last 10 years. In this work, we characterized the *S.* Infantis population found in these regions and placed the local dataset in a broader phylogeny generated for the entirety of Italy. We analyzed *S.* Infantis isolated between 2017 and 2020 during passive surveillance activities of the Regional Reference Laboratory which collects and characterizes pathogenic Enterobacteria data from the Abruzzo and Molise regions as part of a national network of surveillance. We used whole-genome sequencing (WGS)-based tools to identify genomic traits leading to multidrug resistance of the locally isolated strains of *S.* Infantis and compared them with the Italian dataset. In particular, we aimed to describe the ESBL-producing population and identify the genetic features responsible for observed increase in resistance to the 3rd generation cephalosporins in the strains circulating in the studied regions. The results presented in our study will provide additional data that will aid the Italian surveillance system in making informed decisions and form an important part in the antimicrobial resistance monitoring activities of the network. In particular, the study will provide the most recent genomic data regarding the circulating *S.* Infantis strains and the genetic determinants of the antimicrobial resistance currently found in the studied *S.* Infantis population.

MATERIALS AND METHODS

Isolation and Phenotypic Characterization of Bacterial Strains

Strains were collected during routine activities of the Regional Reference Laboratory for Pathogenic Enterobacteria, which included passive surveillance in broiler and turkey farms, controls of the poultry meat destined for human consumption, and groundwater sampling. Isolates were cultured on Rambach agar and incubated overnight at 37°C. Commercial antisera were used to serotype *Salmonella* isolates (Statens Serum Institut, Copenhagen, Denmark) by slide agglutination, as described by Kauffmann–White scheme (Ewing, 1972; Guibourdenche et al., 2010). A set of 103 isolates of *S.* Infantis, which included only one sample per sampled lot, were selected for further analysis.

The antimicrobial susceptibility test was performed using a microdilution method using the Sensititre automated system with TES (Thermo Fisher Scientific Inc., Waltham, MA, United States) and the Sensititre™ EUVSEC (Thermo Fisher Scientific Inc., Waltham, MA, United States). The *Escherichia coli* strain ATCC 25922 was included as a reference and susceptibility values were interpreted using EUCAST breakpoints. A panel of 14 antimicrobials: azithromycin (2–64 μg/mL), ampicillin (1–64 μg/mL), cefotaxime (0.25–4 μg/mL), ceftazidime (0.5–8 μg/mL), chloramphenicol (8–128 μg/mL), ciprofloxacin (0.015–8 μg/mL), colistin (1–16 μg/mL), gentamicin (0.5–32 μg/mL), meropenem (0.03–16 μg/mL), nalidixic acid (4–128 μg/mL), sulfamethoxazole (8–1024 μg/mL), tetracycline (2–64 μg/mL), tigecycline (0.25–8 μg/mL), and trimethoprim (0.25–32 μg/mL) was used.

Whole Genome Sequencing

Total genomic DNA was extracted from 103 bacterial isolates using Maxwell 16 Tissue DNA Purification Kit, according to the standard protocol supplied by the manufacturer. Total DNA was quantified with Qubit DNA HS assay (Thermo Fisher Scientific Inc., Waltham, MA, United States) and sequenced with Illumina NextSeq 500 instrument. Briefly, Nextera XT Library Preparation Kit (Illumina, St. Diego, CA, United States) was used to generate sequencing libraries, which were then sequenced in 300 cycles using NextSeq500/550 Mid Output Reagent Cartridge v2, according to manufacturer's instructions. Paired-end 150 bp reads were generated and after demultiplexing and adapter removal the quality of reads was assessed using FastQC v0.11.5 (Andrews, 2010). The raw reads were trimmed with Trimmomatic v 0.36 (Bolger et al., 2014) using base quality parameters—Leading: 25; Trailing: 25; Slidingwindow: 20:25. Genome scaffolds were assembled using SPAdes v 3.11.1 (using parameters –k 21, 33, 55, 77; –careful) (Bankevich et al., 2012) and the scaffold quality was evaluated using QUAST v 4.3 (Gurevich et al., 2013).

The set of 103 *S.* Infantis genome paired-end sequencing reads from this study was deposited in SRA repository found under Bioproject PRJNA771355. An additional set of 160 publicly available SRA sequences of *S.* Infantis were downloaded on April 7, 2021 and processed as described below. The list of SRA sequences and associated minimal metadata set are shown in **Supplementary Table 1**.

In silico Identification of AMR Genes, Mobile Elements, and Virulence Factors

The set of 263 genomes was characterized *in silico* using ABRicate v 1.0.1, with default settings, in conjunction with four databases, all updated on March 27, 2021 (Seemann, 2020). Specifically, PlasmidFinder (460 sequences) (Carattoli et al., 2014) was used to detect plasmid incompatibility (Inc.) groups, NCBI (5,386 sequences) (Feldgarden et al., 2020) and ResFinder (3,077 sequences) (Zankari et al., 2012) were used for identification of AMR genes and VFBD (2,597 sequences) (Chen et al., 2015) was used for detection of virulence factors. To exclude truncated gene sequences for AMR genes, a positive hit was accepted only if the% coverage of the identified gene was 100%. Mutations in *gyrase A* gene were identified using PointFinder and only known mutations were considered (Zankari et al., 2017). To predict the presence of pESI-like plasmid replicons we screened the genome assemblies for the pESI-like gene pattern proposed by McMillian et al. (2020) which included *ard*D, I1 relaxase, *sog*S, *trb*A, pESI *rep*A, pESI hypothetical backbone sequence, K88, *ybt*, *mer*A, *ipf*, and *pil*L (McMillian et al., 2020). BLAST 2.12.0 + (default parameters) was used to detect the pESI-like sequences, as described previously (McMillian et al., 2020).

Plasmid Sequence Reconstruction

To identify the AMR genes carried on specific plasmids, we used MOB-recon tool v 3.0.0 from MOB-suite package to segregate assembly contigs into predicted plasmid sequences (Robertson and Nash, 2018). Putative plasmid sequence assemblies that contained contigs with assigned Inc. plasmid groups were selected for further analysis. An exception was made for a large putative conjugative plasmid containing between 80 and 110 kbp (Mob ID AA474) that carried MOBP relaxase and partial pESI-like pattern (see "*In silico* identification of AMR genes, mobile elements, and virulence factors"), which was considered a part of the IncFIB plasmid if, additionally, it did not contain IncI sequence. The plasmid assemblies were analyzed by ABRicate v 1.0.1 to identify AMR genes. An AMR gene present on the same contig as Inc. replicon sequence, even if not segregated into a plasmid assembly by MOB-recon, was considered a part of the plasmid.

Multilocus Sequence Typing and Core Genome Multilocus Sequence Typing

Assembled genomes were imported into Ridom SeqSphere+ software, version 6.0.2 (Jünemann et al., 2013) and core genome multilocus sequence typing (cgMLST) profiles were assigned using the default *Salmonella enterica* task template with 3,002 core gene targets created based on EnteroBase *S. enterica* cgMLST v2 scheme,[1] as previously described (Di Marcantonio et al., 2020). Default settings were applied for allele calling and cgMLST complex detection

[1]http://enterobase.warwick.ac.uk

[complex cut-off ≤ 7 loci (Dangel et al., 2019)]. Only genomes containing ≥98% good target sequences were used in further *in silico* analyses. Minimum-spanning tree (MST) was generated by pairwise comparison of cgMLST alleles ignoring missing values. Multilocus sequence typing (MLST) analysis of the set of 103 strains sequenced in this study was performed in Ridom SeqSphere + using the Achtman *Salmonella* seven locus MLST scheme, available at http://enterobase.warwick.ac.uk/species/index/senterica. A novel MLST profile (ST-8528) was generated for strain 2020-CB-4517-1-2 by submitting the sequencing reads directly to EnteroBase.

Phylogenetic Analysis

Core single-nucleotide polymorphisms (SNPs) of 263 *S.* Infantis strains were identified in Ridom SeqSphere + software version 6.0.2. Briefly, the assemblies were imported into the Ridom SeqSphere + and the genomes with fewer than ≥98% good target sequences were discarded. Following assignment of cgMLST profiles, the polymorphisms were identified in the target sequences of alleles from EnteroBase schema and exported as an alignment of concatenated SNPs. Indels and variants present only in the reference sequence were not included in the analysis.

Phylogenetic tree was constructed using the SNPs alignment in IQ-TREE version 1.6.9 (Nguyen et al., 2015). K3P + ASC was determined by the tool as the best suited substitution model and used for reconstruction of phylogeny. Maximum-likelihood tree was midpoint-rooted and visualized in iTOL (Letunic and Bork, 2019).

SNPs in the sequences of IncX1 and IncX4 plasmids were called with *in silico* Genotyper (ISG) version 0.16.10-3 (Sahl et al., 2015) using default parameters, with BWA-MEM version 0.712-r1039 (Li and Durbin, 2009) used as a sequence aligner and GATK version 3.9 (McKenna et al., 2010) used as an SNP caller. IQ-TREE was used to generate plasmid-based phylogenies and the trees were visualized using FigTree version 1.4.1 (Rambaut, 2010).

RESULTS

Clonal Population of *Salmonella* Infantis Predominates in Local Animal Farms

In our study, we analyzed a dataset of 102 *Salmonella* Infantis isolates collected between 2017 and 2020 in the Abruzzo and Molise regions, and one strain collected in the Marche region of Italy (**Figure 1** and **Supplementary Table 1**). MLST analysis showed that all strains, except one, belonged to ST 32 and one strain was assigned a novel ST 8528. The MST based on cgMLST showed that the majority of the strains were closely related and 54

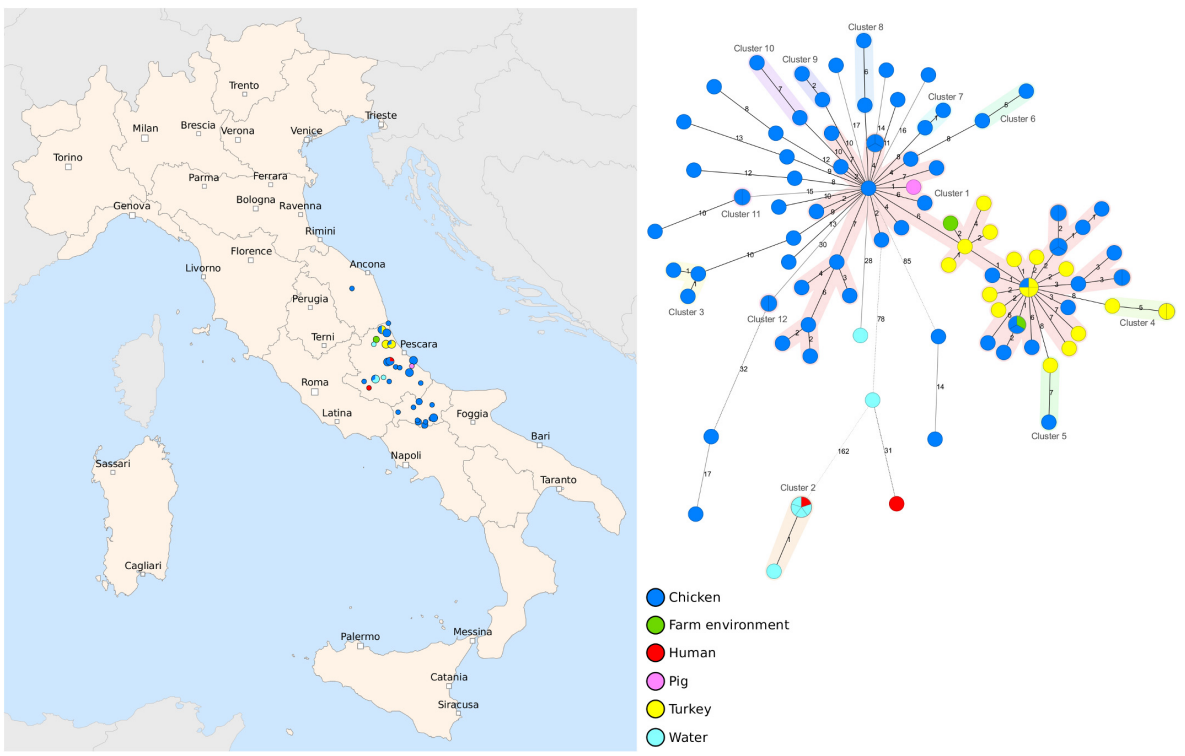

FIGURE 1 | Geographic distribution and genetic diversity of *Salmonella* Infantis strains collected between 2017 and 2020. A set of 103 strains collected in Abruzzo, Molise and Marche regions were sequenced by whole-genome sequencing (WGS) and the core genome multilocus sequence typing (cgMLST) analysis was performed. The MST was generated based on 3,002 target genes, with missing values ignored, and the clusters were assigned using a cut-off of seven core genes and highlighted with different colors. The nodes are colored according to the host and the branch distances correspond to the number of discriminating loci.

strains isolated primarily from broiler and turkeys were assigned to the same cluster (C1) based on the single-linkage cut-off of 7 genes. The maximum distance between the cgMLST profiles in C1 was 24 loci. Other 11 clusters (C2-C12) containing an average of four genomes (min = 2, max = 6) were also identified and, on the contrary to C1, these smaller complexes often contained isolates from the same farms. Interestingly, strains isolated from groundwater and humans were more distantly related to the animal isolates, and the maximum distance of 203 core genes was observed between cluster C2 and a strain obtained from a chicken. Moreover, within C2, the human isolate was assigned the same cgMLST profile as four strains collected from groundwater.

Genomic Characterization of *Salmonella* Infantis Population From Abruzzo and Molise

The antibiotic susceptibility test results demonstrated that over 60% of the strains were resistant to ampicillin, cefotaxime, and ceftazidime and were therefore likely ESBL producers (**Supplementary Table 1** and **Supplementary Figure 1**). In addition, these strains (62 out of 103) often exhibited resistance to other antibiotics including quinolones, sulfamethoxazole, tetracycline, and trimethoprim.

Bioinformatic analysis confirmed the presence of 62 ESBL-producing strains, all of which harbored the $bla_{\mathrm{CTX-M-1}}$ gene (**Figure 2**). Additionally, we detected two common broad-spectrum beta-lactamase genes $bla_{\mathrm{TEM-1B}}$ and $bla_{\mathrm{TEM-1D}}$ that in *Salmonella* spp, confer resistance to penicillins and first generation cephalosporins. The majority of isolates additionally carried multiple AMR genes, most frequently *aad*A1, *dfr*A1, *dfr*A14, *sul1* and *tet*(A), and contained a point mutation (transition A to G) in the chromosomal DNA leading to amino acid change D87G in *gyr*A associated with resistance to quinolones. MDR seen in our dataset suggested the presence of large or multiple plasmids and indeed we identified several Inc. group plasmids carried by the strains. The most frequent plasmid, found in 95 genome drafts, was IncFIB(K), which in eight isolates, was detected together with IncFIB(AP001918) and in six cases with IncFIC replicon (**Supplementary Table 1**). The second most common Inc. group we identified was IncX, harbored by 36 strains, that contained IncX1, IncX3, and IncX4 signatures. The sequences of IncX1 and IncX3 were always located together in the same contig, approximately 500 nt apart, but the percentage coverage and identity of the hits was higher for IncX1 (100% and 98.66% for IncX1 vs. 90.37% and 80.17% IncX3) and therefore we refer to these plasmids as IncX1.

Since previous studies associated IncFIB replicon together with *aad*A1-*dfr*A-*sul1*-*tet*(A) AMR pattern with the presence of pESI-like megaplasmid, we examined if the typical gene pattern proposed by McMillian et al. (2020) could be found in our set of genomes. All of the IncFIB harboring strains contained either a full or a partial set of the genes of the pESI-like pattern. In eight isolates we did not detect IncP replication origin and in six, *merA* gene coding for mercuric reductase was absent and two were missing *pil*L (pilus biogenesis) sequence. Additionally, a partial pESI pattern was detected in the isolate that harbored only IncI plasmid but the specific pESI *rep*A gene, along with seven more targets, were not identified, confirming the lack of pESI megaplasmid. The absence of the megaplasmid was associated with a lower number of virulence factors due to missing *fae*D, *fae*E, *fyu*A, *irp1, irp2* genes and yersiniabactin operon (*ybt*) (**Supplementary Table 1**), as shown previously (García-Soto et al., 2020).

In silico AMR Analysis of Italian *Salmonella* Infantis Strains

Since our dataset contained isolates from a restricted geographical area, we analyzed additional publicly available *S.* Infantis sequences that contained strains collected in Italy between 2001 and 2017 (**Supplementary Table 1**). *In silico* analysis showed a lower proportion of AMR strains circulating before 2011 than those found in the following years (**Figure 3** and **Supplementary Table 1**). In particular, the comparison of the AMR genes (or mutations) identified in the isolates collected before 2011 and between 2011–2015 demonstrated increased resistance to aminoglycosides (from 61.1 to 83.9%), beta-lactams (from 27.8 to 62.5%), quinolones (from 33.3 to 87.5%), sulfonamides and tetracyclines (from 44.4 to 87.5%), and to trimethoprim (from 22.2 to 80.4%). Based on the available WGS data, the current resistance levels of *S.* Infantis in Italy exceeded 80% of resistant isolates for aminoglycosides and trimethoprim, and 90% for quinolones, sulfonamides, and tetracycline. Moreover, a rise in the number of MDR strains was noted, with less than 20% of strains harboring five or more AMR traits before 2011 compared to more than 80% after 2011. While we detected bla_{TEM} genes in 27.8% of strains collected prior 2011, no ESBL-producers were identified in this group of sequences. The number of strains carrying the $bla_{\mathrm{CTX-M-1}}$ gene in the set from 2011–2015 reached 46.4% while between 2016–2017 the number was lower (32.9%). Interestingly, in the dataset from Abruzzo and Molise, the prevalence of ESBL producing strains was more than 20% higher than in the Italian strains from the public dataset from 2011–2017 and exceeded 60% of the analyzed strains. An alarmingly high proportion of human isolates carried bla_{CTX} genes (48%), and a similar prevalence of ESBL producers was observed in the analyzed poultry strains (47%) (**Figure 4** and **Supplementary Table 1**).

Phylogenetic Analysis of *Salmonella* Infantis Population in Italy

The phylogeny reconstructed using concatenated core genome SNPs of Italian *S.* Infantis strains revealed two genetic lineages, one minor, containing primarily strains isolated from groundwater in Abruzzo and one major, which was further divided into separate branches with shared characteristics (**Figure 4**). Isolates collected before 2011 were located in the same part of the tree and displayed a low number of AMR genes and did not express ESBL. These strains originated primarily from humans and swine and none of them harbored IncX plasmids. A partial pattern of pESI was detected in five of them and was attributed to the presence of IncI plasmids. In only two strains, isolated in 2008 and 2009, we detected

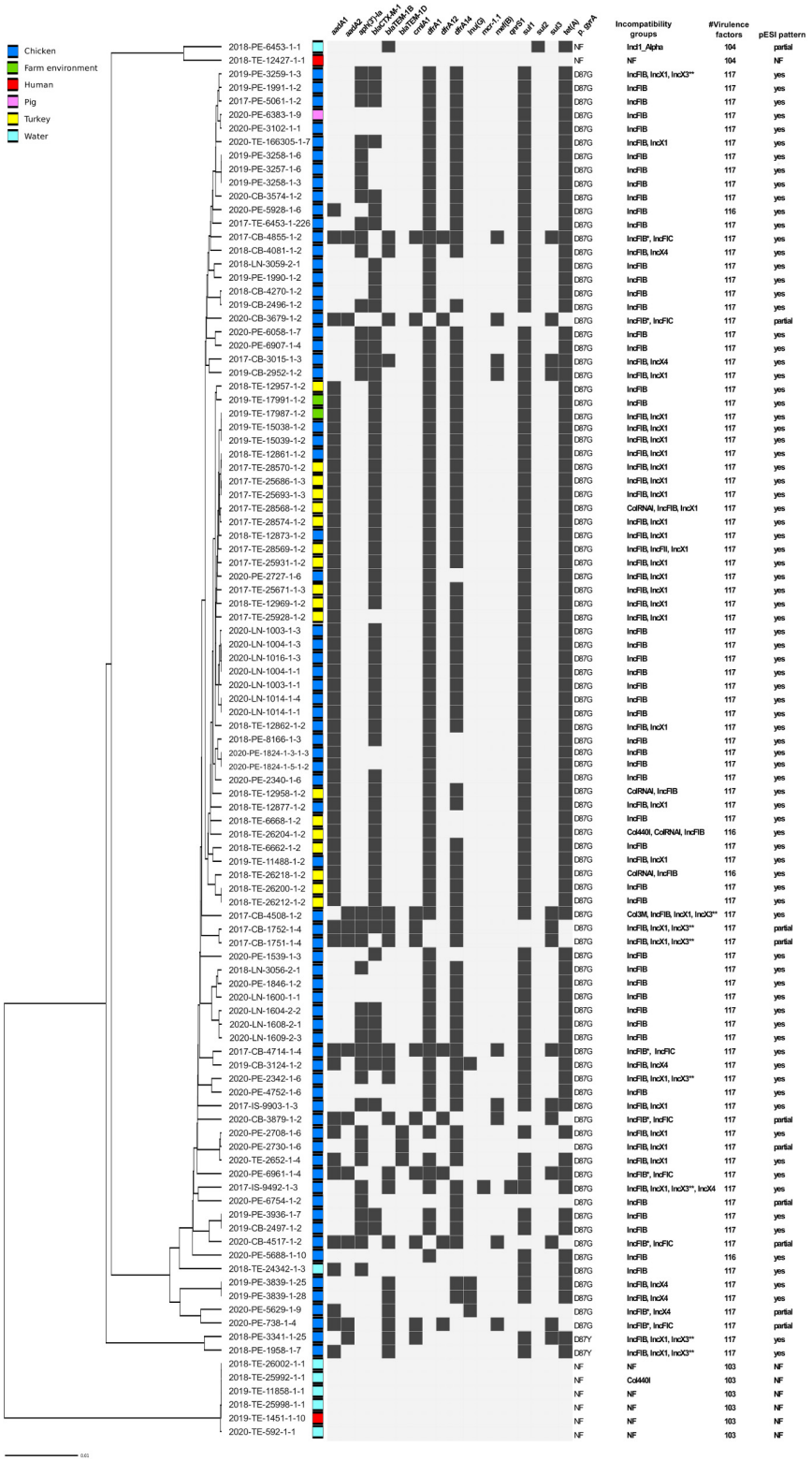

FIGURE 2 | Predicted genetic traits in a set of 103 *Salmonella* Infantis strains from Italy. AMR genes detected *in silico* are shown as black boxes. NF, not found; * more than one IncFIB detected; ** located in the same contig as IncX1.

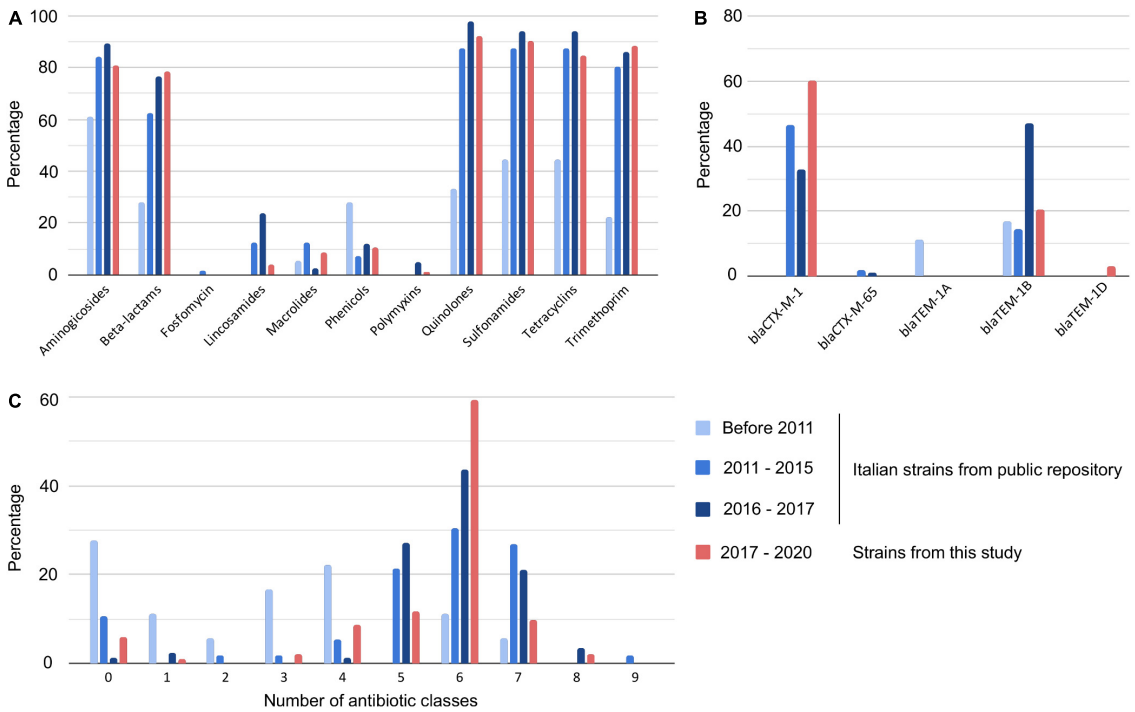

FIGURE 3 | Antibiotic resistance trends of *Salmonella* Infantis collected between 2001 and 2020 in Italy. AMR genes were detected in the genomes *in silico* and assigned into antimicrobial classes. The strains were grouped into four categories and the figure panels show detected AMR traits for each of the groups. **(A)** Percentage of strains resistant to specific antibiotics, **(B)** percentage of strains carrying specific beta-lactam resistance genes, and **(C)** percentage of strains resistant to specified number of antibiotic classes.

a pESI-like plasmid pattern. We also observed three sparsely populated branches (**Figure 4**, highlighted in green) composed of strains from humans and broilers collected between 2007 and 2018 that harbored pESI like plasmid. Two of these strains expressed ESBLs encoded by $bla_{\mathrm{CTX-M-65}}$ gene along with several additional AMR genes.

Two major clusters of genomes, originating from the same branch split into two closely related populations, were widespread in Italy. Both clusters included strains carrying pESI-like IncFIB plasmids, but only one of them consisted primarily of ESBL producers (highlighted in red, Cluster I). The genetic distance between the strains contained in each cluster, based on cgMLST, were 33 (Cluster I) and 56 (cluster in yellow, Cluster II) loci. The majority of the strains sequenced in this study were placed in Cluster I, which would explain the higher genetic uniformness of this population likely caused by the restricted geographic origin of the samples. Interestingly, the distance between the most closely related genomes from these clusters was only 15 genes demonstrating close genetic relationship between the populations.

