Antimicrobial resistance of *Salmonella* Indiana from retail chickens in China and emergence of an *mcr-1*-harboring isolate with concurrent resistance to ciprofloxacin, cefotaxime, and colistin

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*Salmonella enterica* serotype Indiana (S. Indiana) in Chinese poultry meat has aroused widespread concern because of its high prevalence and strong antimicrobial resistance. In consideration of the relationship in our previous study between *S. Indiana* and co-resistance to ciprofloxacin and cefotaxime (CIP-CTX), which were the first-line drug which were used in *Salmonella* infection in clinical, the antimicrobial resistance (AMR) of 224 CIP-CTX co-resistant *S. Indiana* isolated from retail chicken samples in China were investigated, with the aim of characterizing the AMR profiles and related resistance mechanisms to ciprofloxacin and cefotaxime among these CIP-CTX co-resistant *S. Indiana* isolates, all of which showed multi-drug-resistant (MDR) phenotypes. GyrA (S83F and D87N/G) with ParC (T57S and S80R) were identified as common plasmid-mediated quinolone resistance (PMQR)-encoding genes. Five *bla*$_{CTX-M}$ gene subtypes were identified with *bla*$_{CTX-M-65}$ ranking at the top. Equally important, we obtained one isolate CFS664 harboring the *mcr-1* gene was ESBL producer with co-resistance to nine in ten classes of tested drugs including colistin. A single circular chromosome and 3 circular plasmids were found in its genome. Among the 26 AMR genes identified, 24 were located on plasmid pCFS664-1, including three ESBL genes, while plasmid pCFS664-3 owning only the *mcr-1* gene and sharing the same backbone structure with plasmids from Enterobacteriaceae. No insertion sequences were found near the *mcr-1* gene but a relaxase-encoding...
gene in the flank, which could transfer into E. coli J53 at a relatively high frequency. S. Indiana in this study exhibited highly drug-resistant phenotypes, contributing to the acceleration of the dissemination and emergence of this pathogen among different sources. Surveillance and a One Health strategy are needed to limit the emergence of S. Indiana along the food chain.

KEYWORDS
Salmonella Indiana, CIP-CTX co-resistance, resistance gene, poultry, colistin, plasmid, mcr-1

Introduction

Poultry meat is known to be a major source of non-typhoidal Salmonella which causes human salmonellosis and ever-growing food safety concerns across this processing chain (Heredia et al., 2018). In total, there are currently over 2,600 serotypes of Salmonella as described by the World Health Organization (Issenhuth-Jeanjean et al., 2014; Thames et al., 2020). Many of the serovars associated with human infections are frequently found on broiler meats and three serotypes of S. typhimurium, S. enteritidis, and S. Heidelberg were the predominant (Foley et al., 2008; FSIS, 2020). Data reported that the prevalence levels in China differed from high to low among raw poultry meat, including chicken (26.4%), pigeon (22.6%), duck (10.1%), and other poultry meat (15.4%) and chilled poultry meat might be more likely to experience cross-contamination than non-chilled poultry meat in China (Sun et al., 2021). From the public health perspective, Salmonella contamination of poultry products has raised concerns, in particular in regard to the emergence and dissemination of the isolates resistant to fluoroquinolone and/or third-generation cephalosporin (e.g., ciprofloxacin and cefotaxime), the recommended first-line drug treatment for Salmonella infections (Antunes et al., 2016).

Salmonella enterica subsp. enterica serovar Indiana (S. Indiana) is one of the most common foodborne Salmonella serovars, being isolated from poultry meat (Gong et al., 2017; Hu et al., 2018a; Zhang et al., 2022). In recent years, S. Indiana, expressing a high level of antimicrobial resistance (AMR) to many common antimicrobial compounds, has been epidemiologically linked with an increasing tendency of infections (Hu et al., 2018b). It was reported that the high levels of multidrug resistance (MDR) of S. Indiana might be associated with the presence of a variety of mobile elements such as plasmids carrying complex resistance mechanisms and class 1 integrons (Gong et al., 2016). These features increase the risk of treatment failure in the clinical infections caused by serovars of Salmonella.

Following our previous surveillance on the quantitative prevalence of Salmonella contamination in raw whole chicken carcasses at the retail (Zhu et al., 2014; Hu et al., 2017, 2018a), we obtained a subset of 224 ciprofloxacin and cefotaxime (CIP-CTX) co-resistant S. Indiana isolates. This study aimed to sketch an overall view on AMR and related mechanism for CIP and CTX of this set of S. Indiana from chicken sources in parts of China, and to provide an insight into the genomic characteristics of a CIP-CTX co-resistant S. Indiana strain CS664, that expressed concurrent resistance to colistin mediated by mcr-1 was also characterized at the meanwhile.

Materials and methods

Strains study collection

In our previous study (Hu et al., 2018a), 2,629 Salmonella were recovered from 1,680 retail whole chicken carcasses (including freshly slaughtered, chilled and frozen samples) between August 2010 and March 2012 at seven sites located in six provinces of China referring to North, Northeast, Northwest, South and East part of China. All strains were confirmed as belonging to the Salmonella genus and 463 (17.6%, 463/2629) of them were then identified as S. Indiana by serotyping, 224 of which were further identified as CIP-CTX co-resistant S. Indiana by antimicrobial susceptibility testing (AST), and these isolates were distributed as below: Beijing (n = 99); Jiangsu (n = 42); Shaanxi (n = 16); and Guangdong (n = 7).