In addition to IncFIB, multiple Italian *S.* Infantis isolates harbored IncX plasmids. In particular, we noticed that IncX4 were more common within the non-ESBL producer group i.e., in the Cluster II population. Based on *in silico* data, we predicted that IncX plasmids frequently carried $bla_{\mathrm{TEM-1}}$ (**Table 1** and **Supplementary Figure 2**). Phylogenetic trees of IncX1 and IncX4 were largely congruent with the cgMLST based phylogeny. The majority of IncX plasmids harbored by Cluster I and Cluster II isolates were grouped within their respective cluster lineages (**Supplementary Figure 2**). In addition to *bla* genes, IncX4 plasmids from five of the analyzed strains contained *mcr-1.1* genes responsible for resistance to colistin as previously shown by Carfora et al. (2018). Other genes identified on IncX plasmids included *aad*A1, *aad*A2, *cat*B3, *lnu*(G), and *sul3* (**Supplementary Table 2**).

The IncF megaplasmids contained the highest number of AMR genes including *aad*A1, *aph(3')*-Ia, *dfr*A1, *dfr*A14, *sul1*, and *tet*(A). The ESBL gene $bla_{\mathrm{CTX-M-65}}$ was predicted to be found on the IncFIB plasmid however the location of $bla_{\mathrm{CTX-M-1}}$ gene could not be determined as it assembled as a single contig flanked by inverted repeat regions. Blastx analysis showed that this contig, which in most cases contained 4,544 nt, was additionally composed of sequences coding for MFS transporter protein and a serine resolvase. As 64 isolates that carried $bla_{\mathrm{CTX-M-1}}$ gene harbored only IncFIB replicons, it is highly probable that the gene was located on these megaplasmids, as previously shown (Alba et al., 2021).

DISCUSSION

In the last 10 years we have observed a sharp increase in the number of MDR strains of *Salmonella* isolated in the regions of Abruzzo and Molise. This trend has been particularly

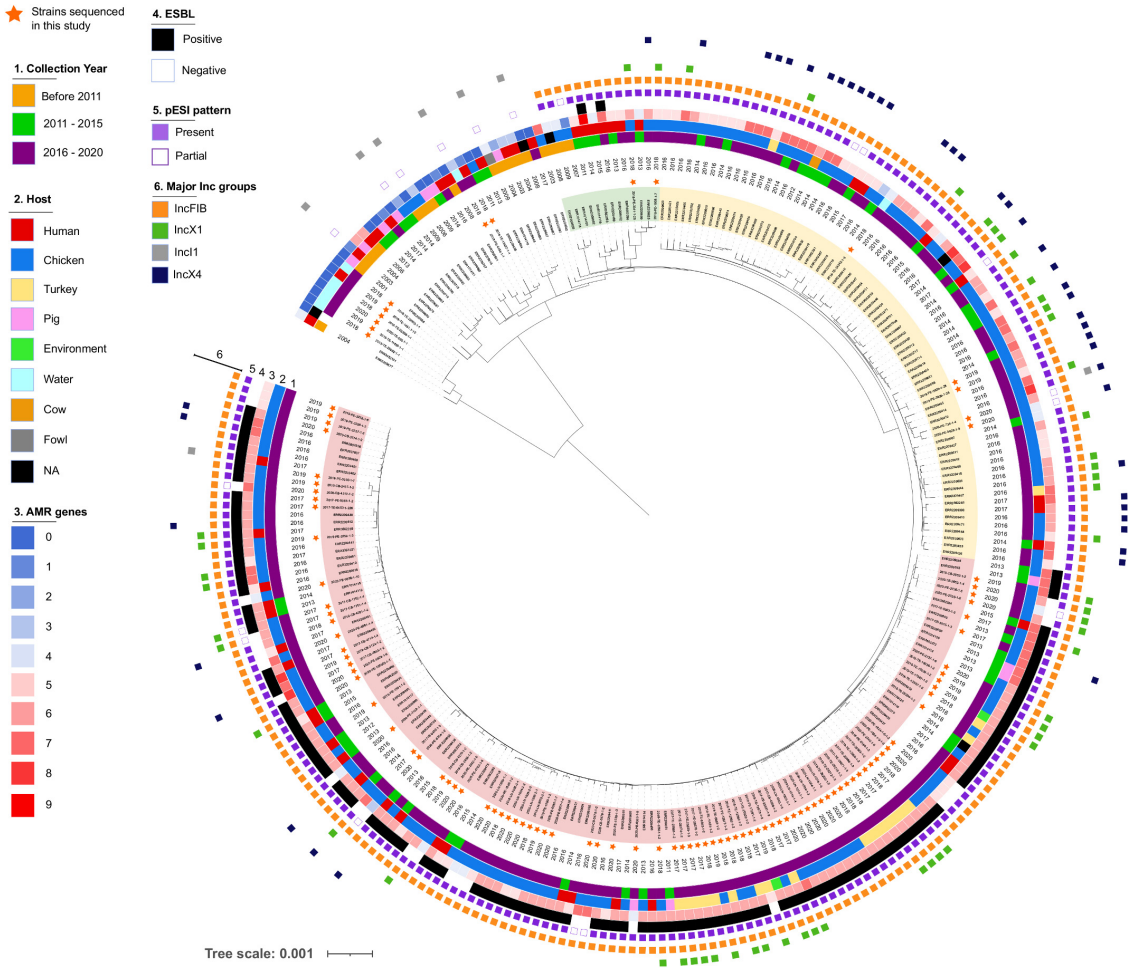

FIGURE 4 | Phylogeny of *Salmonella* Infantis in Italy. *Salmonella* The maximum likelihood tree was constructed based on alignment of 1,826 concatenated core genome SNPs of 263 *S.* Infantis genomes. Strains sequenced in this study are marked with a star and three different clusters are highlighted (Cluster I in red, Cluster II in yellow, Cluster III in green).

pronounced for the serovar Infantis, which has also been more frequently isolated from broilers and turkeys in the current years. In this work, we demonstrated the rise in the number of ESBL-producing *S.* Infantis collected locally, which could be attributed to an expansion of the pESI-like plasmid harboring clone which belongs to one of the two main *S.* Infantis populations circulating in Italy. These strains, in addition to the 3rd-generation cephalosporins, were frequently resistant to aminoglycosides, ciprofloxacin, tetracycline, trimethoprim, and sulfonamides, leaving limited options for the treatment of potential foodborne infections. The larger cluster of *S.* Infantis included isolates from broilers and turkeys. The presence of the same bacterial clone in the broilers from different farms could be explained by the use of the same breeding stock for re-population of the flock. Instead, the spread of *S.* Infantis between broilers and turkeys was likely a consequence of the shared farm environment.

A smaller cluster of isolates collected from groundwater and from humans was genetically diverse from the main population of *S.* Infantis found in the region. Interestingly, the isolates from groundwater were susceptible to all antimicrobials tested, unlike the strains from the major lineage. The division of *S.* Infantis population in Italy into two distinct lineages with different AMR profiles could suggest that these lineages occupy very diverse environmental niches. Since our dataset included samples collected mainly from broilers and turkeys, it is possible that the source of water contamination could be the waste from pastures and farms rearing pigs or ruminants. Indeed, while poultry is a major source of *S.* Infantis in Europe, the serovar has also been occasionally isolated from pigs and pork meat (Schroeder et al., 2015; Borowiak et al., 2017; European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2021). The national control plan for salmonellosis in Italy is focused on poultry farms, including broilers, breeding and laying hens, and fattening and breeding turkeys. Therefore, other animal species, which are not routinely screened, may be an important but overlooked source of *S.* Infantis, genetically and phenotypically diverse from the population found in poultry. Surveillance of *S.* Infantis in animals other than poultry, such as

ruminants and wild birds, and characterization of their antibiotic resistome, would provide additional information about the spread of antimicrobial resistant strains in the environment and about the role of other host species in the maintenance and spread of the AMR gene pool between circulating *S.* Infantis populations.

In our study, we observed a concerningly high number of MDR strains of *S.* Infantis. *In silico* AMR analysis of strains from different years showed a sudden increase in resistance to several antibiotic classes, a change that occurred after 2011. In particular, the number of strains in which we detected genes (or mutations) conferring resistance to beta-lactams, quinolones, sulfonamides, tetracyclines, and trimethoprim doubled in the last decade. Importantly, isolates carrying genes that confer resistance to multiple classes of antimicrobials became considerably more common in the past 10 years. Comparison of our data with the national AMR levels based on the number of identified antimicrobial resistance genes showed that in Abruzzo and Molise, ESBL strains were more frequent than in the Italian public strain collection, however the percentage of MDR strains was similar. In fact, around 2011, a sharp increase in the number of strains resistant to five or more antibiotic classes was observed. It is important to stress that all *S.* Infantis isolated after 2017 analyzed in this study, except for one strain, were collected from these two regions (out of twenty regions of Italy) and therefore the increased carriage of ESBL genes may not be representative of the entire Italian *S.* Infantis population circulating in this time period. Similar trends however have been reported for *S.* Infantis in Europe, Asia, and the Americas, and have frequently been associated with the acquisition of pESI-like megaplasmid (Dionisi et al., 2011; Nógrády et al., 2012; Hindermann et al., 2017; García-Soto et al., 2020; Newton et al., 2020; Tyson et al., 2021).

TABLE 1 | Predicted distribution of antimicrobial resistance genes on plasmids.

	Total	IncA/C	IncF	IncH	IncI	IncX	ND
aac(3)-IVa	2	0	2	0	0	0	0
aac(6')-Ib-AKT	2	0	0	0	0	0	2
aadA1	131	1	105	0	5	6	14
aadA13	1	0	0	0	1	0	0
aadA2	26	0	1	0	3	2	20
aadA5	3	0	0	1	0	0	2
aph(3")-Ib	7	0	0	2	1	0	4
aph(3')-Ia	123	0	120	2	0	0	1
aph(4)-Ia	2	0	2	0	0	0	0
aph(6)-Id	7	0	0	2	1	0	4
blaCTX-M-1	116	0	4	0	1	0	111
blaCTX-M-65	2	0	2	0	0	0	0
blaTEM-1B	72	1	10	1	3	55	2
blaTEM-1D	3	0	0	0	0	3	0
blaTEM-1A	2	0	0	0	0	0	2
catA1	3	1	0	2	0	0	0
catB3	2	0	0	0	0	0	2
cmlA1	25	3	1	0	0	2	19
dfrA1	139	0	127	0	0	0	12
dfrA12	9	0	1	0	0	0	8
dfrA14	178	0	178	0	0	0	0
dfrA17	1	0	0	1	0	0	0
dfrA5	1	1	0	0	0	0	0
dfrA8	7	0	0	0	0	0	7
ere(A)	1	1	0	0	0	0	0
floR	2	0	2	0	0	0	0
fosA3	1	0	1	0	0	0	0
lnu(F)	1	0	0	0	0	0	1
lnu(G)	29	0	0	0	0	8	21
mcr-1.1	5	0	0	0	0	5	0
mef(B)	17	0	1	0	0	0	16
mph(A)	1	0	0	0	0	0	1
qnrB19	2	0	0	0	0	0	2
qnrS1	1	0	0	0	0	0	1
sul1	221	1	215	1	1	0	3
sul2	5	1	0	2	2	0	0
sul3	36	0	1	0	3	2	30
tet(A)	222	1	215	0	1	0	5
tet(B)	2	0	0	2	0	0	0

ND, not determined.
Increasing number of detected genes is highlighted with the red color gradient, white color marking low number of genes and dark red marking 325 large number of genes detected.

The emergence of the *S.* Infantis ESBL clone in Italy was first described by Franco et al. (2016) who noted that the clone carried $bla_{\mathrm{CTX-M-1}}$ gene on the mosaic pESI-like conjugative megaplasmid that additionally rendered the bacteria resistant to multiple classes of antimicrobials, mercury, arsenic, and quaternary ammonium compounds (Franco et al., 2016). According to the authors, this plasmid, which bore a high resemblance to pESI plasmid isolated from *Salmonella* in Israel (Aviv et al., 2014), might have been present in *S.* Infantis in Italy already in 2007. Interestingly, a recent study identified isolates harboring pESI-like plasmids in strains collected in England and Wales as early as 2000 (Lee et al., 2021). Although, we did not detect pESI like signature in the strains isolated before 2011, the publicly available dataset from these years was limited to only 18 isolates and therefore was not fully representative of the strains circulating in Italy at the time. The high proportion of antimicrobial resistant strains carrying $bla_{\mathrm{CTX-M-1}}$ gene in Italian broilers after 2011 is surprising considering that the use of third generation cephalosporins is not licensed in poultry in Italy. According to a recent study, Italy currently has the second highest consumption in the EU of antimicrobials in food producing animals and the most frequently used antibiotics in broilers are penicillins and sulphonamides and in the turkeys are polymyxins, followed by penicillins and tetracyclines (Caucci et al., 2019). It is however likely that a pESI-like plasmid already carrying the $bla_{\mathrm{CTX-M-1}}$ gene was acquired and maintained in the *S.* Infantis population due to the fitness advantages conferred by the other resistance genes and virulence factors present in the plasmid while additionally rendering the strains resistant to beta-lactams (Aviv et al., 2014; Franco et al., 2016). Indeed, Alba et al. (2020) in their recent study that examined epidemiology of *S.* Infantis in Europe, suggested that acquisition of the pESI-like megaplasmid

was a major factor contributing to the rapid transmission of this serovar in Europe.

We noted that only a few strains carried both $bla_{\mathrm{TEM-1}}$ and $bla_{\mathrm{CTX-M-1}}$ genes. The first were most commonly located on IncX1 and IncX4 plasmids and found mainly in the Cluster II population. The loss of $bla_{\mathrm{TEM-1}}$ genes in majority of Cluster I strains, or the loss of entire IncX plasmids, could have been triggered by acquisition of $bla_{\mathrm{CTX-M-1}}$ gene, favored due to its activity against a wider range of beta-lactam antimicrobials.

The overwhelming prevalence of *S.* Infantis resistant to multiple antibiotic classes, including ciprofloxacin and the 3rd- and 4th-generation cephalosporins in the poultry, and consequently, in poultry meat leads to an increased risk of transmission of the resistant strains to humans. The most recent report from EFSA and ECDC that included data for AMR in broilers showed that in 2018, Italy had the largest proportion in the EU of *S.* Infantis strains resistant to cefotaxime and ceftazidime (50.8%) (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2020, 2021). Importantly, at the same time, resistance to these antibiotics in human isolates also exceeded 50%. As Italy is one of the EU members with the highest consumption of third and fourth generation cephalosporins in humans, the pressure constantly exerted on the enteric bacteria will likely lead to selection of the resistant phenotypes rendering the treatment with beta-lactam antibiotics ineffective. Moreover, the persistence of strains carrying MDR-conferring plasmids combined with the ease of horizontal transfer of mobile elements between members of Enterobacteriaceae poses a continuous threat for acquiring resistance genes by strains currently susceptible to the majority of antimicrobials. In line with the *One Health* concept, it is essential to focus on controlling of the spread of the pESI-like plasmid and the MDR strains of *S.* Infantis in poultry populations worldwide. Also, the continuous surveillance of Enterobacteriaceae and the association with specific plasmids both on national and international scales may in the future, impede the emergence and spread of particularly resistant strains. Moreover, ongoing surveillance of antimicrobial resistance within and between the ecosystems is essential to understanding the dynamics of the transmission and persistence of AMR genes at the animal-environment-human interface and to recognize the role that each of the One Health sectors play in the emergence of resistant bacterial strains. As the misuse and abuse of antimicrobials are main factors that drive the development of resistance, the monitoring of antimicrobial usage in livestock and the environment and, consequently, the adaptation of effective stewardship programs in the veterinary medicine sector, are vital in the fight against the global threat of antimicrobial resistance.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AJ, ED, and LD conceived the study. AC, AJ, and LD designed the methodology. AA, FM, LD, KZ, and RR collected, analyzed, and/or interpreted all microbiological data. AJ performed the computational analysis. ED and GG provided funding acquisition, project administration, and resources. AJ and LD wrote the first draft. AC formally reviewed and edited the manuscript. All authors reviewed the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.812481/full#supplementary-material

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Prevalence and Characteristics of *mcr-1*-Producing *Escherichia coli* in Three Kinds of Poultry in Changsha, China

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Colistin is one of the last-line drugs against difficult to treat and multidrug-resistant Gram-negative bacteria. The emergence of mobile colistin resistance gene m*cr-1* increased worldwide attention on colistin resistance. *mcr-1* is the dominant gene that caused resistance to colistin in chicken-derived *Escherichia coli* (*E. coli*) in China; it has a broad resistance spectrum and causes multiple drug resistance problems. There are only few studies on *mcr*-positive *E. coli* (MCRPEC) from laying ducks and quails in China. Here, the molecular and epidemiological characteristics of MCRPEC from three kinds of poultry farms (laying duck, quail, and broiler) were investigated in Changsha, China. A total of 17 *mcr*-positive *E. coli* (MCRPEC) strains were screened in 690 samples from the three kinds of poultry farms. This is the first report on MCRPEC, to our best knowledge, derived from quail. All the MCRPEC strains were resistant to colistin, sulfamethoxazole-trimethoprim, florfenicol, tetracycline, and ciprofloxacin, and mildly resistant to tigecycline, gentamicin, piperacillin/tazobactam, cefotaxime, and ceftiofur. All the strains were sensitive to meropenem and amikacin. By bioinformatics analysis, 17 MCRPEC strains belonging to 11 MLST types were distributed in phylogroups A (58.8%), B1 (23.5%), and phylogroup D (17.6%). *mcr-1* was located in IncI2 plasmid with typical plasmid conjugation transfer part, type IV secretory system, and tellurium-resistant protein, increasing transmission capacity to other bacteria. Monitoring of colistin-resistant bacteria in poultry farms should be strengthened.

Keywords: poultry, quail, laying duck, broiler, colistin, *mcr-1*, *Escherichia coli*

INTRODUCTION

The use of colistin as an antibiotic for the treatment of Gram-negative infections has been gradually reduced because of its nephrotoxicity and neurotoxicity (Koch-Weser et al., 1970). With the emergence of multi-drug-resistant bacteria, especially carbapenem-resistant bacteria, lack of new antibiotics against Gram-negative pathogens has forced the reuse of traditional antibiotic (colistin) (Falagas and Kasiakou, 2005). However, it will be decreasing effectiveness of colistin in clinical after the emergence of mobile colistin resistance gene, *mcr-1*(Liu et al., 2016), and its

variants (*mcr-2* to *mcr-10*) (Xavier et al., 2016; AbuOun et al., 2017; Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017; Wang X. et al., 2018; Yang et al., 2018; Carroll et al., 2019; Wang C. et al., 2020). Colistin-resistant isolates harboring these plasmid-mediated colistin resistance genes had been increasing on human clinical (Li et al., 2021), livestock and poultry farms (Hassen et al., 2020), sewage treatment systems (Yang et al., 2019), meat products retail (Sadek et al., 2021), waterfowl breeding (Shen et al., 2019), and home water purifiers (Chen et al., 2021). *mcr* genes have been mainly found in *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Enterobacter* spp., and *Aeromonas* spp. (Ling et al., 2020). *mcr-1* is the most widely disseminated plasmid-mediated colistin-resistant gene and is mainly carried by the IncI2 and IncX4 types of plasmid (Tang et al., 2021).

The World Organization for Animal Health (OIE) recommends that organizations urgently prohibit colistin use as a growth promoter because of increasing colistin resistance.[1] At the same time, governments began to issue policies to limit colistin in livestock and poultry. The Chinese government formally banned colistin as an animal growth promoter on April 30, 2017 (Walsh and Wu, 2016). Other countries, including India, Japan, Malaysia, and Thailand, have banned or agreed to ban colistin as a feed additive for animal growth (Olaitan et al., 2021). The withdrawal of colistin from animal feed in China has significantly reduced colistin resistance and the prevalence of *mcr-1* in both animals and humans (Wang Y. et al., 2020). It proved that the intervention policies effectively reduced the use of colistin and reduced colistin resistance in animals.

mcr-1 and its variants have been found in various countries and regions but mainly focused on livestock breeding, especially pig and broiler chickens. The prevalence of *mcr-1* in other kinds of poultry is rare. Here, we investigated the prevalence and characteristics of *mcr*-positive *Escherichia coli* (MCRPEC) isolate from three types of poultry farms, namely, quail farm, laying duck farm, and broiler farm, in Changsha, China.

MATERIALS AND METHODS

Sample Collection and Isolation of Bacteria

From May 2019 to May 2020, animal cloacal swabs and surrounding environmental samples were collected from three kinds of poultry farms in Changsha as previously described (Wang et al., 2017; Shi et al., 2021; Zhao et al., 2021). Briefly, 50 animal cloacal swabs per farm were collected using sterile swabs and then suspended in 1 ml phosphate-buffered saline (PBS). Flies were caught using fly glue boards (Green Leaf Co., China) and transferred to the 1 mL PBS with sterile tweezers. Four ml of drinking water and sewage were collected in a clean bottle. Around the farming area, surface soil of 5–10 cm was removed with a shovel, and 5-g soil samples were collected with sterile bags. Five samples from within one square meter were mixed. All the samples were stored in an icebox and transported to the laboratory. The samples were seeded in MacConkey (Solarbio) containing 2 mg/L of colistin and incubated at 37°C overnight; Then, a single pink clone was picked up.

Screening for *mcr* Genes and Species Identification

In all the bacteria, we detected the presence of *mcr-1* to *mcr-10* by polymerase chain reaction (PCR) (Rebelo et al., 2018; Borowiak et al., 2020; Wang C. et al., 2020). Species identification of MCRPEC was confirmed with the 16S rRNA gene (Srivastava et al., 2008). PCR products were sequenced by Tsingke Biological Technology (Changsha, China) using the sanger sequencing method and compared to the GenBank database.

Antimicrobial Susceptibility Testing and Plasmid Conjugation Assay

As recommended by Clinical & Laboratory Standards Institute (CLSI) M100-S30,[2] CLSI VET01-A4 (see text footnote 2), and the European Committee on Antimicrobial Susceptibility Testing (EUCAST),[3] minimum inhibitory concentrations (MICs) of colistin were evaluated by broth microdilution, and 11 antibiotics (meropenem, amikacin, florfenicol, sulfamethoxazole-trimethoprim, piperacillin-tazobactam, tetracycline, ciprofloxacin, gentamicin, tigecycline, cefotaxime, and ceftiofur) were determined with the agar dilution method. *E. coli* ATCC 25922 was used as a quality control strain. The transferability of *mcr-1* was determined by plasmid conjugation assay; streptomycin-resistant *E. coli* C600 serves as the recipient and MCRPEC as the donor strains. Their transconjugants were selected on MacConkey agar with colistin (2 mg/L) and streptomycin (500 mg/L), and confirmed by ERIC-PCR fingerprinting (Amin et al., 2020) for *E. coli* C600 and PCR analysis for the *mcr-1* gene.

Whole-Genome Sequencing of MCRPEC

Genomic DNA was extracted from the MCRPEC strains using a TIANamp Bacteria DNA kit (Tiangen Biotech Company, China) according to the manufacturer's instructions, and 150-bp paired-end reads were generated with a HiSeq X Ten platform (Illumina). Bacterial genome assembly was performed with the SPAdes software (Bankevich et al., 2012).

Bioinformatics Analysis

Multilocus sequence typing (MLST) was performed, antimicrobial-resistant genes were determined by SRST2 (Inouye et al., 2014), and *E. coli* phylogroups were identified with the ClermonTyping scheme (Beghain et al., 2018). The clonal relationship of MCRPEC was evaluated by core genome alignments and with phylogenetic trees, which were constructed with Parsnp (Treangen et al., 2014) and visualized with the online tool iTOL.[4] Plasmid comparisons of the isolates were performed with BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011),

[1] https://www.oie.int/app/uploads/2021/03/book-amr-ang-fnl-lr.pdf

[2] https://clsi.org

[3] https://eucast.org

[4] https://itol.embl.de

and EasyFig 2.2.5 was used for gene-environment analysis (Sullivan et al., 2011).

RESULTS

Detection of MCRPEC

Six hundred ninety samples (250 broiler chicken cloacal swabs, 150 laying duck cloacal swabs, 150 quail cloacal swabs, and 140 environmental samples) were collected from 10 poultry farms in Changsha (**Supplementary Table 1**). After screening, 17 strains were positive for *mcr-1* (**Table 1**), but no other *mcr* variants were detected in this study, indicating that *mcr-1* was the dominant *mcr* gene in poultry farms in Changsha. Among the three kinds of poultry breeding, the isolation rate of *mcr-1*-positive MCRPE was highest in broiler (5.6%, 14/250), followed by quail (0.7%, 1/150), and laying duck (0.7%, 1/150) (**Table 1**). In addition, MCRPEC was detected in the surrounding environment (flies, 1.8%, 1/56) (**Table 1**). MCRPEC was present in five broiler farms, and detection rate was higher than 2% (2, 4, 4, 8, and 10%, respectively), indicating that urgent attention is needed in broiler breeding.

Antimicrobial Susceptibility Profiles and Antimicrobial-Resistant Genes of MCRPEC

The MIC range of poultry MCRPEC against colistin was 4 to >128 mg/L. All the MCRPEC isolates were resistant to sulfamethoxazole, florfenicol, tetracycline, and ciprofloxacin, and sensitive to meropenem and amikacin. We found that some of the MCRPEC strains were tetracycline-resistant, but no tetracycline-resistant gene was detected (**Figure 1**), which may be because of overexpression of the efflux pump gene. In addition, most of the strains were mildly resistant to tigecycline, gentamicin, piperacillin-tazobactam, cefotaxime, and ceftiofur (**Table 2** and **Supplementary Table 2**). Interestingly, different β-lactam-resistant genes (bla_{CTX}, bla_{OXA}, and bla_{TEM}) were found in the 17 MCRPEC strains.

Molecular Typing and Phylogenetic Tree

A total of 11 MLST types (ST10338, ST1403, ST1421, ST156, ST162, ST3941, ST6321, ST69, ST7153, ST93, and ST12735) were identified among the 17 MCRPEC strains (**Figure 1**). MCRPEC isolates predominantly belonged to phylogroup A (58.8%, 10/17), followed by phylogroup B1 (23.5%, 4/17) and phylogroup D (17.6%, 3/17) (**Figure 1**). Strain 20m13, belonging to ST162, was isolated from a quail farm. To the best of our knowledge, this is the first report on detection of *mcr-1* from quail. In another quail farm, one MCRPEC strain (20n54) from a fly belongs to ST6321 ClermontTypingD *E. coli*, which contains seven resistant genes, namely, β-lactam-resistant gene ($bla_{TEM-122}$), tetracycline-resistant gene [*tet*(A)], colistin-resistant gene (*mcr-1*), phenicol-resistant gene (*floR*), fluoroquinolone-resistant gene (*qnrS1*), trimethoprim-resistant gene (*dfrA10*, *dfrA14*), and sulfonamide-resistant gene (*sul1*), which pose a potential risk to quail's health by spreading to quails.

By constructing a phylogenetic tree, Five groups, with two strains per group isolated from the same farm and closely related, possessed a similar ST type, phylogroup (**Figure 1**). In addition, two isolates (20a46 and 20b14) that we found in the proximity of the duck and broiler farms had a close genetic relationship (**Figure 1**).

TABLE 1 | Isolation rate of *mcr*-positive *Escherichia coli* (MCRPEC) from different sources.

Source	Samples	MCRPEC	Farm name (Number of MCRPEC)
Broiler	250	5.6% (*n* = 14)	Liuyang 1 (4); liuyang 4 (1);liuyang 6 (2); liuyang 7 (5); liuyang 8 (2)
Laying Duck	150	0.7% (*n* = 1)	Liuyang 1 (1)
Quail	150	0.7% (*n* = 1)	Changshaxian 1 (1)
Fly	56	1.8% (*n* = 1)	Changshaxian 2 (1)
Soil	44	0	–
Sewage	20	0	–
Drinking water	20	0	–
Total	690	2.5% (*n* = 17)	–

Transferability of MCRPEC and Genetic Environment of the *mcr-1* Gene

mcr-1-bearing plasmid of most strains (82.4%, 14/17) could transfer to streptomycin-resistant *E. coli* C600 by conjugation assay. By bioinformatics analysis, type IV secretion systems (*virB1*, *virB2*, *virB4*, *virB8*, *virB10*, and *virB11*) and plasmid conjugation elements (*pilM*, *pilV*, and *pilL*) were found in almost all the isolates, which are related to the transferability of bacteria and contribute *mcr-1* to other bacteria (**Figure 2**). Besides, 70.6% (12/17) *mcr-1*-bearing plasmids belonged to Incl2 except for the additional five contigs whose plasmid type was difficult to distinguish because of the short sequence. Therefore, we speculated that the Incl2 plasmid was prevalent in local poultry farms. Studies show that IS*Apl1* has been deemed to be the critical element mediating the translocation of *mcr-1* into various plasmid backbones (Sun et al., 2017). However, IS*Apl1* was not found in the 17 isolates, showing a more stable *mcr-1* genetic environment (**Supplementary Figure 1**). In addition, this study also found that some of the strains carry tellurium-resistant proteins (TerA, TerB, and TerD), which are involved in tellurite resistance, colicine resistance, phage inhibition, and pathogenicity (Turkovicova et al., 2016).

DISCUSSION

In this study, colistin was not used in the poultry farms, but the detection rate of the MCRPEC isolates in the poultry farms, especially in the broiler farms, was relatively high. By inquiry, antibiotics are used extensively to promote growth in the broiler; on the contrary, laying ducks are prohibited from using antibiotics during the egg-producing period, because the addition of antibiotics will give rise to veterinary drug residues and reduce the output of egg production. In addition, co-carrier essential resistance genes (*sul*, *floR*, *tet*(M), *bla*, *aph*, etc.) may be related to using other antibiotics (florfenicol, enrofloxacin, doxycycline), along with *mcr-1* which had the

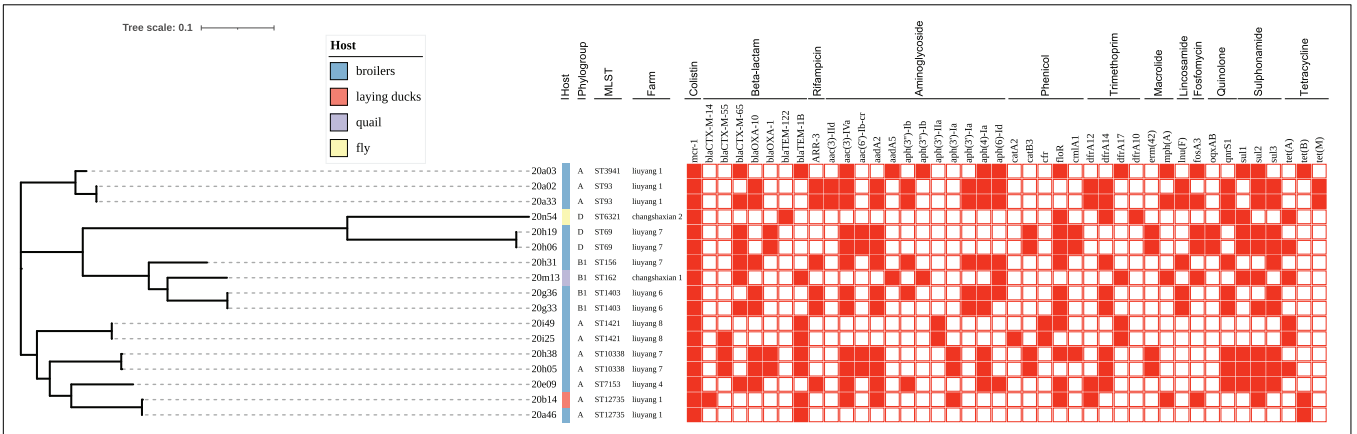

FIGURE 1 | Phylogenetic tree of the 17 *mcr*-positive *Escherichia coli* (MCRPEC) isolates. Phylogroup, multilocus sequence type, and farm origin are shown in text after the corresponding isolate name. Sources of the isolates are differentiated by color. Blue, broiler; Orange, laying duck; Purple, quail; Yellow, fly. Filled squares denote the resistance genes for presence and empty squares for absence.

TABLE 2 | Minimum inhibitory concentration of 11 antibiotics against MCRPEC.

Antibiotics	MIC (mg/L)[a]								Resistance rate (%)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
	≤1	2	4	8	16	32	64	≥128			
Ceftiofur	4	0	1	2	0	4	6	0	70.6	32	64
Colistin	0	0	3	10	3	0	0	1	100	8	16
Gentamicin	2	1	1	6	4	1	2	0	41.2	8	64
Florfenicol	0	0	0	1	0	0	0	16	100	≥128	≥128
Tetracycline	0	0	0	0	0	0	2	15	100	≥128	≥128
Meropenem	17	0	0	0	0	0	0	0	0	≤1	≤1
Ciprofloxacin	0	0	4	6	4	1	0	2	100	8	≥128
Tigecycline	13	1	1	2	0	0	0	0	17.7	≤1	8
Trimethoprim/sulfamethoxazole[b]	0	0	3	0	0	14	0	0	100	32	32
Amikacin	10	4	1	2	0	0	0	0	0	≤1	8
Piperacillin/Tazobactam[c]	1	1	1	2	4	2	4	2	11.8	16	≥128
Cefotaxime	0	1	0	1	0	4	1	10	94.1	≥128	≥128

[a] *Thin vertical lines represent the breakpoints between susceptible and insusceptible isolates. Bold vertical lines represent the breakpoints between non-resistant and resistant isolates.*
[b] *Trimethoprim: sulfamethoxazole at a ratio of 1:19. Breakpoints are expressed as trimethoprim concentration.*
[c] *For susceptibility testing purposes, the concentration of tazobactam was fixed at 4 mg/L.*

potential risk of multi-drug resistant bacteria. Another study showed that *mcr-1*could co-transfer with the bla_{CTX-M}, *fosA3*, *oqxAB*, and *floR* genes (Sun et al., 2017). Therefore, local regulatory authorities should strengthen the supervision of antibiotic usage and monitor bacterial resistance, especially to MCRPEC strains.

Only a strain from laying ducks (farm liuyang 1) had a close relationship with one MCRPEC strain and a high *mcr-1* detection rate (8%, 4/50) in the broiler farm. Two farms are located in different parts of the same land and managed by the same breeder, which is a potential for transmission from broilers to laying ducks. MCRPEC strains with Incl2 plasmid carrying the type IV secretion system and plasmid conjugation elements promote their *mcr-1* transfer to streptomycin-resistant *E. coli* C600. In this way, it makes the Incl2 plasmid become the dominant carrier of *mcr-1*.

To the best of our knowledge, this study is the first to report on the detection of *mcr-1* on quails. Before this, *Salmonella enterica* carrying *tet*A, *tet*B, and *tet*G (Bacci et al., 2012), and multidrug-resistant methicillin-resistant *Staphylococcus aureus* (LA-MRSA) CC398 carrying the *bla*Z, *mec*A, *erm*B, and *erm*C genes (Silva et al., 2021) were found on quails. There was no significant relationship between the *E. coli* isolated from quail farms and that from other poultry farms (**Figure 1**). At the same time, MCRPEC from flies in the quail farms was determined, increasing the transmission probability of *mcr-1* in quails (Wang et al., 2017).

We demonstrated the prevalence of MCRPEC isolates in poultry farms in Changsha, but only *mcr-1* gene was

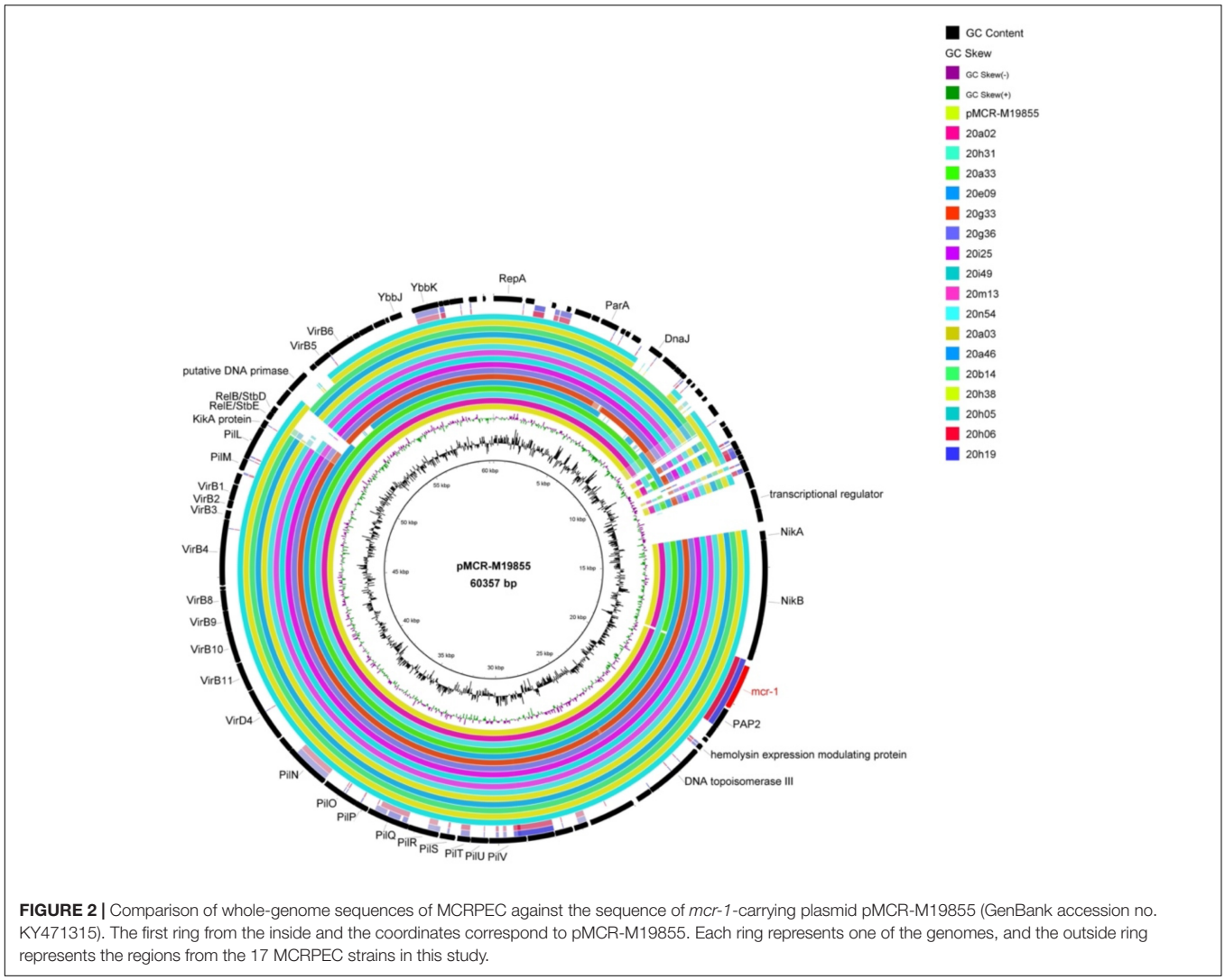

FIGURE 2 | Comparison of whole-genome sequences of MCRPEC against the sequence of *mcr-1*-carrying plasmid pMCR-M19855 (GenBank accession no. KY471315). The first ring from the inside and the coordinates correspond to pMCR-M19855. Each ring represents one of the genomes, and the outside ring represents the regions from the 17 MCRPEC strains in this study.

found. Poultry MCRPEC shows multi-drug resistance when co-harboring with other essential resistant genes. Likewise, comprehensive genomic analyses revealed that the *mcr-1* genes located in the IncI2 plasmid might transfer to a different host, with the transfer element posing a potential risk to humans. Monitoring of colistin-resistant bacteria in poultry farms should be strengthened.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

JL and XC designed the research. JH, QZ, ZL, and HY collected the data. JH, JY, and WC analyzed and interpreted the data. JH drafted the manuscript. JL, JH, ZS, and XC revised the manuscript. All the authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.840520/full#supplementary-material

Supplementary Figure 1 | Genetic environment of *mcr-1* in the 17 *mcr*-positive *Escherichia coli* (MCRPEC) strains. The direction of arrows indicates the direction of transcription.

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Epidemiology, Environmental Risks, Virulence, and Resistance Determinants of *Klebsiella pneumoniae* From Dairy Cows in Hubei, China

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Klebsiella pneumoniae (*K. pneumoniae*) is an opportunistic pathogen, which causes serious infections in humans and animals. To investigate the antimicrobial resistance pattern and virulence profile of *K. pneumoniae*, a total of 887 samples were collected from both the healthy and mastitis cows and the bedding, feed, feces, air, drinking water, spraying water, washing water, and milk cup swabs from five dairy farms in Hubei, China, during 2019 and 2020. *K. pneumoniae* was isolated and identified using PCR of the *khe* and 16S rDNA sequencing. A genotypic characterization was performed for *K. pneumoniae* isolates using *wzi* typing *and* multilocus sequence typing (MLST). Antimicrobial resistances were confirmed using broth microdilution against 17 antimicrobial agents and resistance and virulence genes were determined by PCR. The prevalence of *K. pneumoniae* was 26.94% (239/887) distributed in 101 *wzi* allele types (199/239, 83.26%) and 100 sequence types (STs) (209/239, 87.45%), including 5 new *wzi* allele type and 25 new STs. Phylogenetic analysis showed that *K. pneumoniae* isolated from milk, nipple swab, feed, and feces is classified in the same clone complex. By comparing with the PubMLST database, at least 67 STs have the risk of spreading in different species and regions. Interestingly, 60 STs have been isolated from humans. The isolates were highly sensitive to meropenem and colistin, but resistant to ampicillin (100%), sulfisoxazole (94.56%), cephalothin (47.28%), streptomycin (30.13%), and so on. Noteworthy, multidrug-resistant (MDR) rate was found to be 43.93% in this study. By PCR, 30 of 68 antimicrobial resistance (AMR) genes were identified; the prevalence rate of *blaTEM*, *blaSHV*, *strA*, *strB*, *aadA1*, and *aac(6′)-Ib-cr* was more than 50%. Eleven *CTX-M*-producing *K. pneumoniae* were found. The detection rate of *fimH*,

mrkD, *uge*, *wabG*, *entB*, *iutA*, *iroN*, and *ureA* was over 85%. This study reinforces the epidemiological importance of *K. pneumoniae* in food-producing animals in Hubei. The emergence and spread of environmental MDR *K. pneumoniae* may pose a potential threat to food safety and public health.

Keywords: *Klebsiella pneumoniae*, cow mastitis, antimicrobial resistance, virulence gene, *wzi* gene sequencing, MLST

INTRODUCTION

Cow mastitis is considered to be one of the most common and frequent diseases in dairy herds. This disease only affects the estrus and pregnancy of dairy cows, resulting in the decline of milk production and quality, but also increases treatment costs and causes high economic losses in dairy industries worldwide (Käppeli et al., 2019). The global economic losses per year due to mastitis amount to $35 billion and for the United States dairy industry $2 billion per year (Sathiyabarathi et al., 2016; Li et al., 2019). Besides, mastitis can pose a threat to human and animal health *via* the transfer of antimicrobial resistance bacteria and food poisoning (Li et al., 2019). *Klebsiella pneumoniae* (*K. pneumoniae*) is one of the main environmental pathogens causing mastitis, as well as a pathogen of zoonotic conditions that can cause a series of serious infections such as respiratory tract infections, urinary tract infections, soft-tissue infections, and bloodstream infections (Lee et al., 2017; Massé et al., 2020).

Virulence factors play an important role in the pathogenic mechanism of *K. pneumoniae*. Capsular, iron carriers, pili, and lipopolysaccharide (LPS) have been widely demonstrated to be involved in the adhesion, invasion, and growth of *K. pneumoniae* (Shon et al., 2014; Cheng et al., 2020). Capsular can prevent *K. pneumoniae* from being recognized by the host immune system through some immune escape mechanisms such as antiphagocytosis, inhibition of early inflammatory response, neutralization of antimicrobial peptides to reduce the body's immune response, and inhibition of dendritic cell maturation (Paczosa and Mecsas, 2016). These bacteria can absorb the iron of the host *via* four siderophores such as aerobactin, salmochelin, enterobactin and yersiniabactin for metabolism and enhance the virulence to cause infection (Wang G. et al., 2020). Two common types of fimbriae are found in *K. pneumoniae*: type 1 (*fim*) and type 3 (*mrk*) fimbriae. Type 1 fimbriae can enhance virulence by adhering to mucosal or epithelial surfaces and type 3 fimbriae adhere to the cell surface and promote biofilm formation (Schroll et al., 2010). Lipid A, a component of lipopolysaccharide, reduces the inflammatory response during *K. pneumoniae* infection and prevents the bactericidal effect of cationic antimicrobial peptides. O antigen is the outermost subunit of LPS, which eliminates the lysis of bacteria by the complement membrane attack complex (Paczosa and Mecsas, 2016).

Previous studies have shown that mucoviscosity-associated gene (*magA*), uridine diphosphate galactose 4 epimerase encoding gene (*uge*), regulator of the mucoid phenotype (*rmpA*), iron uptake system gene (*kfu, aerobactin*), and K1 and K2 capsule serotypes are important virulence genes in invasive *K. pneumoniae* strains causing mastitis (Osman et al., 2014; Massé et al., 2020). According to virulence characteristics, *K. pneumoniae* can be divided into classic *Klebsiella pneumoniae* (cKP) and hypervirulence *Klebsiella pneumoniae* (hvKP). Most *K. pneumoniae* infections in the world are cKP infections, but cKP can evolve into hvKP by obtaining virulence factors (such as plasmids) (Wyres et al., 2020). Studies have shown that the genes *peg-344*, *iroB*, *iucA*, $_{p}$*rmpA*, and $_{p}$*rmpA2* can distinguish cKP and hvKP with high accuracy (Harada and Doia, 2018; Russo et al., 2018). Some scholars have found that K1 and K2 capsule serotype is closely related to high virulence (Follador et al., 2016; Guo et al., 2017). Since hvKP was first detected in Taiwan in 1986, the number of clinical infection cases of the bacteria has gradually increased and Wuhan was the city with the highest hvKP prevalence (73.9%) reported among 230 *K. pneumoniae* isolates from 10 cities in China (Liu et al., 1986, 2014; Zhang et al., 2016). More worryingly, with the inappropriate use of antibiotics, multidrug resistant (MDR) has been increased all over the world that is considered as a public health threat. Several recent investigations reported the emergence of multidrug-resistant bacterial pathogens from different origins, including humans, birds, cattle, and fish that increase the need for new potent and safe antimicrobial agents (Algammal et al., 2020a,b, 2021). Epidemics and outbreaks of multidrug-resistant *K. pneumoniae* have also been reported around the world (Gu et al., 2018; Alanezi et al., 2022; Arteaga-Livias et al., 2022). The worldwide spread of extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* remains a critical concern for therapies against multidrug-resistant bacteria (Chong et al., 2018). "Superbug" derived from the combination of virulence and resistance of *K. pneumoniae* will bring great challenges to clinical anti-infective treatment. In addition, hundreds of mobile antimicrobial resistance (AMR) genes have been found in *K. pneumoniae* (Navon-Venezia et al., 2017). *K. pneumoniae* is considered to be a major transporter of resistance genes from environmental sources to clinically important bacteria and some isolates can carry acquired AMR genes or plasmid to move between environmental, human, and animal (Wyres and Holt, 2018).

As the most important measures for disease prevention and control, finding the source of infection and cutting off the transmission route are to block the transmission of pathogens from infected animals/people to susceptible animals/people (Chen et al., 2021). Genotyping technology is mainly used to understand the transmission route of pathogens and trace the source of infection. Multilocus sequence typing (MLST) is widely used because of its good typing ability, comparability, high reproducibility, high throughput, and convenient data sharing (Pérez-Losada et al., 2013). The sequence types (STs)

of *K. pneumoniae* reported to be important and prevalent globally include ST11, ST15, ST23, ST258, ST395, ST512, and so on (Zhang et al., 2019; Gato et al., 2020; Zhou et al., 2020; Di Pilato et al., 2021). Currently, the host and regional epidemiological characteristics of *K. pneumoniae* in various regions are still unclear and surveillance data on resistance and virulence of *K. pneumoniae* in the cow are very scarce. This study aimed to investigate the prevalence, antimicrobial resistance pattern, resistance genes, and virulence genes of *K. pneumoniae* isolated from dairy cows in Hubei, China. *K. pneumoniae* from milk and environmental samples was genotyped using PCR to determine genetic diversity and explore potential reservoirs and transmission.

MATERIALS AND METHODS

Farms and Sample Collection

Samples were collected from five commercial dairy farms in Hubei during 2019 and 2020. The number of lactating cows in farms A, B, C, D, and E are 202, 300, 400, 1,200, and 287, respectively. Five farms participated in monthly Dairy Herd Improvement (DHI). Lactating cows in five farms were milked 2 times/day in milking parlors. The clinical symptoms of cows with clinical mastitis (CM) are elevated body temperature, redness, and pain in the udder. The judgment of subclinical mastitis (SCM) refers to the Chinese agricultural industry standard NY/T 2692-2015 (National standards of the people's Republic of China, 2015) [the number of somatic cell count (SCC) is > 500,000 cells/ml and no visible pathological changes]. Daily care of cows in five farms was provided by the veterinarian; the farms routinely used different antibacterial agents such as ceftiofur, amoxicillin/clavulanic acid, lincomycin, florfenicol, kanamycin, and gentamicin for regular prophylactic and treatment protocols. The sample collection includes animal samples (nipple milk, nipple skin swabs, anal swabs from healthy cows, CM cows, and SCM cows) and environmental samples (bedding, feed, feces, air, drinking water, spraying water, washing water, and milk cup swabs). Lactating cows of parity 3rd to 4th were selected for sample collection. Briefly, milk samples were collected from lactating cows by the milkers after premilking disinfection and the first 3 streams of milk were discarded. Commercial liquid delivery mediums (Haibo Biotech, Qingdao, China) were used to collect nipple skin swabs, anal swabs, and milk cup swabs. All the nipples of each cow were rubbed on the skin with swabs 4 to 5 times from the root to the end of teat after milking. For the anal swab, the swab was inserted into the anus of the cow and rotated for 2 to 3 circles. The swab of the milk cup is collected in milking parlors (5 per farm). Feed, bedding, and feces samples were stored in sterile sampling bags and air samples were collected by natural sedimentation method [suspended sterile sampling tube with 15 ml Trypticase Soy Broth (Haibo Biotech, Qingdao, China) on the column in the barn for 15 to 30 min]. Three sampling points were evenly selected in the barn and the interval between sampling points was not less than 5 m and then mix the samples with equal weight. All the samples were stored at 4°C and transported to the laboratory for bacterial culturing and identification within 24 h. SCC of milk sample was analyzed by DHI Center of Livestock and Poultry Breeding Centre in Hubei.

Isolation and Identification of *Klebsiella pneumoniae*

All the samples were inoculated in 5 ml Trypticase Soy Broth (Haibo Biotech, Qingdao, China) and incubated at 37°C for 18 to 24 h. A loopful of broth culture was streaked onto Columbia Blood Agar and MacConkey Inositol Adonitol Carbenicillin (MIAC) agar (Haibo Biotech, Qingdao, China) and incubated at 37°C for 12 to 18 h. A single pink, slimy, and non-hemolytic suspected colony was picked and purified on MIAC agar (Gao et al., 2010). Purified bacteria were selected for biochemical identification and PCR testing. Gram staining was performed with the Gram Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Biochemical identification tubes (Haibo Biotech, Qingdao, China) were used for motility tests, indole tests, urease tests, oxidase experiments, and citrate utilization tests (Massé et al., 2020). Further identification and confirmation of the isolates were carried out using PCR of *khe* and *16s rDNA* gene (He et al., 2016; Cheng et al., 2021). Positive clones were sequenced by the Tsingke Biotechnology Corporation Ltd. (Wuhan, China). *K. pneumoniae* ATCC 700603 (*khe* positive) and *Escherichia coli* ATCC 25922 (*khe* negative) were used as control strains.

Deoxyribonucleic Acid Extraction

Genomic DNA of *K. pneumoniae* was extracted by boiling method (Kuang, 2015). 3 ml suspended plaque samples were incubated for 15 to 20 min at 99°C. Subsequently, the supernatants were immediately transferred to −20°C for 15 min and centrifuged at 12,000 rpm/min for 15 min at 4°C. The supernatants were diluted by sterile water (1:10) and kept at −20°C for PCR testing.

Capsular Serotyping and Multilocus Sequence Typing

Capsular polysaccharide serotypes were determined by *wzi* gene sequencing (Brisse et al., 2013). MLST detection method refers to the *K. pneumoniae* MLST online database[1]. The seven housekeeping genes (*gapA*, *infB*, *mdh*, *phoE*, *pgi*, *rpoB*, and *tonB*) were amplified by PCR. All the positive products were sequenced by Sanger and sequences were submitted to the website[2] to get the *wzi* allele types and the subtypes of each housekeeping gene of MLST. Submit the allele profile to the MLST website[3] to get ST. *wzi* alleles and STs that had not been previously described were submitted to the curator of the PubMLST database and were assigned new designations. Sanger sequencing was completed by Tsingke Biotechnology Corporation Ltd. (Wuhan, China).

[1] http://www.pasteur.fr/mlst

[2] https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef&page=sequenceQuery

[3] http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public&page=profiles

Phylogenetic Analysis and Comparison of *Klebsiella pneumoniae* From Different Sources and Countries in the Multilocus Sequence Typing Database

The ST phylogeny tree (Tamura 3-parameter model) was constructed using the maximum likelihood method in the MEGA-X software and was modified visually with interactive tree of life (iTOL)[4]. The phylogenetic tree is tested by the bootstrap method and the number of tests is 1,000 times. The information of 726 *K. pneumoniae* isolates from different countries and 724 *K. pneumoniae* isolates from different hosts was downloaded from the PubMLST database (the same 100 STs as in this study). Minimum spanning tree (MST) was constructed by Bionumerics 8.1.

Detection of Virulence Genes

All the 239 *K. pneumoniae* were evaluated for the presence of 23 known virulence genes, including transporter (*peg344*), fimbriae synthesis-related gene (*fimH, mrkD*), lipopolysaccharide-related gene (*uge*, *wabG*), capsular polysaccharide synthesis and synthesis regulation-related gene (*wcaG*, $_{c}$*rmpA*, $_{p}$*rmpA*, $_{p}$*rmpA2*, and *magA*), iron uptake system (*iroB*, *iroN*, *aerobactin*, *iutA*, *irp2*, *iucA*, *ybtA*, *kfu*, and *entB*), urease-related gene (*allS*, *ureA*), tellurite resistance gene (*terB*), and hemolysin (*hly*) by PCR (**Supplementary Table 1**).

Antimicrobial Susceptibility Testing

The susceptibility of *K. pneumoniae* isolates to 17 antimicrobials, which are commonly used on dairy farms and become clinically important broad-spectrum antimicrobials, was determined using the microbroth dilution method recommended by the Clinical and Laboratory Standardization Institute [Clinical and Laboratory Standards Institute [CLSI] (2021)], including penicillins: ampicillin (AMP); β-lactam/β-lactamase-inhibitor combinations: amoxicillin/clavulanic acid (AMC); cephalosporins: cephalothin (CEP), ceftiofur (EFT), and ceftriaxone (CRO); carbapenems: meropenem (MEM); aminoglycosides: streptomycin (STR), kanamycin (KAN), and gentamicin (GEN); tetracyclines: tetracycline (TET) and doxycycline (DOX); amphenicols: florfenicol (FFC) and polymyxins: colistin (CT), rifamycins: rifaximin (RFX), fluoroquinolones: ciprofloxacin (CIP); and sulfonamides: sulfisoxazole (SOX) and sulfamethoxazole/trimethoprim (SXT). Results were interpreted according to the CLSI M100-Ed31 and VET01S-Ed5 standards [Clinical and Laboratory Standards Institute [CLSI] (2020, 2021)]. No CLSI interpretative criteria for ceftiophene and streptomycin are currently available and the resistance breakpoint of ceftiophene and streptomycin refers to ceftiofur and kanamycin, respectively. The criteria for resistance of rifaximin were analyzed by Ecofinder software recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Turnidge et al., 2006). The phenotypic resistance patterns are categorized into MDR, extensively drug-resistant (XDR), and pandrug-resistant (PDR) as previously described by Magiorakos et al. (2012). The quality control strain is *K. pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922.

[4]https://itol.embl.de

Detection of Antimicrobial Resistance Genes

Antimicrobial resistance genes that confer resistance to β-lactams ($bla_{CTX-M-2}$, $bla_{CTX-M-10}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}, and bla_{OXA-1}), carbapenems (*IMP*, *VIM*, bla_{OXA-48}, $bla_{OXA-181}$, *NDM*, and *KPC*), AmpCs (*MOX*, *CIT*, *DHA*, *ACC*, *EBC*, and *FOX*), aminoglycosides [*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *npmA*, *aadA1*, *aadA2*, *aadB*, *aacC1*, *aacC2*, *aac(3)-IV*, *aacA4*, *aphA1*, *aphA2*, *aphA6*, *strA*, and *strB*], plasmid-mediated quinolone resistance (PMQR) genes [*aac(6′)-Ib-cr*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*], tetracyclines [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G)], sulfonamides (*sul1*, *sul2*, and *sul3*), macrolides (*mefA*, *ereA*, *ereB*, *ermB*, *mphA*, and *mphB)*, polymyxins (*mcr-1* to *mcr-5*), and phenicols (*floR*, *cmlA*, and *Cat1*) were investigated by PCR (**Supplementary Table 2**). For each positive gene, some PCR products were selected and sent to Quintara Biotechnology Corporation Ltd. (Wuhan, China) for Sanger sequencing for further confirmation.

Amplification and Sequencing of the Quinolone Resistance-Determining Regions Mutation Genes Fragments

All of ciprofloxacin-resistant *K. pneumoniae* were selected to determine the DNA sequence of quinolone resistance-determining region (QRDR) genes (*gyrA*, *gyrB*, *parC*, and *parE*) (Horii et al., 2008; Pitondo-Silva et al., 2015; Cheng et al., 2018). Three ciprofloxacin-intermediate and 7 ciprofloxacin-sensitive isolates were randomly selected as controls. Genomic DNA was extracted by the Universal Genomic DNA Kit of ComWin Biotechnology Corporation Ltd. (Beijing, China). PCR products were purified and sequenced in both the directions by Quintara Biotechnology Corporation Ltd. (Wuhan, China). The nucleotide sequences and the deduced amino acids were compared with that of *K. pneumoniae* ATCC 13883 (GenBank JOOW00000000.1) using the Bioedit (Azargun et al., 2019).

Statistical Analyses

SPSS statistics version 26 and GraphPad Prism version 8.0.1 software were used for statistical analysis and the chi-squared test (χ^2) was used to compare the statistical significance between the different groups. $p < 0.05$ means significant difference and $p < 0.01$ indicates extremely significant difference. Correlations were assessed by calculating the Spearman's rank correlation coefficient (r). $|r| \geq 0.8$ means high correlation, $0.5 \leq |r| < 0.8$ means moderate correlation, $0.3 \leq |r| < 0.5$ means low correlation, and $|r| < 0.3$ means no correlation.

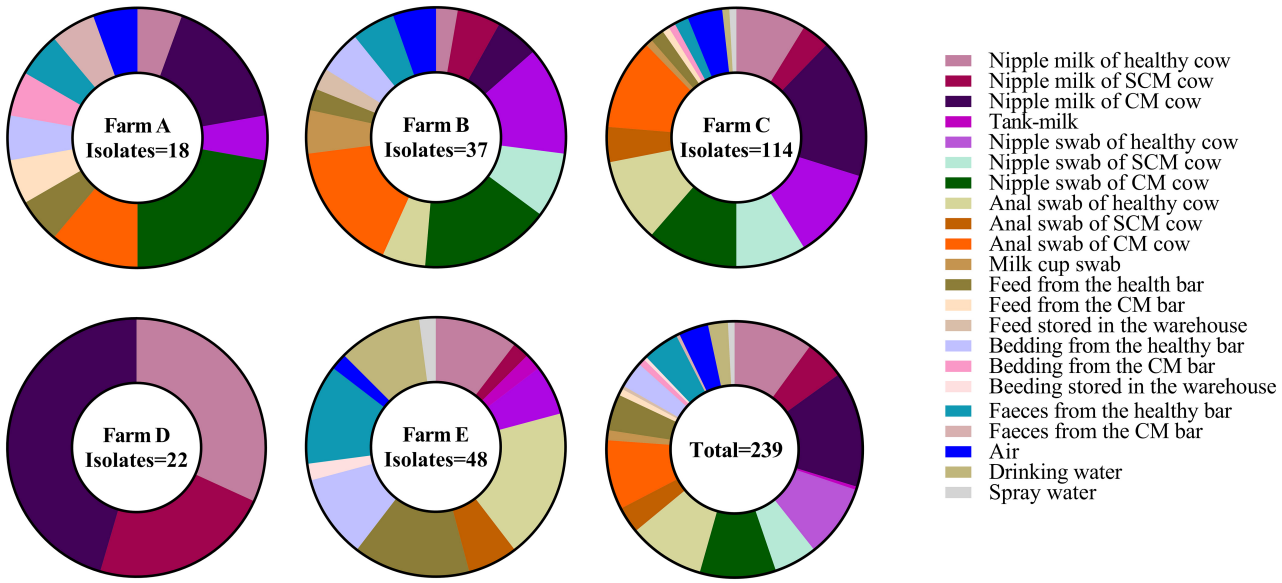

FIGURE 1 | The percentage of *Klebsiella pneumoniae* (*K. pneumoniae*) in different sample types in five farms.

RESULTS

Phenotypic Characteristics of *Klebsiella pneumoniae* Isolates

Klebsiella pneumoniae isolates grew well on MIAC agar due to resistance to carbenicillin and gave characteristic pink colonies as a result of inositol and adonitol fermentation. On Columbia Blood Agar, the colonies are not hemolytic and tend to be viscous and stringy. *K. pneumoniae* was observed as Gram-negative bacillus under a light microscope. Besides, it showed the characteristics of non-motility, indole negativity, production of urease, negative oxidase testing, and utilization of citrate.

Prevalence of *Klebsiella pneumoniae* in Five Dairy Farms

A total of 239 *K. pneumoniae* (26.94%) were identified in 887 samples through biochemical identification, *khe* amplification, and 16s rDNA sequencing (**Figure 1** and **Supplementary Table 3**). The samples collected in farm C (114/338, 33.73%) showed a significantly higher *K. pneumoniae* prevalence than farm A (18/109, 16.51%), farm B (37/179, 20.67%), and farm E (48/189, 25.40%). The *K. pneumoniae* prevalence in farm D (22/72, 30.56%) was also significantly higher than farm A (18/109, 16.51%) ($p < 0.05$). There was no significant difference in the *K. pneumoniae* prevalence of nipple milk, skin swabs, and anal swabs between healthy and mastitis cows ($p > 0.05$). Besides, *K. pneumoniae* was found in a variety of environmental sources, such as milk cup swab, feed, bedding, feces, air, drinking water, and spray water and the separation rate is between 15.79 and 47.83%.

Capsular Serotyping and Multilocus Sequence Typing

Based on the *wzi* gene sequences, 239 isolates were classified as 101 different *wzi* allele types (**Figure 2**). *wzi*150-KL163KL27KL46 (40/239, 16.74%) accounted for the most and *wzi*706, *wzi*727, *wzi*728, *wzi*729, and *wzi*730 were the new *wzi* allele. The *wzi* genotypes of 40 strains were unknown, which might be new alleles. K1 and K2 capsule serotypes commonly found in hvKP were not detected in this study. The results of MLST on these strains showed that 100 different STs were obtained (**Figure 2**), among which ST2854 (30/239, 12.55%) accounted for the largest proportion, followed by ST5960 (15/239, 6.28%) and ST5915 (13/239, 5.44%) and ST5367, ST5369, ST5370, ST5372, ST5855, ST5874, ST5875, ST5876, ST5914, ST5915, ST5917, ST5918, ST5919, ST5920, ST5947, ST5949, ST5951, ST5952, ST5953, ST5954, ST5955, ST5957, ST5959, ST5960, and ST5961 were the new STs. There are still 30 strains of unknown ST, which might be a new allele. We did not find STs such as ST11 and ST23 that have been reported to have a global epidemic of highly toxic in humans. According to the ST phylogeny tree of 239 isolates (**Figure 2**), *K. pneumoniae* isolated from five farms in Hubei has a rich variety of serotypes and genotypes. Strains of the same serotype may have differences in the genome and strains of the same genotype may also have different serotypes, such as ST2584 and ST5370 are *wzi*150, isolates of ST5960 have at least 10 different *wzi* types.

Minimum Spanning Tree Analysis and Prevalence of 100 Sequence Types Among Different Sources and Countries

The MST of 100 different STs showed that there was diversity in the population structure of STs in this study (**Figure 3**). The

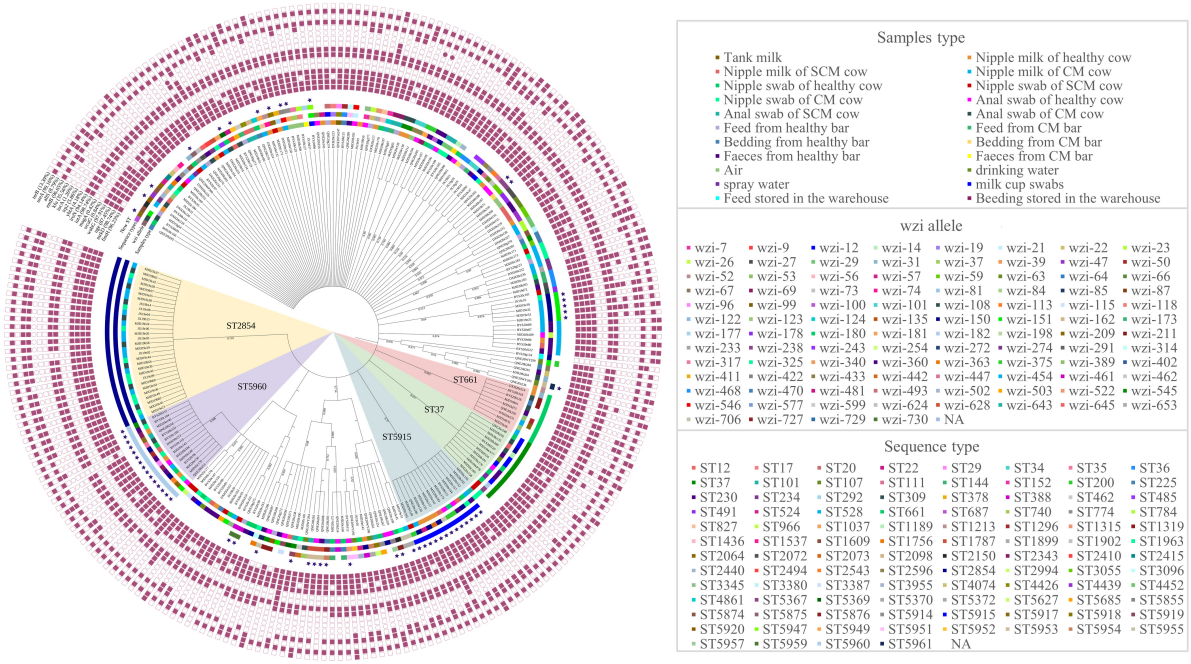

FIGURE 2 | Sequence type (ST) phylogenetic tree, *wzi* allele, and detection of virulence of 239 *K. pneumoniae*. The blue stars represent the new STs. PaleVioletRed squares represent the presence of virulence genes and white squares represent the absence of virulence genes, respectively. NA: No allele number assigned.

founder ST20 and ST17 and ST22 with single locus variants (SLVs) comprise clonal complex 20 (CC20); the founder ST200 and ST292, ST966, ST1537, and ST5627 with SLVs comprise CC200. *K. pneumoniae* isolated from milk, nipple swab, feed, and feces is classified in the same clone complex, which indicated that the contamination of milk may have a great correlation with the hygiene of nipple surface and environment.

Compared with the PubMLST database, we found that 58 STs were isolated in other countries (**Figure 4A**). These genotypes are mainly from Italian isolates; ST17, ST20, ST29, ST34, ST36, ST37, and ST101 have a wide range of the region. Furthermore, the host sources of 67 STs were not only cows, but also humans, pigs, chickens, dogs, cats, horses, rhizosphere, environment, and so on. According to **Figure 4B**, there are 66 STs known to come from multiple hosts, of which 60 STs had existed in humans.

Virulence Genes

Sixteen of 23 virulence genes were positively detected by PCR (**Figure 2**). The detection rate of fimbriae synthesis-related gene (*fimH, mrkD*), lipopolysaccharide-related gene (*uge*, *wabG*), iron uptake system (*entB*, *iutA*, and *iroN*), and urease-related gene (*ureA*) was over 85% and others were between 0.42 and 35.56%. Notably, there are 3 isolates [ST34 (n = 2), ST2410 (n = 1)] carrying *iucA* (**Figure 2**), which are related to high virulence. Besides, 99.16% (237/239) *K. pneumoniae* carried at least 5 virulence genes and three isolates were positive for 12 virulence genes (**Figure 2**). There are differences in the virulence genes carried by strains of the same serotype or ST; no significant correlation has been found between the virulence genes and the serotypes or genotypes in this study.

Antimicrobial Susceptibility Testing

The results of the susceptibility test to 17 antimicrobials are shown in **Figure 5** and **Supplementary Table 4**. *K. pneumoniae* isolates were highly sensitive to meropenem (99.16%) and colistin (99.58%). However, *K. pneumoniae* was completely resistant to ampicillin and highly resistant to sulfisoxazole (94.56%); the resistance rate to cephalothin, streptomycin, gentamicin, tetracycline, doxycycline, florfenicol, and sulfamethoxazole/trimethoprim was between 20.08 and 47.28%. It is worth noting that 43.93% (105/239) of the isolates were MDR strains and neither XDR nor PDR strains were found in this study (**Figure 6A**). The resistance rate of *K. pneumoniae* to ceftriaxone, streptomycin, and gentamicin in mastitis milk (nipple milk of SCM and CM cows) is significantly higher than that in healthy milk ($p < 0.05$) (**Figure 6B**). Ninety-three resistance profiles have been identified and the dominant ones were AMP-SOX (57/239, 23.85%), followed by AMP-CEP-SOX (26/239, 10.88%), AMP-STR-GEN-SOX (11/239, 4.66%), and AMP-CEP-STR-GEN-SOX (11/239, 4.66%) (**Supplementary Figure 1**).

Antimicrobial Resistance Genes and Quinolone Resistance-Determining Region Mutation

Thirty of 68 AMR genes were identified by PCR (**Figure 7**). The detection rate of bla_{TEM}, bla_{SHV}, *strA*, *strB*, *aadA1*, and *aac(6′)-Ib-cr* was more than 50% and others were between 0.42 and 38.08%. Eleven *CTX-M*-producing *K. pneumoniae* were found. Among the isolates resistant to β-lactam or aminoglycoside antibiotics, more than 90% of isolates have detected at least

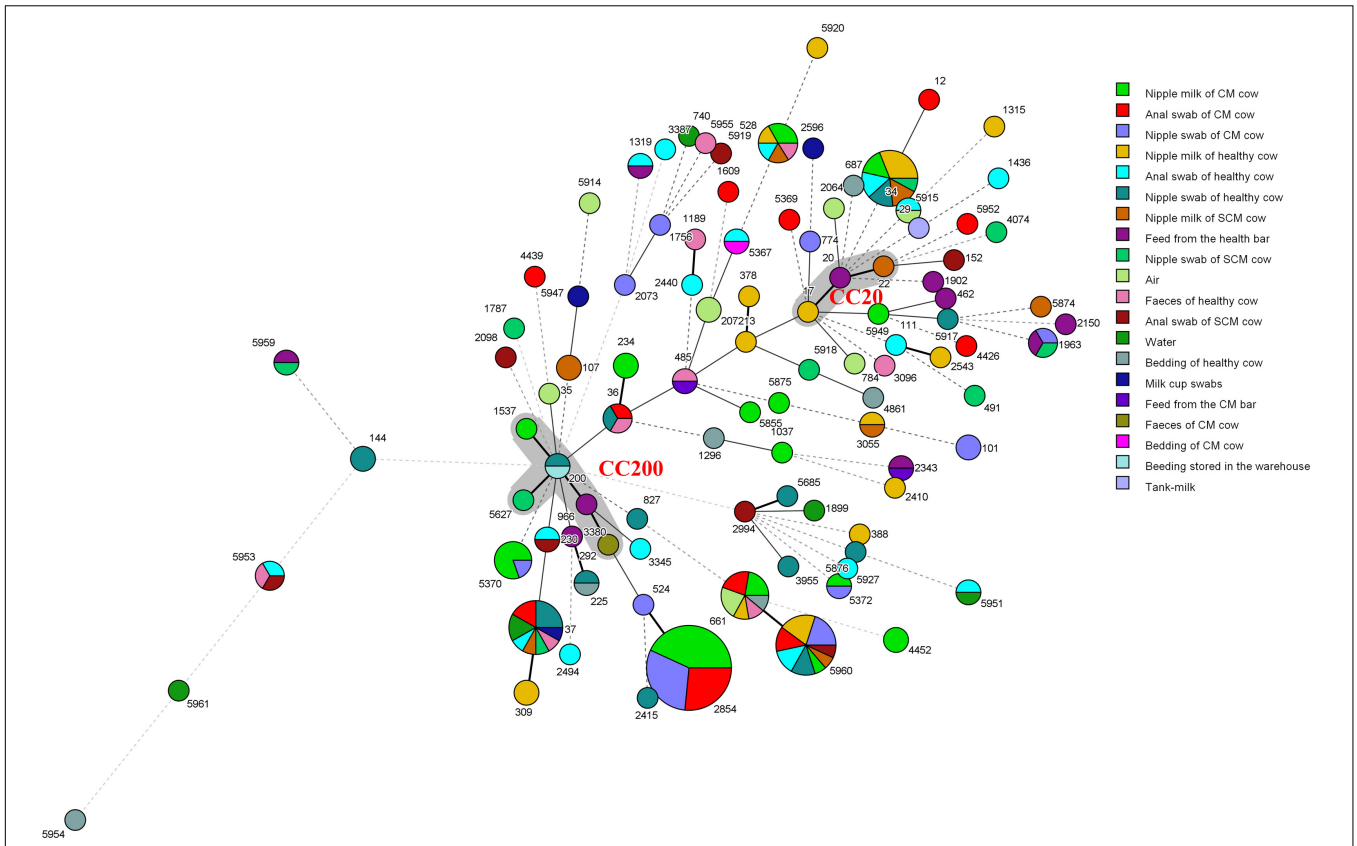

FIGURE 3 | Minimum spanning tree (MST) of 239 *K. pneumoniae*. The gray area represents a clonal complex and the number represents ST. Each ST is grouped by different sample types. One allele difference is represented by a bold line, two allele differences are represented by a straight line, and three or more allele differences are represented by a dotted line.

one β-lactam resistance gene or aminoglycoside resistance gene; among the isolates resistant to TET, DOX, FFC, CIP, and SXT, more than 60% of isolates have detected at least one resistance gene corresponding to the resistance phenotype (**Supplementary Figure 1** and **Supplementary Table 5**). The correlation analysis performed between different phenotypic antibiotic resistance and the antibiotic resistance genes is shown in **Figure 8**. Among β-lactam antibiotic phenotypes and antibiotic resistance genes, the obtained results revealed low positive correlations between EFT, CRO, and $bla_{CTX-M-15}$ (r = 0.492–0.474) and AMC and bla_{OXA-1} (r = 0.590). Moreover, a low positive correlation was observed between CEP and EFT (r = 0.405). Among aminoglycosides, a high positive correlation was found between GEN and *aacC2* (r = 0.823); moderate positive correlation was observed between STR and GEN (r = 0.606), STR and *strB* (r = 0.501), and KAN and *aphA1* (r = 0.568); low positive correlation was observed between STR and *aacC2* (r = 0.488) and KAN and *aacA4* (r = 0.428). Among tetracyclines, moderate positive correlations were observed between TET, DOX, and *tetA* (r = 0.580–0.686). Among sulfonamides and amphenicols, low positive correlation was observed between *sul1* and *sul2* (r = 0.482), *sul2* and *sul3* (r = 0.303), FFC, *floR*, and *cmlA* (r = 0.316–0.457). No significant association was found between fluoroquinolone resistance phenotype and PMQR genes. DNA sequencing of QRDR of *gyrA*, *gyrB*, *parC*, and *parE* demonstrated that no amino acid changes were found in these genes whether *K. pneumoniae* isolates were resistant, moderately resistant, or sensitive to ciprofloxacin and silent mutations found are shown in **Supplementary Table 6**.

DISCUSSION

Klebsiella pneumoniae is an important opportunistic pathogen that has attracted global attention due to the difficult clinical cure of mastitis caused by *K. pneumoniae*, the low effectiveness of antibiotic treatment, and the lack of advancement in preventive measures (Yang et al., 2019b). The prevalence and transmission of multidrug-resistant and hypervirulent *K. pneumoniae* have brought unexpected harm and loss to humans and animals. At present, most of the study on *K. pneumoniae* is in the field of human medicine, while the study data in the field of mastitis is very scarce. This study showed the prevalence and molecular characteristics of virulence, resistance, capsular serotyping, and genotype of *K. pneumoniae* in five cattle farms in Hubei, which will help to track the infection trend of *K. pneumoniae* and emphasized the importance of environmental sanitation

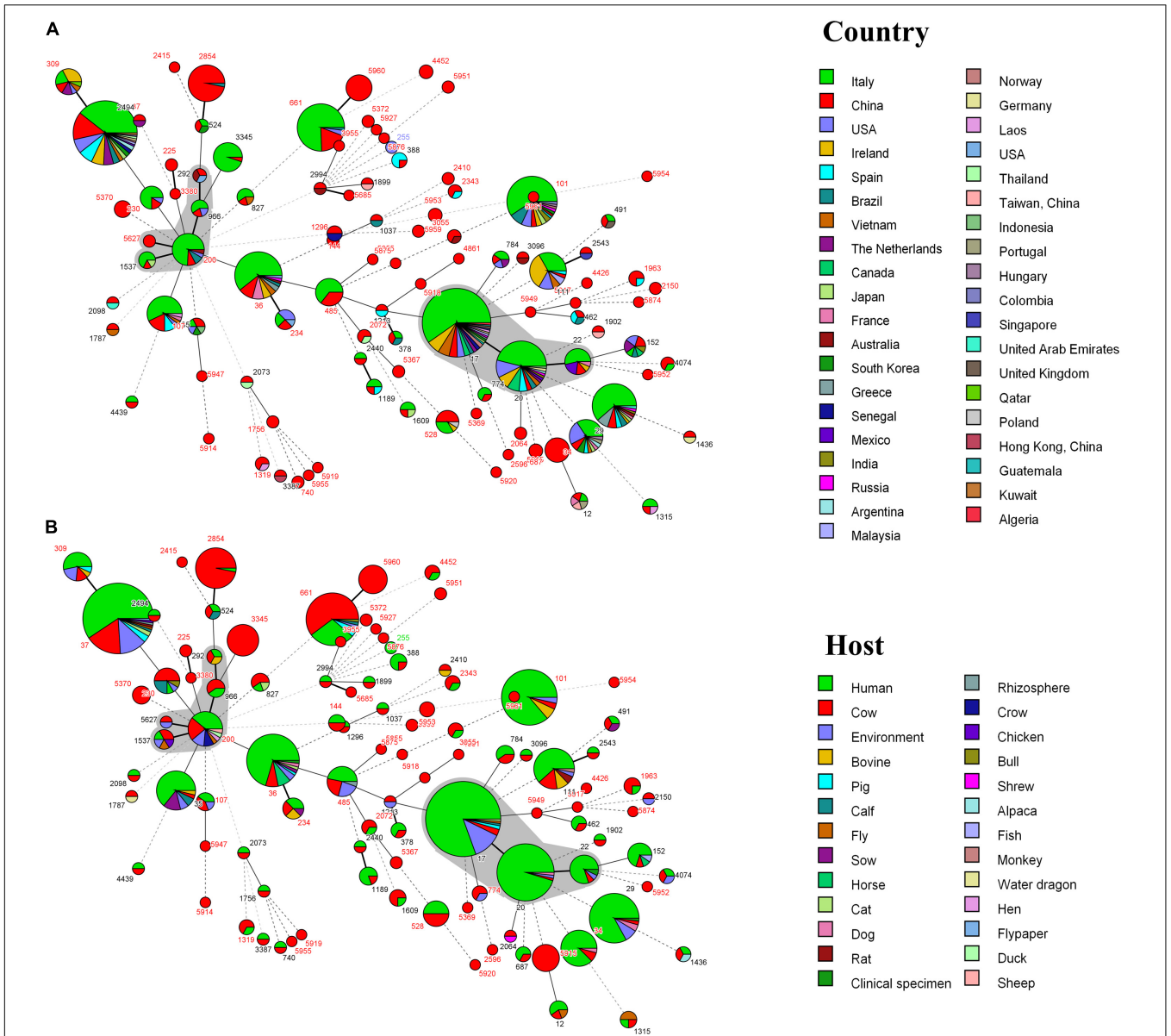

FIGURE 4 | (A) MST of 239 *K. pneumoniae* in this study and 722 isolates with the same 104 ST from the PubMLST database. Each ST is grouped by different countries. **(B)** MST of 239 *K. pneumoniae* in this study and 720 isolates with the same 104 ST from the PubMLST database. Each ST is grouped by different hosts. The gray area represents a clonal complex. One allele difference is represented by a bold line, two allele differences are represented by a straight line, and three or more allele differences are represented by a dotted line.

in the breeding process. The prevalence of *K. pneumoniae* varied among the five farms involved in this study, which may be related to stocking density and farm environment. The *K. pneumoniae* isolation rate in milk from CM cows was 25.36% (35/138), which was similar to the study by Yang et al. (2021). However, we found that there was no significant difference in the *K. pneumoniae* prevalence of nipple milk, skin swabs, and anal swabs between healthy and mastitis cows, which was different from the previous study (Koovapra et al., 2016). This may be due to that most of the CM cows are in the period of treatment or recovery. The results also confirmed that *K. pneumoniae*, as an environmental pathogen, is also ubiquitous in the environment (Wareth and Neubauer, 2021).

At present, the hvKP was reported to be most common in *K. pneumoniae* with ST11, serotype K1 or K2 (Wang et al., 2021). No strains of these serotypes and sequence types were found in this study, but K54 (*wzi*66, *wzi*115, $n = 2$) and K57 (*wzi*57, $n = 1$) were found, which may also be related to high virulence (Zhong et al., 2020). The genetic diversity of *K. pneumoniae* is very rich and its serotypes and sequence types are not in a one-to-one correspondence, which is similar to previous studies (Benulič et al., 2020; Lepuschitz et al., 2020). Many studies

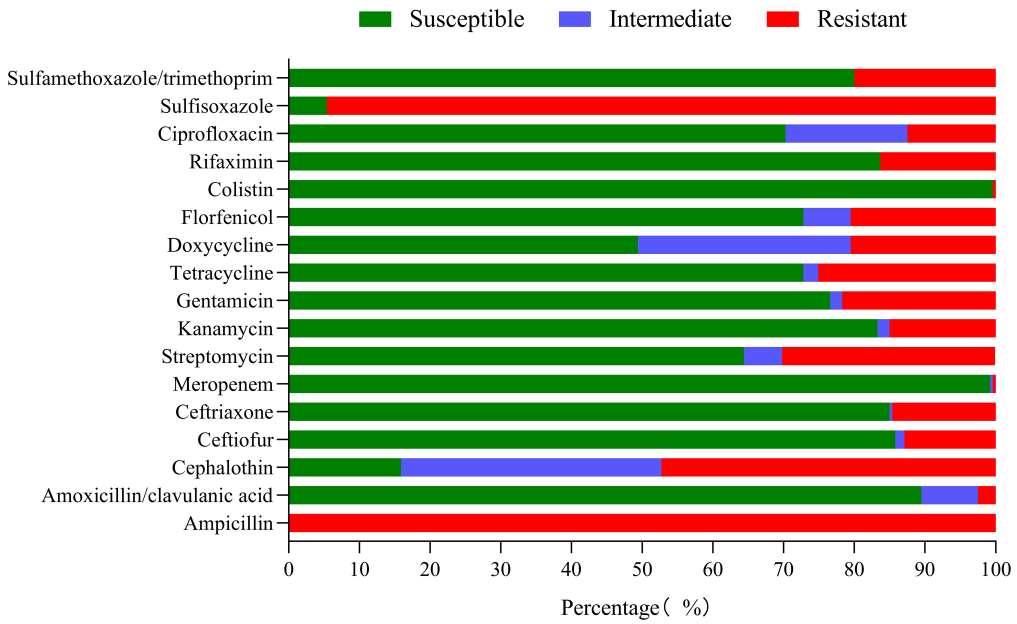

FIGURE 5 | Resistance rate of *K. pneumoniae* to 17 antimicrobials.

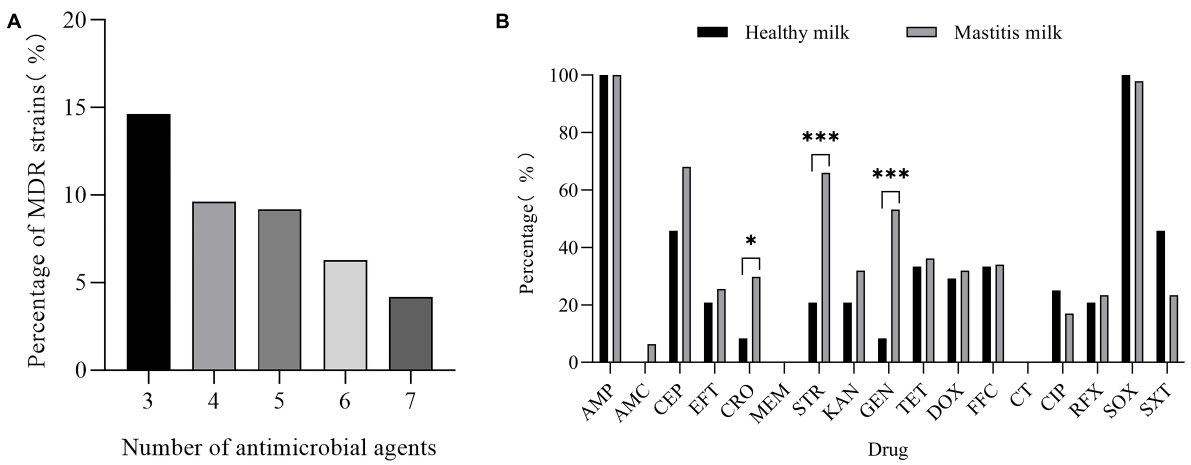

FIGURE 6 | (A) Proportion of multidrug-resistant (MDR) *K. pneumoniae* resistant to different amounts of antibiotics. **(B)** Resistance rate of healthy milk and mastitis milk [milk from CM and subclinical mastitis (SCM) cows] to 17 antimicrobials. "*" indicates $p < 0.05$ and "*" indicates $p < 0.001$.

confirmed that the occurrence of *K. pneumoniae* is related to the environment of farms and *K. pneumoniae* can be spread through contaminated feed, feces, drinking water, and so on (Wareth and Neubauer, 2021; Zhao et al., 2021). Similar results were also observed in our MST analysis. *K. pneumoniae* isolated from milk, nipple swab, feed, and feces is closely related and 58 STs exist in different countries and 66 STs have been found in different hosts. Strains of these genotypes have potential harm of cross-species and regional transmission, which cannot be ignored. Enhancing the sanitation of the breeding environment and disinfection of the cattle stalls may be an effective ways to prevent mastitis.

The pathogenicity of *K. pneumoniae* is inseparable from the role of virulence factors. In this study, *peg-344*, *iroB*, $_{p}$*rmpA*, and $_{p}$*rmpA2* related to high virulence were not found, but 3 strains were detected carrying the *iucA*; further study is needed to verify the virulence, such as animal experiments. In addition, PCR detection results showed that fimbriae-related genes (*fimH*, *mrkD*), iron uptake system (*iutA*, *iroN*, and *entB*), urease-related genes (*ureA*), and the lipopolysaccharide-related genes (*uge*, *wabG*) were widely distributed in the isolates, which were similar to the previous results (Zhang et al., 2018; Yang et al., 2019a). These results can preliminarily elucidate the pathogenic mechanism of the *K. pneumoniae* that may include:

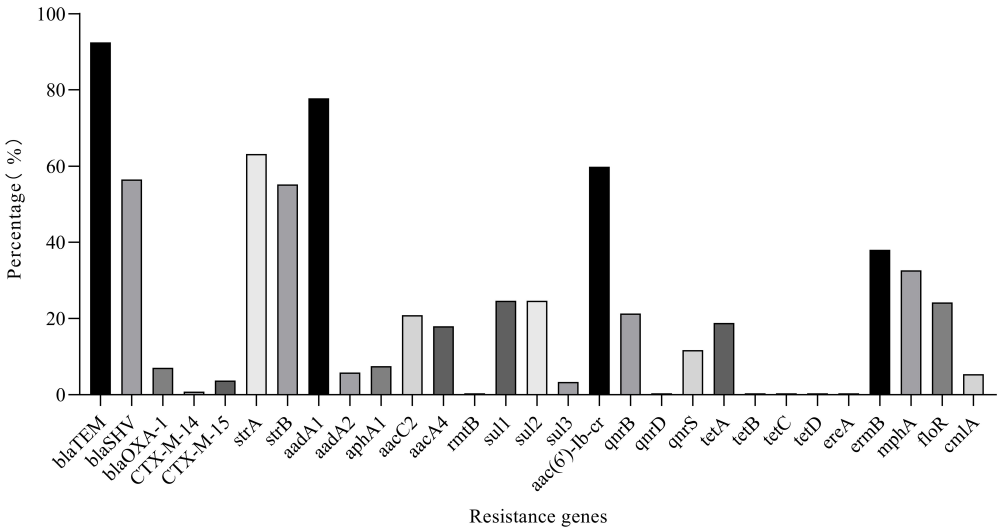

FIGURE 7 | Detection rate of resistance genes.

synthesizing fimbriae to adhere to the surface of host cells or form biofilms for virulence, secreting siderophores to absorb iron in the host for metabolism to enhance virulence, evading serum killing of phagocytes and suppress host immunity by utilizing capsular polysaccharides, and lipopolysaccharide aggregates into complexes on the surface of the *K. pneumoniae* so that the bacteria can escape or resist the killing of the host's innate immunity (Schroll et al., 2010; Li et al., 2014; Liu et al., 2019).

Antibiotic resistance has always been a key and difficult issue of global concern. The results of antibiotic susceptibility test showed that *K. pneumoniae* isolates were highly sensitive to meropenem and colistin. It is mainly because carbapenems are strictly forbidden to be used in animals; the Chinese government has officially banned polymyxin as an animal growth promoter on 30 April 2017 (Wang Y. et al., 2020; Yang et al., 2021). However, *K. pneumoniae* has different degrees of resistance to other antibiotics and has a complex antimicrobial spectrum. As previously reported, *K. pneumoniae* shows intrinsic resistance to ampicillin (Fu et al., 2007). The rate of MDR (43.93%) and resistance to SOX (94.56%) are higher than that of the study by Zhang et al. (2020). β-lactam and aminoglycoside antibiotics have long been used to treat mastitis in five farms, which may explain why the resistance rate of isolates in mastitis milk to CEP, STR, and GEN was significantly higher than that of healthy milk. The development of antibiotic resistance is inseparable from the existence and spread of resistance genes. Our results show that bla_{TEM} and bla_{SHV} are present in mostly *K. pneumoniae* and $bla_{CTX-M-15}$ is the main *CTX-M* gene detected, which is similar to the results of previous studies (Timofte et al., 2014; Carvalho et al., 2021). These extended-spectrum β-lactamase (ESBL) resistance genes can lead to resistance by hydrolysis of penicillins and cephalosporins (Algammal et al., 2020a). Correlation analysis showed that the resistance of *K. pneumoniae* to AMC, EFT, and CRO may be caused by hydrolysis mediated by bla_{OXA-1} and $bla_{CTX-M-15}$. A variety of aminoglycoside-modifying enzyme genes (*aadA1*, *aacc2*, *aacA4*, and *aphA1*) were detected in the isolates. These enzymes can modify the active antimicrobial drugs that enter the cell and make them inactive (Belaynehe et al., 2017). The resistance of STR is mainly mediated by *strA* and *strB* and correlation analysis shows that the resistance of the STR of *K. pneumoniae* in this study may be more related to *strB*. It has been reported that *tetA* and *floR*/*cmlA* mediate the efflux of tetracycline and florfenicol, respectively, which can pump the drug out of the cell and reduce the intracellular drug concentration to generate resistance (Acosta-Pérez et al., 2015; Wang et al., 2018; Graesbøll et al., 2019). Notably, *tetA* and *floR* are the most frequently observed AMR genes in *K. pneumoniae* resistant to tetracycline and amide alcohols, respectively, which are consistent with the study by Nobrega et al. (2021). Although the three dihydrofolate synthase genes (*sul1*, *sul2*, and *sul3*) were detected to varying degrees, no significant correlation was found between them and SOX and SXT. Other resistance mechanisms, such as the expression of dihydrofolate reductase gene (*dfr*) and permeability barriers, are the causes of sulfonamide resistance (Skold, 2001). *K. pneumoniae* can be against quinolones through several mechanisms, including mutations in quinolone resistance-determining regions, plasmid-mediated quinolone resistance (PMQR), increased activity of efflux pumps, and decreased cellular permeability. Among them, mutation of QRDR is the main mechanism mediating quinolone resistance, especially *gyrA* and *parC* (Kareem et al., 2021). However, no amino acid changes were found in this study, which is different from the study by Saiful Anuar et al. (2013) and Fu et al. (2008), but similar to the study by Kim et al. (2018). Although the PMQR gene was detected to varying degrees in the isolates, its correlation with the ciprofloxacin resistance phenotype was not significant. We speculate that the activity of efflux pumps and the reduction of cell permeability may be the main reasons for mediating *K. pneumoniae* resistance to ciprofloxacin, but further verification is needed.

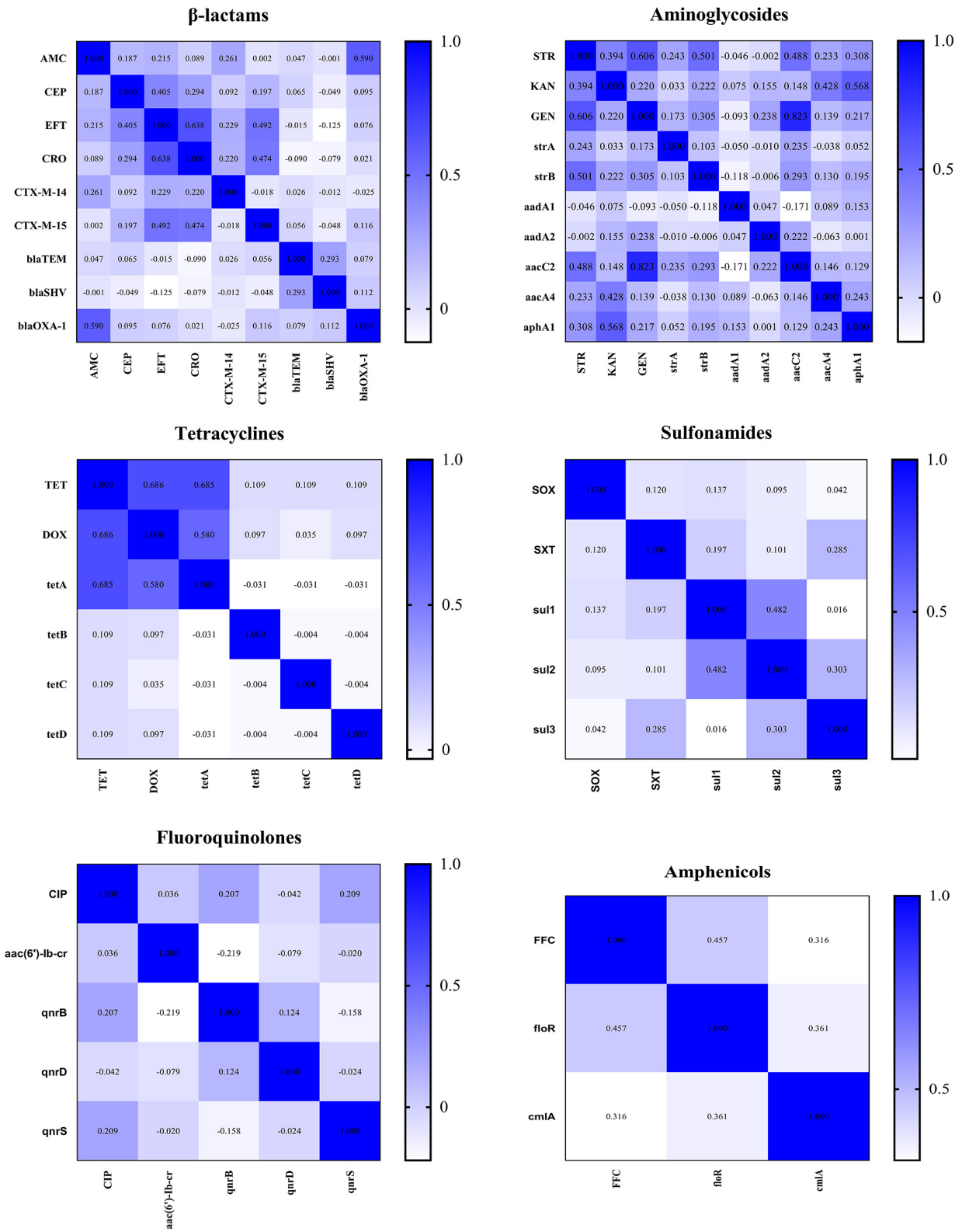

FIGURE 8 | Correlations between different classes of antimicrobials and resistance genes. The intensity of the color indicates the numerical value of the correlation coefficient (r).

CONCLUSION

The occurrence of mastitis may be closely related to environmental hygiene. Virulence factors such as fimbriae, iron uptake, and lipopolysaccharide may play important roles in the pathogenesis of *K. pneumoniae*. The MDR of *K. pneumoniae* is a serious public health problem that still needs to be paid attention to, especially, the high resistance caused by the frequent use of β-lactams, aminoglycosides, and sulfonamides. The resistance of some antibiotics is attributed to the existence of resistance genes and other mechanisms such as efflux pumps and decreased permeability may be involved in the resistance of *K. pneumoniae* to sulfonamides and fluoroquinolones. Multiple sequence types of *K. pneumoniae* have the risk of cross-species and regional transmission. It is recommended to strengthen and regularly conduct surveillance, antibiotic resistance investigation, and traceability study on this strain. Further studies will be required to clarify whether the resistance and virulence characteristics of these isolates are affected by some movable genetic elements (such as plasmids) and whether they pose a risk of transmission.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YW conceived the study and directed the study. XW, JL, JF, YF, RG, SH, and MZ performed the experiments. GW performed the somatic cell count of milk. XW performed the data analysis and wrote the manuscript. YW, HH, GC, and MS revised the manuscript. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.858799/full#supplementary-material

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Prevalence, Characteristics, and Clonal Distribution of *Escherichia coli* Carrying Mobilized Colistin Resistance Gene *mcr-1.1* in Swine Farms and Their Differences According to Swine Production Stages

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Global spread of *Escherichia coli* strains carrying the mobilized colistin resistance gene *mcr-1.1* (MCR1-EC) poses serious threats to public health. Colistin has been generally prescribed for swine colibacillosis, having made swine farms as major reservoirs of MCR1-EC. The present study aimed to understand characteristic differences of MCR1-EC, including prevalence, antimicrobial resistance, and virulence, according to swine production stages. In addition, genetic relatedness was evaluated between MCR1-EC isolated from this study as well as pig-, human-, and chicken-derived strains published in the National Center for Biotechnology Information (NCBI), based on the multi-locus sequence types (MLSTs) and whole-genome sequences (WGS). Individual fecal samples ($n = 331$) were collected from asymptomatic weaning-piglets, growers, finishers, and sows from 10 farrow-to-finishing farms in South Korea between 2017 and 2019. The weighted prevalence of MCR1-EC was 11.6% (95% CI: 8.9%–15.0%, 55/331), with the highest prevalence at weaning stage. The 96.2% of MCR1-EC showed multi-drug resistance. Notably, weaning stage-derived MCR1-EC showed higher resistance rates (e.g., against extended-spectrum β-lactams or quinolones) than those from other stages. MCR1-EC with virulence advantages (e.g., intestinal/extraintestinal pathogenic *E. coli* or robust biofilm formation) were identified from all pig stages, accounting for nearly half of the total strains. WGS-based in-depth characterization showed that intestinal pathogenic MCR1-EC harbored multi-drug resistance and multiple virulence factors, which were highly shared between strains isolated from pigs of different stages. The clonal distribution of MCR1-EC was shared within swine farms but rarely across farms. The major clonal type of MCR1-EC from swine farms and NCBI database was ST10-A. Core genomes of MCR1-EC isolated from individuals within closed environments (same farms or human hospitals) were highly shared

(genetic distance < 0.01), suggesting a high probability of clonal expansion of MCR1-EC within closed environments such as livestock husbandry. To the best of our knowledge, this is the first study to analyze the differences in the characteristics and clonal distribution of MCR1-EC according to production stages in swine farms, an important reservoir of MCR1-EC. Our results highlight the need to establish MCR1-EC control plans in swine farms based on an in-depth understanding of MCR1-EC characteristics according to swine production stages, focusing especially on the weaning stages.

Keywords: colistin, *mcr-1.1*, intestinal pathogenic *E. coli*, extra-intestinal pathogenic *E. coli*, extended-spectrum β-lactamase, swine production stages

INTRODUCTION

Colistin is regarded as a last resort for the treatment of multi-drug resistant (MDR) bacterial infections in humans and has been classified as a critically important antimicrobial agent by the World Health Organization (WHO, 2018). Before 2016, colistin resistance was mainly considered to be associated with mutational and regulatory changes in chromosomal genes, including *pmrAB* and *phoPQ* (Liu et al., 2016). The mobilized colistin resistance gene *mcr-1* was first described in a plasmid carried by *Escherichia coli* strains in 2016 (Liu et al., 2016), and has since been found in more than 50 countries across six continents (Wang et al., 2018b), highlighting the global spread of colistin resistance *via mcr-1*.

Swine colibacillosis is a major disease in pigs that causes huge economic losses for the global swine industry (Luppi, 2017). Colistin has been generally used for the treatment of swine colibacillosis, leading to an increased prevalence of *E. coli* strains carrying *mcr-1* (MCR1-EC) in swine farms (Malhotra-Kumar et al., 2016; Tong et al., 2018; Liu et al., 2020; Nakano et al., 2021). In pig production systems, pigs at different stages of growth, referred to as weaning piglets, growers, finishers, and pregnant pigs, are usually raised in separate barns (Kyriazakis, 2006). However, as pigs age and transition to the next growth stage and next stage barn, bacterial transmission can occur between animals at different swine production stages within farms, which has been reported to be a significant risk factor for the high prevalence of MDR bacteria in swine farms (Fromm et al., 2014; Schmithausen et al., 2015). Since *mcr-1* is mainly mediated by plasmids, the important role of genetic transferability of *mcr-1* in the spread of MCR1-EC has been continuously highlighted in various studies (Garcia et al., 2018; Wang et al., 2018b; Wu et al., 2018b; Soliman et al., 2021). However, genetic transfer essentially presupposes the transfer of strains and bacteria-to-bacteria interactions under favorable conditions (e.g., physical distance between strains, nutrition, and environmental conditions, etc.; Virolle et al., 2020), which suggests that bacterial transmission also provides a crucial basis for the spread of MCR1-EC. Understanding the genetic characteristics and distribution of MCR1-EC considering swine production stages, which is an important reservoir of MCR1-EC, could be a cornerstone to establish strategies for the control of colistin resistance in the swine industry. However, despite its importance, the characteristics and distribution of MCR1-EC based on different swine production stages within farms have rarely been studied.

Given that colistin has been considered a recommended treatment option for swine colibacillosis and that intestinal pathogenic *E. coli* (InPEC) comprises major causative pathogens of swine colibacillosis (Malhotra-Kumar et al., 2016; Tong et al., 2018; Liu et al., 2020; Nakano et al., 2021), the presence of intestinal pathogenic MCR1-EC in pig husbandry represents a severe challenge for the swine industry. Colistin administration during the treatment of swine colibacillosis caused by intestinal pathogenic MCR1-EC can lead to disease treatment failure, as well as complications, resulting in serious economic losses for pig farms (Garcia et al., 2018). To establish suitable strategies to control intestinal pathogenic MCR1-EC in swine farms, an in-depth characterization of intestinal pathogenic MCR1-EC should be performed, and whole-genome sequence (WGS)-based analysis might provide valuable insights.

The present study aimed to understand the characteristic differences and clonal distribution of MCR1-EC in swine farms according to production stages. For this, first, the prevalence, antimicrobial resistance, and genetic and phenotypic virulence characteristics of MCR1-EC isolated from swine farms were investigated, and differences according to swine production stages were analyzed. Second, we performed WGS for all intestinal pathogenic MCR1-EC isolated in this study and conducted an in-depth genetic characterization. Finally, to understand spread characteristics of MCR1-EC, genetic relatedness analysis based on the clone types and WGS were conducted for MCR1-EC strains isolated in this study, as well as MCR1-EC isolated from various sources published in the National Center for Biotechnology Information (NCBI) GenBank database.

MATERIALS AND METHODS

Sample Collection

We collected individual swine fecal samples from 10 "farrow-to-finishing" swine farms located in the five provinces with the highest number of pig farms in South Korea, specifically Gyeonggi-do, Chungcheong-nam-do, Jeolla-nam-do, Jeolla-buk-do, and Gyeongsang-buk-do (**Figures 1, 2**; **Supplementary Table 1**). The number of pig farms by province in South Korea was obtained from the 2017 demographic report of the Korean Statistical Information Service of Statistics Korea (Korea, 2017). Each swine

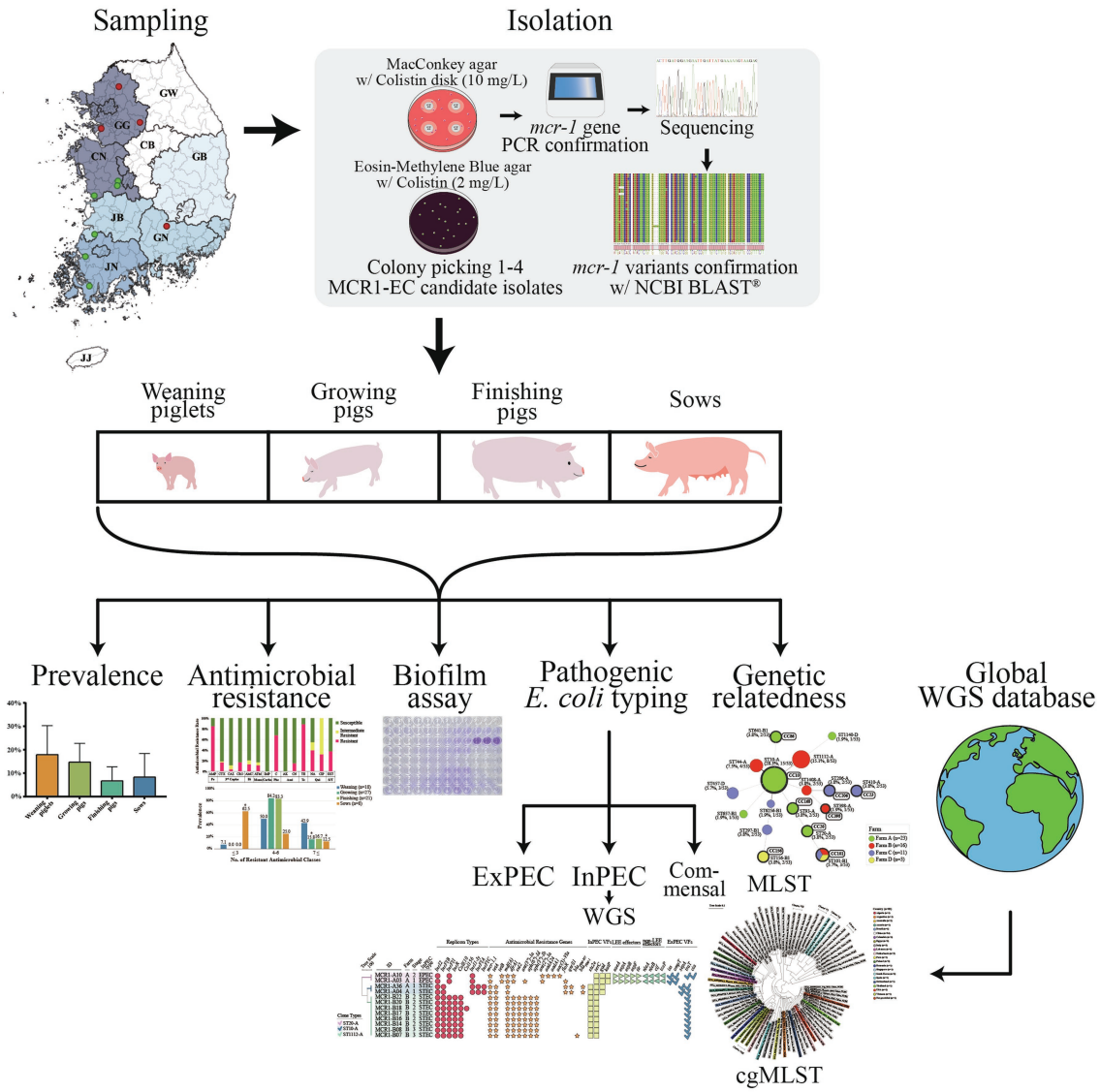

FIGURE 1 | Flow chart of the present study design.

farm was visited once between May 2017 and July 2019, and fecal samples were randomly collected from 26 to 34 asymptomatic pigs for each farm, including 5–6 weaning piglets (4–7-week-old), 9–11 growing pigs (7–14-week-old), 8–11 finishing pigs (14–24-week-old), and 3–6 sows, and immediately transported to the lab. In total, 331 fecal samples (59 from weaners, 108 from growers, 107 from finishers, and 57 from pregnant sows) were included.

Isolation of MCR1-EC

The isolation of MCR1-EC was conducted following previously described protocols for the isolation of antimicrobial resistant *E. coli*, with slight modifications (Bartoloni et al., 1998, 2006; Wedley et al., 2011, 2017). Approximately, 1 g of each sample was resuspended in 9 ml of *Escherichia coli* broth (BD Biosciences, New Jersey, United States) and incubated overnight at 37°C. Thereafter, 100 µl of culture suspension was spread on MacConkey agar (BD Biosciences), and a colistin disk (10 µg/ml, Oxoid, Cheshire, United Kingdom) was placed on the plate. After overnight incubation at 37°C, 1–4 colistin-resistant *E. coli* candidate isolates grown inside the colistin-resistant zone (≤10 mm) were selected and streaked on Eosin Methylene Blue agar (BD Biosciences) containing 2 mg/L colistin (Sigma Aldrich, Massachusetts, United States) for further confirmation. The diameter (≤10 mm) of the candidate colistin-resistant zone was set with reference to the disk diffusion quality control range of *E. coli* reference strain ATCC 25922 described in the Clinical Laboratory Standard Institute (CLSI) guidelines M100S 31th Edition (2021). Then, the presence of *mcr-1* and *mcr-1*-encoded replicon types was determined *via* PCR and sequencing as previously described (Wu et al., 2018b). The sequenced PCR amplicons were compared with the reference sequences from

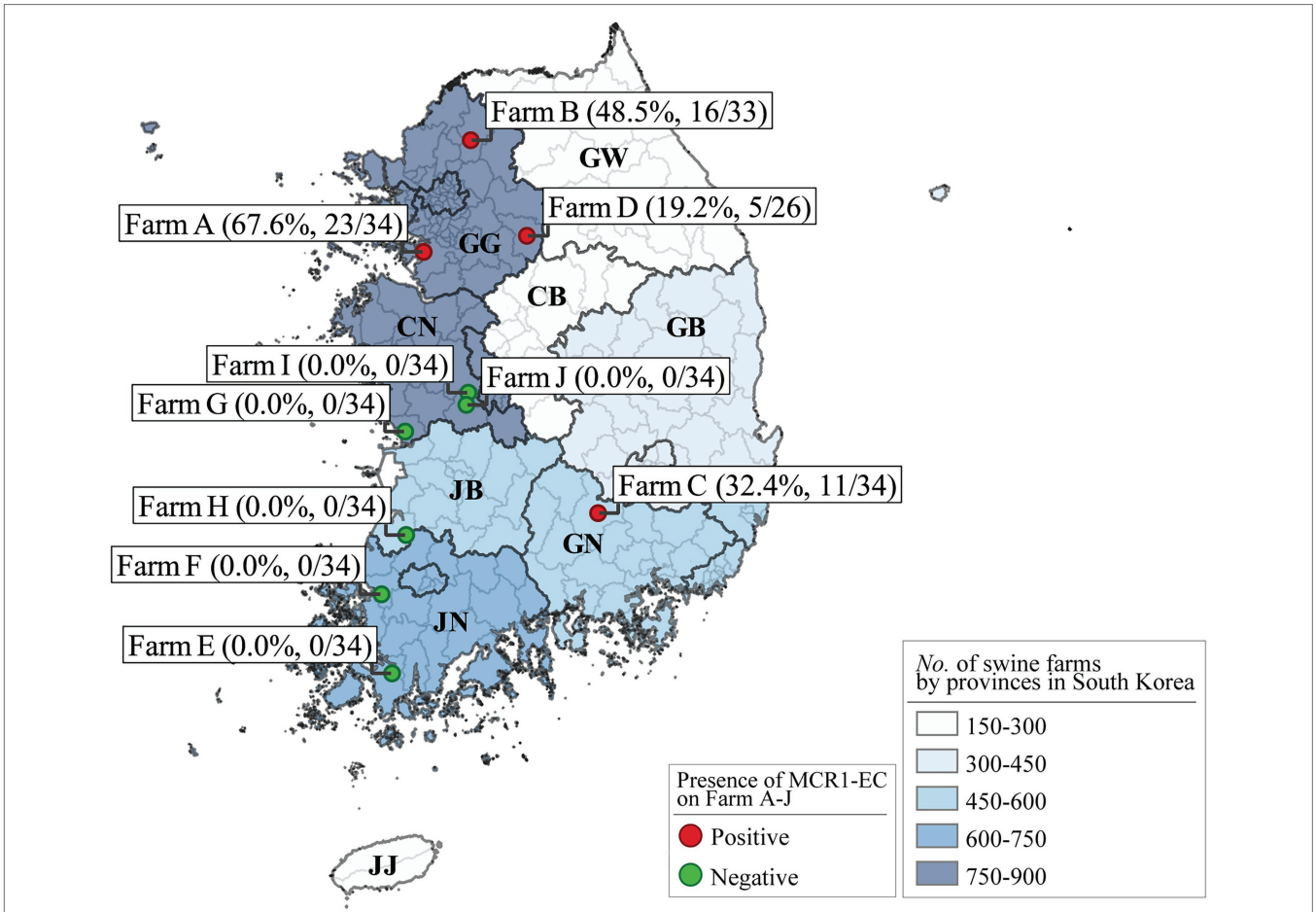

FIGURE 2 | *Escherichia coli* strains carrying *mcr-1* (MCR1-EC) prevalence in Farms A–J and number of swine farms by South Korean province. The numbers in parentheses show the unweighted prevalence of MCR1-EC and the number of MCR1-EC-positive samples versus that of total samples for each farm. The number of pig farms by province in South Korea was obtained from the 2017 demographic report of the Korean Statistical Information Service of Statistics Korea. Visualization was conducted using the QGIS geographic information system program (v3.16.15). MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; GG, Gyeonggi-do; CN, Chungcheong-nam-do; JB, Jeolla-buk-do; JN, Jeolla-nam-do; GN, Gyeongsang-nam-do; GB, Gyeongsang-buk-do; CB, Chungcheong-buk-do; and GW, Gangwon-do; JJ, Jeju-do.

the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the replicon types, as well as *mcr-1* variants from *mcr-1.1* to *mcr-1.32*. The PCR was performed using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Massachusetts, United States), and sequencing was performed using an ABI PRISM 3730XL DNA analyzer (Thermo Fisher Scientific). *Escherichia coli* isolates carrying *mcr-1* were confirmed as MCR1-EC, and one MCR1-EC strain per sample was randomly selected if more than one isolates were identified from a sample. Primer sequences and reaction conditions are summarized in **Supplementary Table 2**.

Antimicrobial Resistance

Antimicrobial Susceptibility Assay

Minimum inhibitory concentration (MIC) tests were conducted to evaluate colistin resistance using the Trekstar Sensititre KNIHCOL custom panel (colistin test range: 0.25–128 μg/ml, Trek Diagnostic Systems, Ohio, United States) according to the manufacturer's instructions. Each isolate was tested in duplicate for the MIC of colistin. Kirby-Bauer disk diffusion susceptibility tests (KBTs) were conducted for 11 antimicrobial classes comprising 14 antimicrobial agents using antimicrobial disks from Oxoid (Cheshire, United Kingdom) as follows: ampicillin (10 μg/ml), cefotaxime (30 μg/ml), ceftazidime (30 μg/ml), ceftriaxone (30 μg/ml), amoxicillin/clavulanate (20/10 μg/ml), aztreonam (30 μg/ml), imipenem (10 μg/ml), chloramphenicol (30 μg/ml), amikacin (30 μg/ml), gentamycin (10 μg/ml), tetracycline (30 μg/ml), nalidixic acid (30 μg/ml), ciprofloxacin (5 μg/ml), and sulfamethoxazole/trimethoprim (1.25/23.75 μg/ml). The MIC tests and KBs results were interpreted according to the CLSI guidelines M100S 31th Edition (2021), and the *E. coli* reference strain ATCC 25922 was used for quality control. When the isolate was resistant to at least one antimicrobial agent belonging to the antimicrobial class,

we determined that this isolate was resistant to this antimicrobial class. Then, we calculated the average number of antimicrobial classes to which MCR1-EC strains were resistant. Extended-spectrum β-lactamase (ESBL) phenotypes were determined *via* a standard double-disk test according to CLSI guidelines using four antimicrobial disks from BD Bioscience (New Jersey, United States) as follows: cefotaxime (30 μg/ml), ceftazidime (30 μg/ml), cefotaxime/clavulanate (30/10 μg/ml), and ceftazidime/clavulanate (30/10 μg/ml).

Antimicrobial Resistance Genes and Replicon Typing

The presence of genes conferring resistance to β-lactams, chloramphenicol, aminoglycoside, quinolones, and sulfonamide/trimethoprim was determined by PCR. The ESBL genotypes were determined by PCR and sequencing as previously described (Jouini et al., 2007). PCR-based replicon typing was conducted as previously described (Carattoli et al., 2005; Johnson et al., 2012; Lv et al., 2013). Primer sequences and reaction conditions are summarized in **Supplementary Table 2**.

Classification of Pathogenic *Escherichia coli*

To analyze the genotypic virulence characteristics of MCR1-EC, we investigated the presence of virulence factors associated with InPEC, extra-intestinal pathogenic *E. coli* (ExPEC), and uro-pathogenic *E. coli* (UPEC). The classification of InPEC was conducted by PCR for the following five InPEC types: shiga toxin-producing *E. coli* (STEC) carrying *stx1* or *stx2*, enteropathogenic *E. coli* (EPEC) carrying *eaeA* or *bfpB*, enteroaggregative *E. coli* (EAEC) carrying *aggR*, enteroinvasive *E. coli* (EIEC) carrying *ipaH*, and enterotoxigenic *E. coli* (ETEC) carrying *lt*, *sta*, or *stb*. The carriage of 21 ExPEC-associated virulence factors associated with adhesion (*csgA*, *fimH*, *sfa/focDE*, *afa/draBC*, *papC*, *papAH*, *yfcV*, and *iha*), toxins (*hlyF*, *astA*, *pic*, *vat*, and *aat*), protectin/serum resistance (*traT*, *ompT*, *iss*, and *kpsMTII*), and siderophores (*fyuA*, *iroNE.coli*, *iutA*, and *chuA*) were investigated using PCR. The classification of ExPEC was conducted following the previously described criteria, specifically positive for ≥2 of five key markers as follows: *papA* and/or *papC*, *sfa/focDE*, *afa/draBC*, *iutA*, and *kpsMTII* (Johnson et al., 2003). The classification of UPEC was conducted following previously described criteria, specifically positive for ≥3 of four key markers as follows: *vat*, *fyuA*, *chuA*, and *yfcV* (Spurbeck et al., 2012). Finally, since all pigs included in this study were healthy, without showing any disease symptoms, *E. coli* isolates that not classified as InPEC, ExPEC, or UPEC were then classified as commensal *E. coli* strains. Primer sequences and reaction conditions are summarized in **Supplementary Table 3**.

Phenotypic Assay

Conjugation Assay

Conjugation assays were conducted to evaluate the horizontal genetic transferability of *mcr-1* with the *E. coli* J53-Azi^R strain as the recipient and 53 MCR1-EC strains as the donors. The conjugation assay was conducted following a previously described protocol with modifications (Kim et al., 2019). Briefly, overnight cultures of donor and recipient strains in Luria-Bertani broth were mixed at a ratio of 1:1, followed by incubation at 37°C for 18 h with constant shaking. Then, 100 μl of the mixture of donor and recipient cells were spread on LB agars supplemented with 2 mg/L colistin (Sigma Aldrich, Massachusetts, United States) and 100 mg/L sodium azide (Sigma Aldrich), followed by overnight incubation at 37°C. The presence of *mcr-1* in conjugants was confirmed *via* PCR.

Biofilm Assay

To analyze the phenotypic virulence characteristics of MCR1-EC, biofilm production assays were performed following a previously described protocol with modifications (Nandanwar et al., 2014). Briefly, overnight M9 minimal medium [200 ml/L of M9 media (5X, Sigma-Aldrich), 0.4 g/L of glucose (Sigma-Aldrich), 2 ml/L of $MgSO_4$ solution (1 M, Sigma-Aldrich), and 100 μl/L of $CaCl_2$ solution (1 M, Sigma-Aldrich)] culture was diluted in fresh M9 minimal medium to a McFarland scale of 0.5. Approximately, 100 μl of this dilution was added into a 96-well microtiter plate and incubated for 24 h at 28°C under stationary conditions. Each bacterial suspension was inoculated into three wells of a microtiter plate. Growth optical densities (ODs) were measured at $\lambda = 595$ nm with a multiplate reader (Bio-Rad, California, United States). The wells were then washed once with 200 μl of phosphate-buffered saline, dried for 20 min, and stained with 100 μl of 1% crystal violet for 1 h. This was followed by gentle washing with 200 μl of distilled water four times and air-drying for 1 h. The absorbed dye was solubilized in 100 μl of absolute ethanol, and ODs were read at 595 nm. The extent of biofilm formation was calculated using the following formula: SBF = (AB−CW)/G, where SBF is the specific biofilm formation index, AB is the OD595 of the stained bacteria, CW is the OD595 of the stained control wells containing absolute media without bacteria, and G is the OD595 corresponding to cell growth in the media. *Escherichia coli* ATCC 25922 was used as the positive control, whereas the culture medium was used as the negative control. The degree of biofilm production was classified into three categories, weak (SBF < 0.5), moderate ($0.5 \leq$ SBF < 1.0), and strong (SBF ≥ 1.0).

Genetic Relatedness Analysis and WGS

Clonal Distribution Analysis of MCR1-EC Based on Multi-Locus Sequence Typing and *E. coli* Phylogroup Typing

Multi-Locus Sequence Typing (MLST) was performed as previously described (Wirth et al., 2006). A detailed scheme describing gene amplification, allelic type, and sequence type (ST) assignment methods is available on the pubMLST website.[1] The minimum spanning tree (MST) based on allelic profiles of seven MLST housekeeping genes was constructed using BioNumerics software (v6.6, Applied Maths, Sint-Martens-Latem, Belgium). The PCR-based PG typing was conducted as previously

[1]https://pubmlst.org/

described (Clermont et al., 2013), and primer sequences and reaction conditions are summarized in **Supplementary Table 3**.

Further, we analyzed the clonal distribution of 1,652 MCR1-EC strains, of which WGS was publicly available in the NCBI database (accessed on 07 Jan 2020, https://www.ncbi.nlm.nih.gov/pathogens/isolates/), including strains isolated from humans ($n = 940$), chickens ($n = 446$), and pigs ($n = 226$). In addition, we also analyzed the clonal distribution of 17 South Korean-derived MCR1-EC strains, of which WGS was available in the NCBI database, including strains isolated from humans ($n = 13$), chickens ($n = 2$), a pig ($n = 1$), and a dog ($n = 1$). The *in silico* MLST and *E. coli* phylogenetic typing were performed using the MLST 2.0 (v2.0.4) program at the CGE website and the Clermont typing program (v21.03) provided by the website http://clermontyping.iame-research.center/ (Beghain et al., 2018). The assembly accession numbers of strains used in this study are summarized in **Supplementary File 1**.

In-depth Characterization of Intestinal Pathogenic MCR1-EC Strains Based on WGS

We conducted WGS for all intestinal pathogenic MCR1-EC strains isolated in this study. Total genomic DNA was extracted using the Nucleospin Microbial DNA kit (Macherey-Nagel, North Rhine-Westphalia, Germany) following the manufacturer's instructions. Genomic DNA was sequenced *via* NextSeq® 500 technology (Illumina, California, United States). The nucleotide sequences have been submitted to the NCBI sequence read archive with the assigned Bioproject no. PRJNA757225. The sequence reads were assembled into contigs using the CLC Genomics Workbench program (Qiagen, Hilden, Germany) with default setting. The assembled contigs were analyzed using the bioinformatics tools of the Center for Genomic Epidemiology[2] for the presence of resistance genes (ResFinder V4.1.), virulence factors (VirulenceFinder v2.0.), and plasmid replicon types (PlasmidFinder 2.1).

Genetic Relatedness Analysis Based on WGS

For genetic relatedness analysis based on WGS, we conducted core genome multi-locus sequence typing (cgMLST) to focus on the genetic relatedness between the core genomes of strains, not the genetic difference that occurs through the acquisition or loss of accessory genomes such as plasmids. The cgMLST was performed using the Ridom SeqSphere+ program (v8.2.0; Junemann et al., 2013). In this analysis, first, we conducted cgMLST among all 12 intestinal pathogenic MCR1-EC strains isolated from this study and 17 MCR1-EC strains isolated in South Korea published in the NCBI database to assess the genetic relatedness among strains isolated in South Korea. Second, for genetic relatedness analysis of global MCR1-EC strains, we performed cgMLST on MCR1-EC isolated from humans, pigs, and chickens worldwide and harboring a major clone type. Based on clonal distribution analysis, 154 strains carrying the major clone type ST10-A were identified among 1,652 MCR1-EC strains published in the NCBI database. Moreover, 80 strains were selected among 154 MCR1-EC isolates of clone type ST10-A using a simple random sampling procedure with Statistical Package for the Social Sciences (SPSS) program (v27.0, IBM SPSS Statistics for Windows, New York, United States). Then, the genetic relationships among 82 MCR1-EC strains harboring ST10-A (two intestinal pathogenic MCR1-EC strains isolated in this study and 80 MCR1-EC strains published in the NCBI database) were analyzed based on cgMLST. Then, we clustered strains with a genetic relatedness distance of less than 0.01 in cgMLST, and a total of eight clusters were identified.

[2]http://www.genomicepidemiology.org/

Statistical Analysis

All statistical analyses included in this study were conducted using the SPSS program (IBM SPSS Statistics for Windows). For analysis of MCR1-EC prevalence, we performed the weighted prevalence analysis of MCR1-EC [complex samples crosstabs (CSC) and complex samples logistic regression model (CSLRM)] based on the unbiased Horvitz-Thompson estimator (Horvitz and Thompson, 1952), setting farm as a cluster parameter since sampling probabilities for each swine farm were not equal. Weighted prevalence of MCR1-EC by stage and 95% confidence interval (95% CI) were calculated using CSC. In addition, differences in the prevalence of MCR1-EC according to swine stage were evaluated using the CSLRM setting stage as a covariate parameter.

For comparative analyses of antimicrobial resistance and virulence factors of MCR1-EC isolates by swine stages, the generalized estimating equation (GEE) was used for the calculation of odds ratios (ORs) and 95% CIs setting weaning stages as a reference. To adjust the farm-induced factors, farm was set as the "subject variable" and number of MCR1-EC strains per each farm was set as "within subject variables." If the zero value of the cross-tab caused a problem in the GEE-based OR calculation, Fisher's exact test was performed by adding 0.5 to each cell instead of GEE (Pagano and Gauvreau, 2018). To evaluate the correlation between antimicrobial resistance genes and the expected phenotypic resistance, Spearman's correlation test (SCT) was performed.

RESULTS

Prevalence of MCR1-EC Isolates According to Four Swine Production Stages

Escherichia coli strains carrying *mcr-1* strains were isolated from 55 of 331 pigs (16.6%), from four of 10 swine farms (**Figure 2**; **Supplementary Table 1**). The weighted prevalence of MCR1-EC was 11.6% (95% CI: 8.9%–15.0%), and weaning piglets had the highest weighted prevalence of MCR1-EC (17.9, 95% CI: 9.9%–30.3%). The second highest weighted prevalence MCR1-EC was identified in growing pigs (14.7, 95% CI: 9.2%–22.7%), followed by sows (8.3, 95% CI: 3.5%–18.4%), and finishing pigs (6.7, 95% CI: 3.4%–12.7%). There were no significant differences in the prevalence of MCR1-EC based on the four swine stages (CSLSM, $p > 0.05$).

We included 53 MCR1-EC stains for further analysis, since two MCR1-EC isolates were not recovered. All 53 MCR1-EC strains were found to carry *mcr-1.1* among 32 *mcr-1* variants (*mcr-1.1–mcr-1.32*), and *mcr-1.1* was encoded on either IncI2 (94.3%, 50/53) or IncX4 (5.7%, 3/53). MCR1-EC carrying both *mcr-1.1*-carrying IncI2 and IncX4 was not identified. In the conjugation assay of MCR1-EC strains, *mcr-1.1* was transferred from 90.6% (48/53) of donor strains to the recipient strain J53-Azi^{R}.

Among 53 MCR1-EC isolates, 16 strains (30.2%, 16/53) were identified as pathogenic *E. coli*, including InPEC (22.6%, 12/53) or ExPEC (7.5%, 4/53; **Figure 3**). Among 12 InPEC strains, 10 MCR1-EC (18.9%, 10/53) was identified as STEC and two strains (3.8%, 2/53) were identified as EPEC. Ten STEC were isolated from two weaning piglets, six growing pigs, and two finishing pigs. Two EPEC were isolated from one weaning piglet and one growing pig. Four ExPEC were isolated from one weaning piglet, one growing pig, one finishing pig, and one sow.

Antimicrobial Resistance of MCR1-EC Isolates From Swine Farms

All 53 MCR1-EC isolates were resistant to colistin, with MICs of 4 μg/ml (17.0%, 9/53) or 8 μg/ml (83.0%, 44/53). Through KBTs for 11 antimicrobial classes, 96.2% (51/53) of MCR1-EC strains exhibited MDR, showing resistance to three or more antimicrobial classes (average: 4.8 classes; **Figure 3**). Among the 14 antimicrobial agents tested, the resistance rate of tetracycline was highest (86.8%, 46/53), followed by that of ampicillin (81.1%, 43/53) and chloramphenicol (66.0%, 35/53; **Figure 4A**). Nine MCR1-EC strains (17.0%, 9/53) were resistant to cefotaxime and had a typical phenotype of ESBL. Imipenem- or amikacin-resistant MCR1-EC isolates were not found. In comparison by pathogenic *E. coli* types, ExPEC strains showed resistance to average 7.0 antimicrobial classes, and InPEC strains showed resistance to average 4.0 antimicrobial classes. The resistant rate of ExPEC strains against third generation

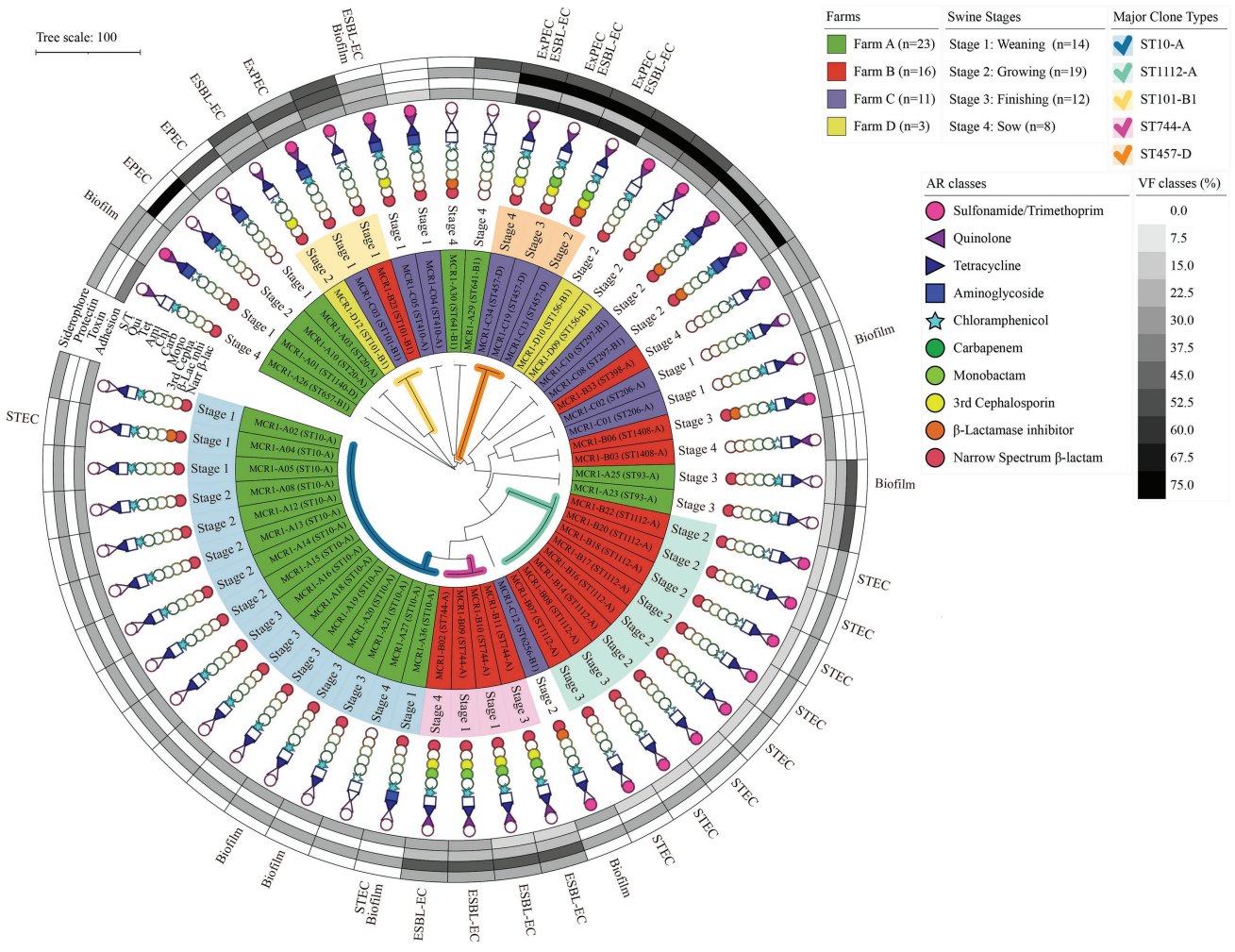

FIGURE 3 | Antimicrobial resistance, genotypic/phenotypic virulence factors, and clone types of 53 MCR1-EC isolates from swine farms. Visualization was conducted using the online visualization tool iTOL (v6, https://itol.embl.de/). MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; EPEC, enteropathogenic *E. coli*; STEC, shiga toxin-producing *E. coli*; and ESBL-EC, extended-spectrum β-lactamase producing *E. coli*, Biofilm, *E. coli* with moderate/strong biofilm formation ability.

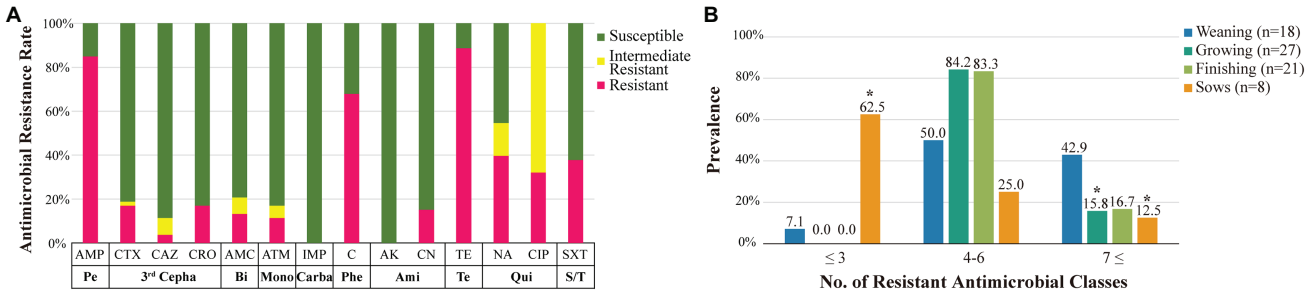

FIGURE 4 | Antimicrobial susceptibility of MCR1-EC strains and prevalence of resistant MCR1-EC. Antimicrobial susceptibility of MCR1-EC from swine farms **(A)** and prevalence of MCR1-EC isolates resistant to different numbers of antimicrobial classes by swine production stage **(B)**. MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; Pe, broad spectrum penicillin class; third Cepha, third-generation cephalosporin class; Bi, β-lactamase inhibitor class; Mono, monobactam class; Carba, carbapenem class; Phe, phenicol class; Ami, aminoglycoside class; Te, tetracycline class; Qui, quinolone class; S/T, sulfonamide/trimethoprim class; AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; AMC, amoxicillin/clavulanate; ATM, aztreonam; IMP, imipenem; C, chloramphenicol; AK, amikacin; CN, gentamycin; TE, tetracycline; NA, nalidixic acid; CIP, ciprofloxacin; and SXT, sulfamethoxazole/trimethoprim. *$p < 0.05$, significantly different prevalence relative to that of weaning piglets, calculated *via* GEEs.

cephalosporins was 75.0% (3/4), whereas, all InPEC strains were susceptible to third cephalosporins. The antimicrobial susceptibility results of InPEC, ExPEC, and commensal *E. coli* were described in **Supplementary Table 4**.

In the comparative analysis based on the four swine stages, the prevalence of isolates showing resistance to seven or more antimicrobial classes was highest in the weaning stage (42.9%, 6/14) compared to that in other stages, which was statistically significant compared to that in finishing pigs (OR: 3.8, 95% CI: 1.73–8.11, $p < 0.05$, GEE) and sows (OR: 5.25, 95% CI: 2.04–13.50, $p < 0.05$, GEE; **Figure 4B**). Meanwhile, the prevalence of isolates showing resistance to three or fewer antimicrobial classes was highest in pregnant sows (62.5%, 5/8), and it was significantly higher than that in weaning piglets (OR: 21.7, 95% CI: 8.77–53.50, $p < 0.05$, GEE). Compared to that in weaning pigs, the resistance rate of aminoglycoside was significantly lower in growing pigs (OR: 0.3, 95% CI: 0.12–0.51, $p < 0.05$, GEE), and the resistance rate of quinolone was significantly lower in finishing pigs (OR: 0.1, 95% CI: 0.07–0.18, $p < 0.05$, GEE; **Table 1**). Compared to those in weaning pigs, the resistance rate of chloramphenicol (OR: 0.1, 95% CI: 0.04–0.28, $p < 0.05$, GEE) and tetracycline (OR: 0.03, 95% CI: 0.001–0.78, $p < 0.05$, Fisher's exact test) were significantly lower in pregnant sows.

Escherichia coli strains carrying *mcr-1* carried a variety of antimicrobial resistance genes, including *tetA* (79.2%, 42/53, against tetracyclines), *floR* (69.8%, 37/53, against phenicols), $bla_{\text{TEM-family}}$ (58.8%, 31/53, against narrow-spectrum β-lactams), *sul2* (50.9%, 27/53, against sulfonamides), *qnrS1* (41.5%, 22/53, against quinolones), and $bla_{\text{CTX-M-55}}$ (17.1%, 9/53, against third Cephalosporins; **Supplementary Table 5**). Resistance genes were strongly associated with expected phenotypic resistance to all antimicrobial classes included in this study ($p < 0.05$, SCT), with the exception of quinolones. Among the 14 replicon types investigated in this study in 53 MCR1-EC isolates, the predominant replicon types were IncI2 (94.3%, 50/53), IncFIB (84.9%, 45/53), IncFII (67.9%, 36/53), and IncFIC (43.4%, 23/53; **Supplementary Table 6**).

Genotypic and Phenotypic Virulence of MCR1-EC Isolates From Swine Farms

Among the four investigated virulence factor classes, all 53 MCR1-EC strains carried one or more adhesion-associated virulence factors, including *fimH* (90.6%, 48/53) and *csgA* (84.9%, 45/53) (**Table 2**). Toxin virulence factors were identified in 54.7% of MCR1-EC (29/53), with *hlyF* (26.4%, 14/53) and *astA* (7.5%, 4/53) present. Protectin virulence factors were identified in 90.6% of MCR1-EC (48/53), with *traT* (88.7%, 47/53), *ompT* (26.4%, 14/53), and *iss* (13.2%, 7/53) present. Siderophore virulence factors were identified in 35.8% of MCR1-EC (19/53), with *iutA* (26.4%, 14/53) and *iroNE.coli* (9.4%, 5/53) present. In addition, four (7.5%, 4/53) MCR1-EC were identified as having two UPEC-associated virulence factors, although this did not satisfy the criteria of UPEC (≥3 UPEC virulence factors). In the comparison based on the four swine stages, no significant differences were identified in the prevalence of the four virulence factor classes between stages ($p > 0.05$, GEE; **Supplementary Table 7**). In the biofilm assay, eight MCR1-EC strains (15.1%, 8/53) showed medium-to-strong biofilm formation (**Figure 3**), including four strains with strong biofilm formation and four strains with moderate biofilm formation. In contrast, 84.9% (45/53) of MCR1-EC showed weak biofilm formation.

WGS-Based In-depth Characterization of Intestinal Pathogenic MCR1-EC Strains

All 10 STEC isolates harbored *stx2e*, and two EPEC strains harbored the locus of enterocyte effacement (LEE), including *eae*, *tir*, *esp*, and *nle* (**Figure 5**). Intestinal pathogenic MCR1-EC carried a variety of InPEC-associated virulence factors, including *terC* (100.0%, 12/12), *gad* (33.3%, 4/12), and *katP* (16.7%, 2/12). In addition, ExPEC-associated virulence factors, including *traT* (91.7%, 11/12), *ompT* (16.7%, 2/12), *iss*, (16.7%, 2/12), *sepA* (167%, 2/12), and *cia* (8.3%, 1/12), were also identified.

In the analysis of antimicrobial resistance genes, all 12 intestinal pathogenic MCR1-EC strains carried resistance genes to five or more antimicrobial classes, including *tetA/B* (100%,

TABLE 1 | Antimicrobial resistance rate of *Escherichia coli* strains carrying *mcr*-1 (MCR1-EC) according to the pig productions stages.

Anti-microbial classes	Weaning piglets (reference)			Growing pigs			Finishing pigs			Pregnant sows		
	Rate (%)	OR (95% CI)	*p* value	Rate (%)	OR (95% CI)	*p* value	Rate (%)	OR (95% CI)	*p* value	Rate (%)	OR (95% CI)	*p* value
Pe	78.6	–	–	94.7	4.9 (0.62–39.16)	0.13	100.0	7.6[a] (0.35–163.83)	0.19	50.0	0.3 (0.05–1.41)	0.12
Third Cepha	28.6	–	–	10.5	0.3 (0.02–3.64)	0.34	8.3	0.2 (0.01–5.24)	0.36	25.0	0.8 (0.15–4.64)	0.84
Carba	0.0	–	–	0.0	0.7[a] (0.01–39.73)	0.88	0.0	1.2[a] (0.02–62.85)	0.94	0.0	1.7[a] (0.03–94.11)	0.79
Mono	21.4	–	–	5.3	0.2 (0.01–8.01)	0.40	8.3	0.3 (0.01–16.66)	0.58	12.5	0.5 (0.23–1.20)	0.13
Bi	7.1	–	–	21.1	3.5 (0.12–104.32)	0.47	8.3	1.2 (0.05–27.38)	0.92	12.5	1.9 (1.00–3.45)	0.05
Phe	85.7	–	–	63.2	0.3 (0.01–6.71)	0.44	75.0	0.5 (0.01–19.33)	0.71	37.5	0.1 (0.04–0.28)	<0.01*
Ami	42.9	–	–	15.8	0.3 (0.12–0.51)	<0.01*	0.0	0.5[a] (0.002–1.06)	0.05	0.0	0.1[a] (0.003–1.59)	0.10
Te	100.0	–	–	89.5	0.2[a] (0.01–5.44)	0.37	100.0	0.9[a] (0.02–46.71)	0.94	50.0	0.03[a] (0.001–0.78)	0.03†
Qui	64.3	–	–	31.6	0.3 (0.03–1.97)	0.19	16.7	0.1 (0.07–0.18)	<0.01*	50.0	0.6 (0.21–1.49)	0.24
S/T	28.6	–	–	57.9	3.4 (0.43–27.61)	0.25	33.3	1.3 (0.06–28.35)	0.89	12.5	0.4 (0.03–4.30)	0.42

The odds ratio (OR), including 95% of confidential interval (95% CI) and p value, was calculated by generalized estimating equations (GEE).

[a]*Where zeros cause problems in calculating OR or 95% CI, Fisher's exact test was used in the calculations instead of GEE.*

**p <0.05, statistically significant based on GEE.*

†*p <0.05, statistically significant based on Fisher's exact test.*

Pe, broad spectrum penicillin class; third Cepha, third cephalosporin class; Carba, carbapenem class; Mono, monobactam class; Bi, β-lactamase inhibitor class; Phe, phenicol class; Ami, aminoglycoside class; Te, tetracycline class; Qui, quinolone class; S/T, sulfonamide/trimethoprim; and class Poly, polymyxin class.

12/12, against tetracyclines), *mdf(A)* (100%, 12/12, against macrolides), *dfrA1* (83.3%, 10/12, trimethoprims), *ant(3")-Ia* (75.0%, 9/12, aminoglycosides), *sul2* (66.7%, 8/12, sulfonamides), *floR* (25.0%, 3/12, phenicols), and $bla_{\text{TEM-family}}$ (25.0%, 3/12, narrow-spectrum β-lactams). All carried IncI2, accompanying by a variety of replicon types, including IncFIB (83.3%, 10/12), IncFII (83.3%, 10/12), IncR (66.7%, 8/12), ColE10 (66.7%, 8/12), and IncI1-Iγ (33.3%, 4/12).

In the comparative genomic analysis based on swine production stages, intestinal pathogenic MCR1-EC showed highly shared virulence factor characteristics between strains with the same clone type. In addition, the patterns of replicon types and antimicrobial resistance genes were also identical with slight differences between strains with the same clone types.

Genetic Relatedness Analysis of MCR1-EC Strains Based on Clone Types and WGS

Clonal Distribution Analysis of MCR1-EC From This Study and the NCBI Database

Among 53 MCR1-EC strains, 38 strains were identified as *E. coli* phylogenetic group A (71.7%, 38/53), 11 strains (20.8%) were identified as group B1, and four strains (7.5%) were identified as group D (**Figure 6**). In total, 17 clone types were identified among 53 MCR1-EC strains isolated in this study, and the major clone types were ST10-A (28.3%, 15/53), ST1112-A (15.1%, 8/53), ST744-A (7.5%, 4/53), ST101-B1 (5.7%, 3/53), and ST457-D (5.7%, 3/53). The other clone types included only one or two MCR1-EC strains. In a comparison by swine farm, all clone types were not shared between pig farms with the exception of ST101-B1, which was isolated from three pig farms. In a comparison by swine production stage, the clone types were shared between pigs of different stages within farms (**Figure 3**).

In the clonal distribution analysis of MCR1-EC published in the NCBI database, 17 MCR1-EC strains derived from South Korea harbored 15 clone types, including ST10-A (11.8%, 2/17) and ST11124-A (11.8%, 2/17; **Supplementary File 1**). In the clonal distribution analysis of human-, pig-, and chicken-derived 1,652 MCR1-EC, 248 clone types were identified among 940 human-derived MCR1-EC, and major clone types were ST10-A (9.6%, 90/940), ST152-A (3.5%, 33/940), ST206-A (3.0%, 28/940), and ST101-B1 (2.9%, 27/940). Among 266 pig-derived MCR1-EC strains, 101 clone types were identified, and major types were ST10-A (11.3%, 30/268), ST206-A (4.1%, 11/266), and ST101-B1 (3.8%, 10/266). Among 446 chicken-derived MCR1-EC isolates, 118 clone types were identified and major types were ST10-A (7.6%, 34/446), ST156-B1 (6.7%, 30/446), and ST93-A (4.7%, 21/446).

Genetic Relatedness Analysis Based on cgMLST Between MCR1-EC Strains From This Study and the NCBI Database

In the cgMLST-based genetic relatedness analysis of intestinal pathogenic MCR1-EC isolated from this study and South Korea-derived MCR1-EC published on the NCBI database, the genetic

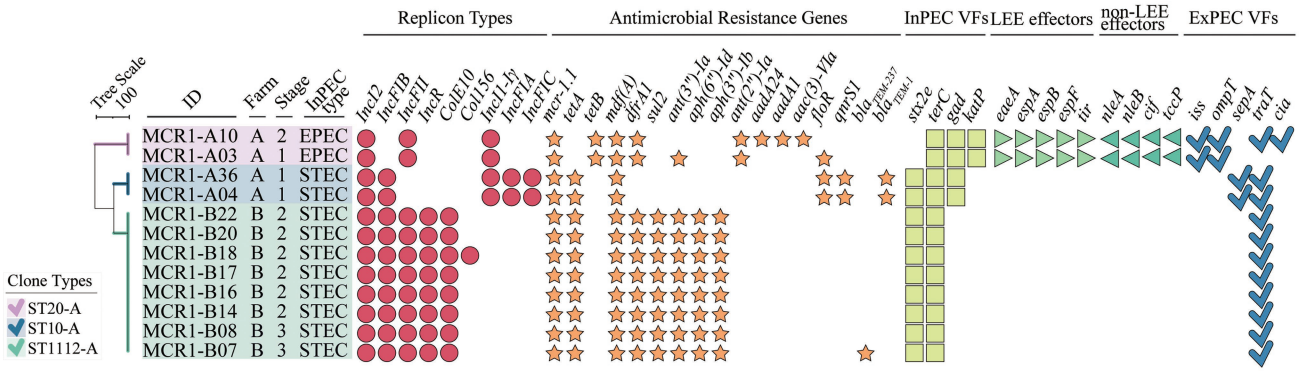

FIGURE 5 | Whole-genome sequence (WGS)-based in-depth genetic characterization of 12 intestinal pathogenic MCR1-EC strains. The phylogenetic tree was constructed based on allele profiles of multi-locus sequence types (MLSTs) of 12 intestinal pathogenic MCR1-EC strains *via* the Unweighted Pair Group Method with Arithmetic means (UPGMA) method, calculating dice coefficients in the Bionumerics program (v6.6). Visualization was conducted using the online visualization tool iTOL (v6, https://itol.embl.de/). MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; Stage 1, weaning piglets; Stage 2, growing pigs; Stage 3, finishing pigs; Stage 4, pregnant sows; InPEC type, intestinal pathogenic *E. coli* type; EPEC, enteropathogenic *E. coli*; STEC, shiga toxin-producing *E. coli*; InPEC VFs, intestinal pathogenic *E. coli*-associated virulence factors; LEE effectors, locus of enterocyte effacement (LEE)-encoded effectors; non-LEE effectors, non-LEE-encoded effectors; and ExPEC VFs, extra-intestinal pathogenic *E. coli*-associated virulence factors.

relatedness distances between strains ranged from 0.000 to 0.961 (average 0.720, 95% CI: 0.694–0.746; **Figure 7**). We clustered strains with a genetic relatedness distance of less than 0.01 in cgMLST, and a total of four clusters (clusters I–IV) were identified. Cluster I included two ST20-A MCR1-EC strains (MCR1-A03 and MCR1-A10) isolated from one weaning piglet and one growing pig in Farm A. Cluster II included eight ST1112-A MCR1-EC strains (MCR1-B07, B08, B14, B16, B17, B18, B20, and B22) isolated from six growing and two finishing pigs in Farm B. Cluster III included two ST10-A MCR1-EC strains (MCR1-A04 and MCR1-A36) isolated from two weaning piglets in Farm A. Cluster IV included two ST11124-A MCR1-EC strains (GCA_013390695.1 and GCA_013391045.1) published in the NCBI database. All strains of four clusters were identified as being isolated from individuals from the same farm or hospital. According to the metadata in the original report, two South Korean-derived ST11124-A MCR1-EC strains in cluster IV were reported to be isolated from two patients in the same hospital but at different collection times for each strain (Kim et al., 2021). Except for MCR1-EC strains belonging to four clusters, the genetic relatedness distance was confirmed to have an average value of 0.771 (95% CI: 0.752–0.790), and the average value was 0.397 (95% CI: 0.317–0.477) even among six MCR1-EC isolates carrying the same clone type, ST10-A.

In the genetic relatedness analysis of 82 ST10-A MCR1-EC strains from humans, chickens, and pigs worldwide, the genetic relatedness distance between strains ranged from 0.000 to 0.525 (average 0.309, 95% CI: 0.305–0.312; **Figure 8**). We clustered strains with a genetic relatedness distance of less than 0.01 in cgMLST, and a total of five clusters (cluster III, V, VI, VII, and VIII) were identified. Cluster III included two MCR1-EC strains (MCR1-A04 and MCR1-A36) isolated from Farm A in this study. Cluster V included two chicken-derived strains (GCA_013072745.1 and GCA_013072725.1) from China. Cluster VI included two human-derived strains (GCA_003290855.1 and GCA_003290875.1) from China. Cluster VII included two human-derived strains (GCA_003291515.1 and GCA_003290695.1) from China. Cluster VIII included two chicken-derived strains (GCA_014900955.1 and GCA_014900935.1) from China. According to the metadata in the original report, MCR1-EC, belonging to the four clusters V, VI, VII, and VIII, was isolated from individuals in the same hospital or farm, with strains in the same cluster (Shen et al., 2018; Soliman et al., 2021). Except for MCR1-EC isolates belonging to four clusters, the genetic relatedness distance between the other MCR1-EC isolates was confirmed to have an average value of 0.309 (95% CI: 0.305–0.313).

DISCUSSION

The global emergence and spread of MCR1-EC represent a serious threat for public health (WHO, 2018). Although the use of colistin for the prevention of swine colibacillosis has been banned from multiple countries worldwide since 2016, colistin has been generally used for the treatment of swine diseases, leading to an increased prevalence of MCR1-EC in swine farms worldwide including South Korea (Malhotra-Kumar et al., 2016; Tong et al., 2018; Kyung-Hyo et al., 2020; Liu et al., 2020; Mechesso et al., 2020; Nakano et al., 2021). In this study, the weighted prevalence of MCR1-EC was 11.6% (95% CI: 8.9%–15.0%) and it was comparable with that in previous reports conducted from Belgium (13.2%; Malhotra-Kumar et al., 2016), Japan (20.4%; Nakano et al., 2021), Taiwan (29.2%; Liu et al., 2020), and China (76.2%; Tong et al., 2018). Comparison of the four swine production stages showed that weaning piglets exhibited the highest prevalence of MCR1-EC compared with pigs at other stages. This result was consistent with that of previous studies conducted worldwide, in which

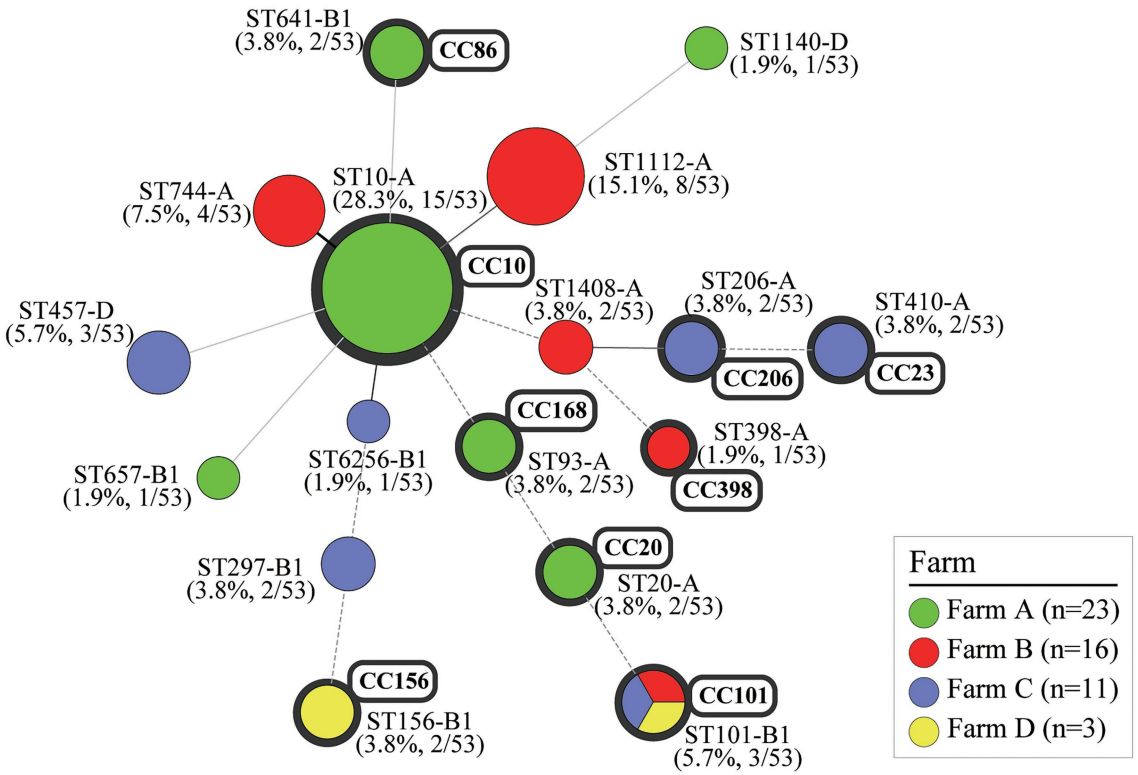

FIGURE 6 | Clonal distribution of MCR1-EC isolates: Minimum spanning tree (MST) based on MLST allele profiles. The MST was constructed using the Bionumerics program (v6.6). The colors of nodes correspond to the four swine farms. The upper number shows the sequence type of each node, and the lower number in parentheses indicates percentages for each node. The size of the node indicates the number of strains belonging to the sequence type (ST)-phylogroup (PG) type. The gray shaded area represents the clonal complex (CC). The branch line types represent differences in the number of alleles as follows: bold solid line (one allele), thin solid line (2–3 alleles), dashed line (four alleles), and dotted line (above five alleles). MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; MLST, multi-locus sequence typing.

MCR1-EC was isolated mainly from weaning piglets (Malhotra-Kumar et al., 2016; Tong et al., 2018; Wang et al., 2018b; Kyung-Hyo et al., 2020; Liu et al., 2020; Mechesso et al., 2020; Nakano et al., 2021). Recent studies on the occurrence of MCR1-EC in pigs following the cessation of colistin use have proposed a positive correlation between colistin administration and prevalence of MCR1-EC in swine farms (Randall et al., 2018; Shen et al., 2020). Considering that colistin has been reported to be mainly prescribed in weaning stages for the treatment of swine colibacillosis, which exhibits higher incidence during the weaning stage (Callens et al., 2012; Sjolund et al., 2016; Korsgaard et al., 2020), high colistin at weaning stage could be one of the important causes for the high prevalence of MCR1-EC at this stage.

Notably, 96.2% of MCR1-EC exhibited MDR, with resistance against average 4.8 antimicrobial classes. Furthermore, all MCR1-EC showed intermediate-to-resistance to ciprofloxacin and 17.0% of strains showed an ESBL phenotype as well as carried $bla_{\text{CTX-M-55}}$. The $bla_{\text{CTX-M-55}}$ gene has been globally reported as an ESBL genotype that confers resistance to third-generation cephalosporins, and it has been found in various hosts including humans and food-animals (Lv et al., 2013). In the comparative analysis based on the four swine stages, it was found that the weaning piglets exhibited significantly higher resistance rates to various antimicrobial agents than other stages, especially sow. This result suggests that the antimicrobial resistance found in weaning piglets may not be inherited from sow. According to studies on the use of antibiotics throughout the swine production cycle, over than 70% of the total antimicrobial agents used in the swine industry have been prescribed between birth and 10 weeks of age (Callens et al., 2012; Sjolund et al., 2016; Korsgaard et al., 2020). Antibiotic selective pressure has been reported to play significant role in the increasing prevalence of resistant bacteria (Randall et al., 2018). Wu et al. (2018a) suggested that colistin and β-lactam antibiotics have been commonly prescribed together in food-animal husbandry, and resistance to colistin and third generation cephalosporins emerged and increased together under the heavy selection pressure of antibiotics over the last few decades. In our previous study, we investigated the prevalence of ESBL-producing *E. coli* (ESBL-EC) in pig farms, which revealed that the prevalence of ESBL-EC was significantly higher in weaning piglets compared with that in other stages and sow showed the lowest prevalence of ESBL-EC (Lee et al., 2021). In addition, interestingly, the prevalence of

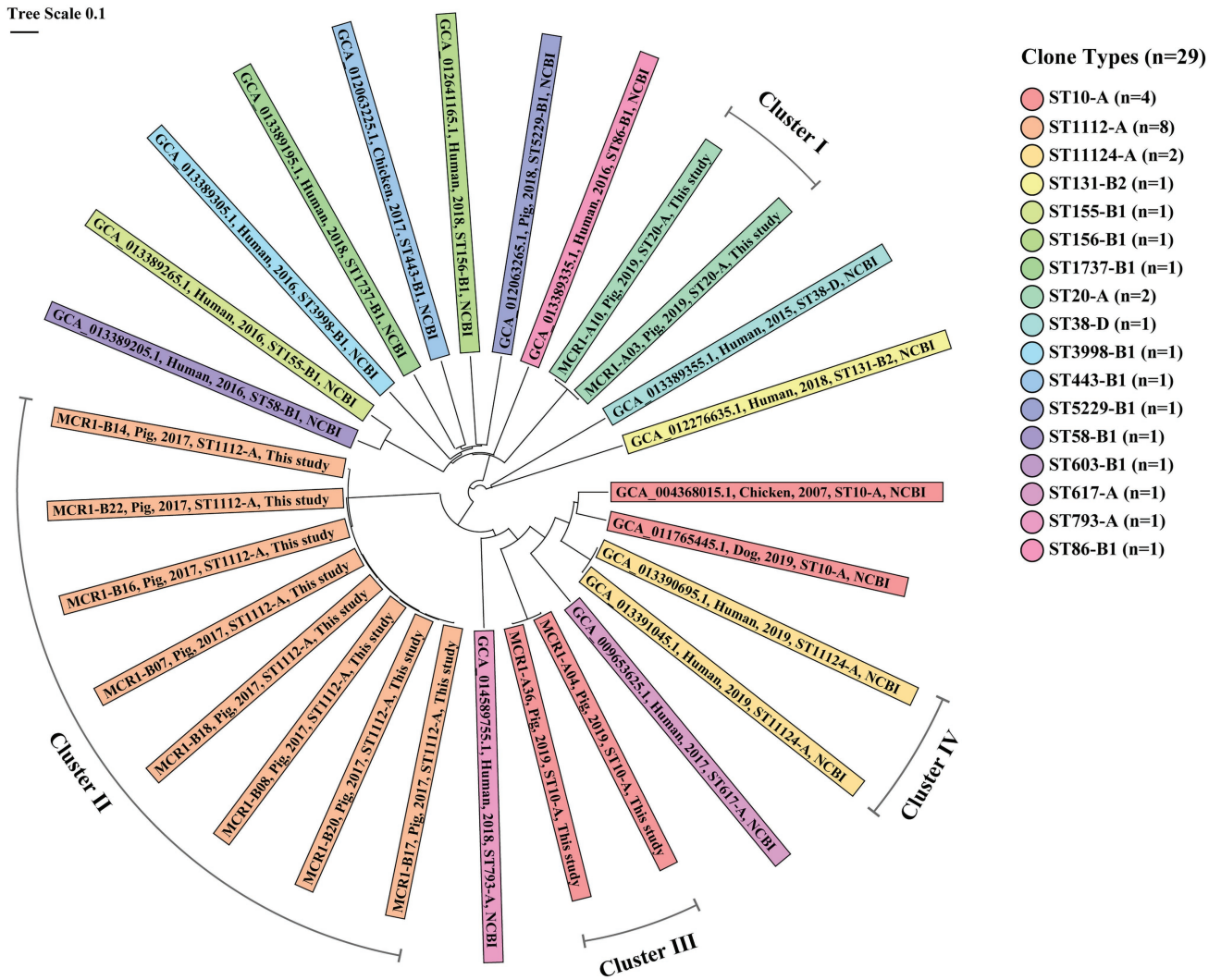

FIGURE 7 | Core genome MLST (cgMLST)-based genetic relatedness between 29 MCR1-EC isolates from South Korea. Data comprise 12 intestinal pathogenic MCR1-EC strains isolated in this study and 17 MCR1-EC strains isolated from South Korea, published in the National Center for Biotechnology Information (NCBI) GenBank database. The phylogenetic tree based on cgMLST was constructed using the neighbor-joining algorithm with default parameters implemented in the Ridom SeqSphere+ program (v8.2.0). The color of shades corresponds to each clone type. The contents of shades include, from left to right, the assembly accession number, host, isolation date, clone type, and data source (this study or NCBI database) for each strain. Four clusters consist of MCR1-EC strains with a relatedness distance value less than 0.01. MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; MLST, multi-locus sequence typing.

ESBL-EC in the four farms, where MCR1-EC was found in the present study, was significantly higher (76.4%; OR: 3.8, 95% CI: 1.73–8.14, $p < 0.05$, GEE) than in farms where MCR1-EC was not identified (46.2%). Hence, these findings were consistent with the conclusion of Wu et al. (2018a). Our study suggests that weaning piglets could act as an important reservoir for MDR bacteria, including ESBL-EC and MCR1-EC. The potential of MDR bacterial transmission from food-animal husbandry to humans and vice versa has been continuously proposed by various studies (Liu et al., 2016; Hadjadj et al., 2017; Wu et al., 2018a). Considering that both third generation cephalosporins and colistin are classified as critically important antimicrobial agents for livestock and humans (WHO, 2018), the high prevalence of MDR bacteria carrying both *mcr-1.1* and $bla_{\text{CTX-M-55}}$ implies the potential for the emergence of MDR pathogens, which can hardly be treated, even by last resort antimicrobials. Collectively, we suggest that pig farms, which are important reservoirs of MDR bacteria, require special attention at the weaning stage to control crucial bacteria, such as MCR1-EC and ESBL-EC.

Swine colibacillosis is one of the major swine diseases impacting the global swine industry and is associated with huge economic losses; edema disease (ED) and post-weaning diarrhea (PWD) belong to the classification of swine colibacillosis (Luppi, 2017). Given that colistin has been

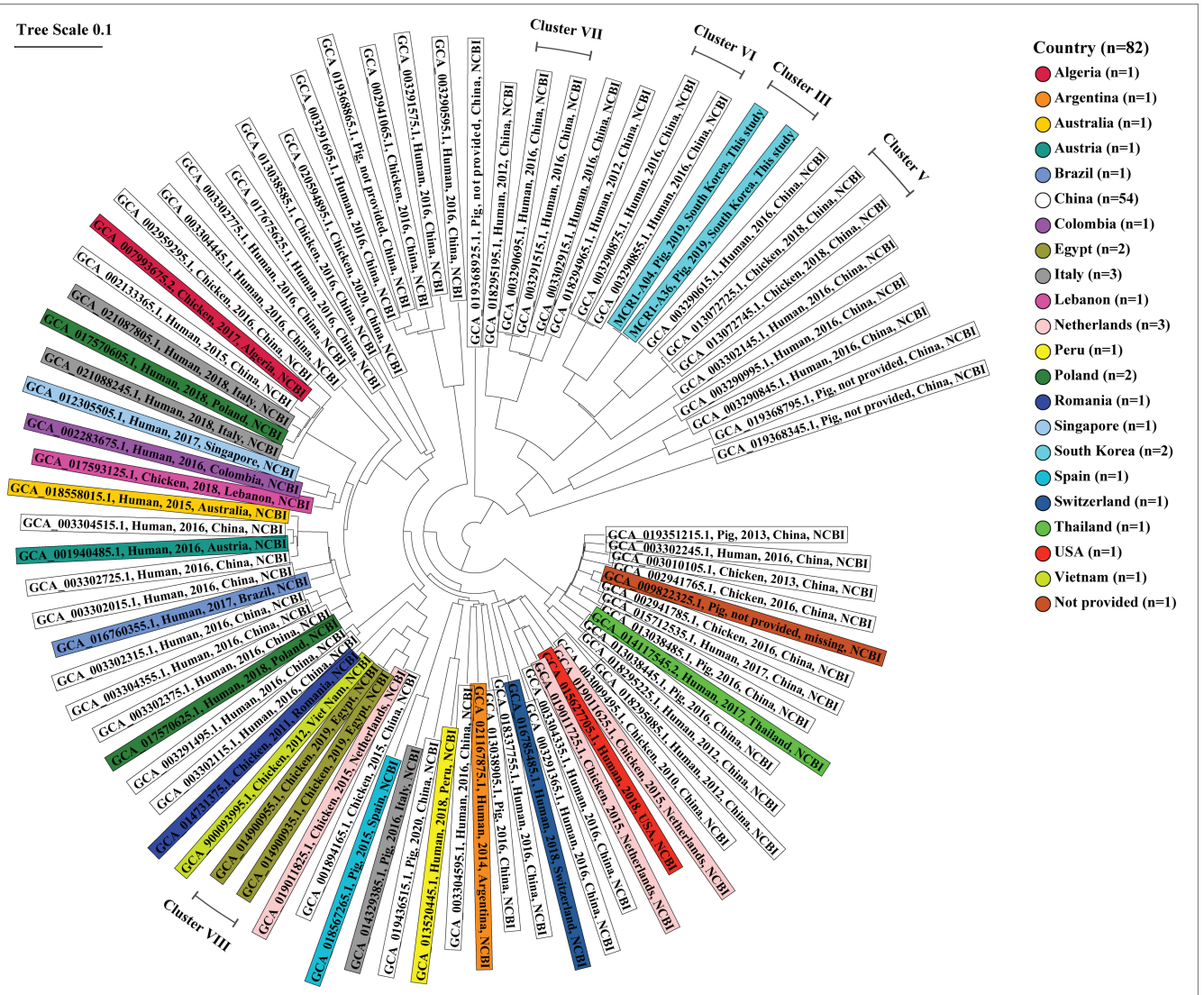

FIGURE 8 | Core genome multi-locus sequence typing-based genetic relatedness of 82 ST10-A MCR1-EC strains isolated from humans, pigs, and chickens worldwide. Strains were derived from the NCBI GenBank database. The phylogenetic tree based on cgMLST was constructed using the neighbor-joining algorithm with default parameters implemented in the Ridom SeqSphere+ program (v8.2.0). The shaded color corresponds to the country where the strain was isolated. The contents of shades include, from left to right, the assembly accession number, host, isolation date, isolation country, and data source (this study or NCBI database) for each strain. Five clusters consist of MCR1-EC isolates with a relatedness distance value of less than 0.01. MCR1-EC, *Escherichia coli* carrying the mobilized colistin resistance gene *mcr-1.1*; cgMLST, core genome multi-locus sequence typing.

considered a recommended treatment for swine colibacillosis and InPECs are major causative bacteria of swine colibacillosis (Luppi, 2017), the presence of intestinal pathogenic MCR1-EC in pig husbandry could represent a major challenge for the swine industry. In this study, 22.6% of MCR1-EC strains were identified as InPECs including STEC carrying *stx2e* and EPEC carrying LEE-encoded virulence factors. The *stx2e* gene is key virulence factor causing damage to arterioles and edema at various sites, eventually leading to death associated with ED (Casanova et al., 2018). The LEE-encoded VFs are responsible for the characteristic histopathological lesion of PWD, termed attaching/effacing lesions (Rhouma et al., 2017). Among various identified virulence factors from intestinal pathogenic MCR1-EC strains, the presence of *katP* might especially increase the risk presented by the strains, since it has been reported to promote the virulence of InPECs by supporting their colonization of the host intestine (Brunder et al., 1996). In addition to virulence factors, all intestinal pathogenic MCR1-EC strains were identified as MDR bacteria harboring five or more antimicrobial class resistance genes. Comparative genomic analysis according to the stage of pig development revealed that the genetic characteristics of the intestinal pathogenic MCR1-EC strains were highly shared among pigs at different stages, suggesting that there is a high potential for the transmission of intestinal pathogenic MCR1-EC within farms.

TABLE 2 | Analysis of pathogenic *E. coli*-associated virulence factors in MCR1-EC from swine farms.

Virulence factor classes	Virulence factors	Prevalence (%)	No. of positive MCR1-EC/No. of total MCR1-EC
Intestinal pathogenic *E. coli* (InPEC)	*stx2*	18.9	10/53
	eaeA	3.8	2/53
	stx2 or *eaeA*	22.6	12/53
Extra-intestinal pathogenic *E. coli* (ExPEC)	*kpsMTII*[a]	9.4	5/53
	papC[a]	7.5	4/53
	papAH[a]	7.5	4/53
	sfa/focDE[a]	1.9	1/53
	afa/draBC[a]	0.0	0/53
	Two and more ExPEC VFs	7.5	4/53
Uropathogenic *E. coli* (UPEC)	*fyuA*[b]	7.5	4/53
	chuA[b]	7.5	4/53
	yfcV[b]	5.7	3/53
	vat[b]	3.8	2/53
	Two and more UPEC VFs	7.5	4/53
Adhesion	*fimH*	90.6	48/53
	csgA	84.9	45/53
	papC[a]	7.5	4/53
	papAH[a]	7.5	4/53
	yfcV[b]	5.7	3/53
	sfa/focDE[a]	1.9	1/53
	afa/draBC[a]	0.0	0/53
	iha	0.0	0/53
	Total adhesion (at least one)	100.0	53/53
Toxin	*hlyF*	26.4	14/53
	astA	7.5	4/53
	vat[b]	3.8	2/53
	pic	0.0	0/53
	aat	0.0	0/53
	Total toxin (at least one)	32.1	17/53
Protectin	*traT*	88.7	47/53
	ompT	26.4	14/53
	iss	13.2	7/53
	kpsMTII[a]	9.4	5/53
	Total protectin (at least one)	90.6	48/53
Siderophore	*iutA*[a]	26.4	14/53
	iroNE.coli	9.4	5/53
	fyuA[b]	7.5	4/53
	chuA[b]	7.5	4/53
	Total siderophore (at least one)	35.8	19/53

[a]*Virulence factors used for criteria of ExPEC; if positive for ≥2 of five key markers, including papA and/or papC, sfa/focDE, afa/draBC, iutA, and kpsMTII.*
[b]*Virulence factors used for criteria of UPEC; if positive for ≥3 of four key markers, including vat, fyuA, chuA, and yfcV.*

Although *E. coli* is a major organism carrying *mcr* genes, other *Enterobacterales* species have also been reported to carry the *mcr* genes and inhabit the intestinal tract of pigs (Lima et al., 2019; Phetburom et al., 2021). In addition, *mcr* genes have been reported to be highly transferred from *E. coli* to other pathogens, causing swine diseases, such as *Salmonella*, *Klebsiella*, and *Pseudomonas* (Kim et al., 2019). Thus, in cases of swine diseases caused by pathogens harboring these multiple virulence factors and MDR, the prescription of colistin may simply impose selection pressure, leading to disease treatment failure and the spread of colistin resistance in swine farms. To the control these highly virulent and MDR pathogens, it may be necessary to establish a strategy based on in-depth characterization, such as WGS analysis, rather than blindly using antibiotics for the treatment of swine diseases.

In the investigation of genotypic and phenotypic virulent characteristics, MCR1-EC isolates carried multiple ExPEC-associated virulence factors, including *traT*, *hlyF*, and *kpsMTII*, and four MCR1-EC isolates were identified as ExPEC. A high rate of ExPEC-associated virulence factors has been reported to correspond with high potential for survival in the harsh environments and pathogenicity of the bacteria against the host immune system (Pitout, 2012). The expression of TraT protein, an outer membrane lipoprotein, has been linked to improved serum resistance (Nilius and Savage, 1984). The hemolysin production regulator *hlyF* create pores in the membrane of host cells, which increasing the permeability of host cells and ending cell lysis (Bhakdi et al., 1988). The *kpsMTII* has been reported to encoding capsular polysaccharides acting protect the bacteria from environment by covering bacteria and helping to form biofilm (Antao et al., 2009). In addition to genotypic virulence, eight MCR1-EC strains showed moderate-to-strong biofilm formation capacity. Biofilm formation has been reported to confer a fitness advantage to bacteria by enhancing their survivability, increasing their virulence, and facilitating their ability to acquire virulence and antibiotic resistance genes during horizontal gene transmission owing to their high microbial density (Donlan and Costerton, 2002; Schroeder et al., 2017) Based on fitness advantages, such as strong biofilm formation or harboring multiple ExPEC virulence factors, MCR1-EC could survive better in an environment of swine farm husbandry and continuously exist through a repeated cycle, which involves the shedding from swine through feces, survival in the farm environment, and reintroduction to swine. In addition, although MCR1-EC might not be directly transmitted from pig farms to humans through the food-chain, these fitness advantages could provide MCR1-EC strains possibility to survive better in the food-chain and serve as an important source of *mcr-1.1* for various other bacteria in food-chains through genetic transmission mechanisms, such as conjugation.

In the analysis of clonal distribution of MCR1-EC, ST10-A was the most prevalent clone type of MCR1-EC strains in this study, as well as in the human, pig, and chicken-derived MCR1-EC strains described on the NCBI database. However, ST10-A represented only 28.3% of the MCR1-EC samples isolated in this study and 9.8% of 1,562 MCR1-EC samples described in the NCBI database. Other clone types, such as ST101-B1, ST744-A, and ST206-A, also accounted for a significant proportion of total strains. Consistently, the epidemiological analyses of MCR1-EC global clonal distribution revealed that ST10-A was the most prevalent

clone type of MCR1-EC in humans and food-animals, whereas the other clone types also accounted for a significant proportion among total strains (Malhotra-Kumar et al., 2016; Matamoros et al., 2017; Garcia et al., 2018; Tong et al., 2018; Liu et al., 2020; Nakano et al., 2021). Furthermore, one recent study in Thailand showed that the dominant clone type of MCR1-EC in swine farms was ST101, followed by ST10 (Khanawapee et al., 2021). Interestingly, the results of clonal distribution analysis of MCR1-EC isolated from pig farms in this study revealed that the clone types were highly shared among MCR1-EC strains isolated from the same farm, but not between farms. Comparison by swine farms showed that all clone types, including the most predominant clone type ST10-A, were not shared between pig farms with the exception of ST101-B1, which was identified in three pig farms. Collectively, our study suggests that clonal types of MCR1-EC may vary widely between studies, and that it may be shared within closed environments such as a pig farm, but not between environments such as different pig farms or food-chains. Hence, this suggestion may imply that that clonal expansion alone may not have a direct role in MCR1-EC propagation between environments.

The cgMLST-based genetic relatedness analysis of intestinal pathogenic MCR1-EC strains isolated in this study, as well as those published in the NCBI database, revealed that MCR1-EC strains isolated from individuals within closed environment (such as hospitals or farms) were highly clustered, showing a genetic distance lower than 0.01. Noteworthy, clustered strains were isolated within the same hospitals or farms, but in separate spaces or at different time points. According to the original metadata of the two strains in cluster IV, they were isolated from patients in the same hospital but with a time interval of 2 months (Kim et al., 2021). In addition, two strains in cluster I and eight strains in cluster II, isolated in the present study, were isolated from different swine stages, which mean that they were isolated from pigs living in separate barns, including weaning, growing, and finishing barns. These results suggest that the clonal expansion may have a relatively high contribution to the propagation of MCR1-EC between individuals in closed environments. Since *mcr-1* is mainly transmitted by plasmids, the important role of genetic transferability of *mcr-1* in the spread of MCR1-EC has been continuously highlighted in various studies. However, genetic transfer essentially presupposes the transfer of strains and bacteria-to-bacteria interactions under favorable conditions, such as physical distance between strains, nutrition, and environmental conditions, among others (Virolle et al., 2020), which suggests that bacterial transmission also provides a crucial basis for the spread of MCR1-EC. It was previously reported that bacterial transmission between swine production stages within farms may probably occur through farm worker/veterinarian handling, equipment contamination, and transference of manure excretions between different stage barns (Fromm et al., 2014; Schmithausen et al., 2015). Our results suggest that bacterial cross-infection between different stages, pigs may act as an important risk factor for the prevalence of MCR1-EC. Swine farms have been continuously reported as an important reservoir of MCR1-EC (Malhotra-Kumar et al., 2016; Tong et al., 2018; Liu et al., 2020; Nakano et al., 2021). Our findings highlight that efforts to reduce bacterial cross-infection between stages are imperative to control MCR1-EC prevalence in swine farms, one of major reservoir of MCR1-EC.

Among reported *mcr* variants, the present study focused on the most predominant variant type, *mcr-1*. Recent studies have shown that the mobile genetic elements associated with the *mcr* genes may differ between variant types, which may lead to different genotypic and phenotypic traits in bacteria (Yin et al., 2017; Wang et al., 2018a,b,c; Lu et al., 2019; Yang et al., 2021). In this study, we conducted the comparative analysis of prevalence, characteristics, and clonal distribution of MCR1-EC according to swine production stages by excluding other *mcr* variants, which could be potential confounding factors. For further study, it would be interesting to analyze the characteristic differences of the other major *mcr* variants, such as *mcr-3* or *mcr-9*, according to food-animal production stages in livestock husbandry. Based on the 2017 demographic report of the Korean Statistical Information Service of Statistics, we analyzed the prevalence, characteristics, and clonal distribution of MCR1-EC according to four swine production stages in 10 swine farms, which were located in the provinces with the largest number of farms in South Korea. Overall, MCR1-EC was identified in four farms among the 10 swine farms investigated, of which three farms with MCR1-EC incidence were located in Gyeonggi-do. This result suggests that this study may not reflect the national prevalence of MCR1-EC, but regional characteristics. Further studies based on the national antimicrobial monitoring system by expanding the target farms and sampling size may help further describe the nationwide characteristics of MCR1-EC incidence and prevalence according to swine production stages.

CONCLUSION

In conclusion, our study results showed that MCR1-EC isolates having MDR (e.g., against quinolones and ESBLs) were distributed throughout swine production stages in farms, with the highest prevalence at the weaning stage. Weaning stage-derived MCR1-EC showed a significantly higher resistance rate than those from other stages. MCR1-EC with pathogenic advantages (e.g., InPEC/ExPEC-associated virulence factors or robust biofilm formation) were identified from all pig stages and accounted for nearly half of the total strains. Genetic relatedness analysis based on MLST and cgMLST proposed a high potential for cross-infection of MCR1-EC within closed environment such as livestock farms as well as human hospitals. Our results highlight the need to establish MCR1-EC control plans in swine farms based on an in-depth understanding of MCR1-EC characteristics according to swine production stages, focusing especially on the weaning stages.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI sequence read archive repository, with bioproject accession no. PRJNA757225.

AUTHOR CONTRIBUTIONS

SL was a major contributor, both in experiments and writing the manuscript. SL and SC conceived and designed the study. SL, J-UA, HS, and SY performed the sampling and experiments. SL, J-UA, JW, J-HL, and SR analyzed the data. SL, W-HK, and SC prepared and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.873856/full#supplementary-material

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