Antimicrobial susceptibility testing

A panel of fourteen antimicrobial agents, representing ten classes, were selected for these 224 selected CIP-CTX co-resistant S. Indiana isolates for AST by broth micro-dilution, the drugs included ampicillin (AMP), cefotaxime (CTX), ceftazidime (CAZ), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), imipenem (IPM), nalidixic acid (NAL), ampicillin-sulbactam (SAM), trimethoprim-sulfamethoxazole (SXT), tetracycline (TET), meropenem (MEM), florfenicol (FFC), and colistin (CT). Minimal inhibitory concentration (MICs) value data for all compounds were recorded manually, and these data obtained were interpreted following the recommendations of CLSI (M100-S28 and M31-A3) and EUCAST (version 2018); extended-spectrum beta-lactamase (ESBL) production was then
screened according to the protocol and breakpoints described in CLSI (M100-S28) as previously described (Hu et al., 2017). *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC™700603 were included as quality control microorganisms in AST assays and ESBL confirmation, respectively.

**CIP and CTX resistance mechanism analysis**

Molecular methods were used to determine AMR genes and to show the relevant resistance mechanism. Genomic DNA was extracted with a TIANamp bacterial DNA kit (DP302, Tiangen Biotech) from all isolates. PCR detections for both quinolone resistance determinant regions (QRDRs) including *gyrA*, *gyrB*, *parC*, and *parE* genes and PMQR-encoding genes including *qnrABCDS*, *qepA*, *oxaAB*, and *aac-(6′)-ib-cr* were carried out according to the study reported previously (Ling et al., 2003; Park et al., 2006; Jacoby et al., 2008; Liu et al., 2008; Yamane et al., 2008; Cavaco et al., 2009; Kim et al., 2009; Bai et al., 2016). Additionally, two multiplex PCR reactions were used for the detection of ESBL genes including *blaCTX-M* groups and subtyping was also performed based on the methods reported previously (Eckert et al., 2004; Xu et al., 2005).

**mcr-1–mcr-10 gene screening**

All 224 CIP-CTX-resistant *S. Indiana* isolates were screened to investigate the presence of *mcr-1–mcr-10* genes by multi-target PCR methods as previously reported (Rebelo et al., 2018; Borowiak et al., 2020; Lei et al., 2020). To achieve the aim of addressing the full resistance phenotypic pattern for any *mcr* gene positive *S. Indiana* isolate screened in this study, an extended AST was carried out, using additional selected antimicrobial agents relevant to Enterobacteriaceae, including 13 classes (composing 27 compounds) in total and all procedures of AST were processed according to the AST processing workflow described above.

**Whole-genome sequencing**

A single colony of each CIP-CAZ-CT co-resistant isolate was cultured overnight in brain heart infusion broth (Beijing Landbridge, China) at 37°C. Genomic DNA was extracted and purified using a TIANamp Bacterial DNA extraction kit (DP302, TIANGEN BIOTECH, China) and then sequenced with the SMRT® Pacific Biosciences (PacBio) RS II platform (Tianjin Biochip Corporation, Tianjin, China) with a 10-kbp template library preparation step with PacBio® Template Prep Kit. SMRT Analysis v2.3.0 was used for *de novo* assembly according to RS Hierarchical Genome Assembly Process (HGAP) workflow v3.0. Subsequently, Consed version 28.0 was used to manually inspect and trim duplicate ends to generate single, complete, and closed sequences for each chromosome and plasmid. For data error correction, Pilon v1.23 was used with Illumina MiSeq sequencing read data, for which a library was prepared with a NEBNext Ultra DNA Library Prep Kit for Illumina (NEB#E7370) followed by sonication fragmentation (350bp insert), and then loaded on the Illumina HiSeq platform with PE 150 sequencing strategy (Novogene, Beijing, China) using a HiSeq X Ten Reagent Kit v2.5 (Illumina, San Diego, CA). The closed genomes were deposited in National Center for Biotechnology Information (NCBI) and automatically annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAP, version 4.8).

**Bioinformatic analysis**

The predicted serotype and multi-locus sequence typing (MLST) types were identified using the *Salmonella In Silico* Typing Resource (SISTR), Plasmid replicon types (Incompatibility groups or Inc. groups) and antimicrobial resistance genes were identified through the Center for Genomic Epidemiology (CGE) website with PlasmidFinder (v2.0) and ResFinder (v3.0), respectively. Virulence factors and related virulence genes were predicted with VFanalyzer of the VFDB (virulence factor database, current status of August 10, 2022). All genes, plasmids, and chromosome sequences used in this study were managed, aligned, and analyzed by Geneious prime (v2019.2.3) software. Plasmids referring to several publicly published *S. Indiana* sequences were selected for genomic structure comparison against the three plasmids of *S. Indiana* CFSAspect664 in this study, and map generation was performed by BRIG (v0.95). Genetic environments were analyzed and displayed using Easyfig (v2.2.2).

**Plasmid conjugal transfer**

In line with a previous study (Zhang et al., 2021), the transferability and frequency of colistin resistance were investigated by broth mating conjugation experiments with plasmid-free and sodium azide-resistant *E. coli* J53 as the recipient strain. The transconjugants were selected on MacConkey agar plates (Beijing Landbridge, China) supplemented with 100mg/L sodium azide (Sigma-Aldrich) and 2mg/L colistin (Sigma-Aldrich). Two different conjugation temperatures (30°C and 37°C) were used for the transfer of *mcr-1*-carrying plasmid in this study. Transfer frequencies were calculated as the number of transconjugants after confirmation by PCR. The colistin MIC values of the *E. coli* J53 and transconjugants were tested according to the description above.

**Results**

**Antimicrobial resistance phenotypes of 224 isolates to 14 tested compounds**

The percentages related to AMR for all 244 CIP-CTX co-resistant *S. Indiana* recovered from poultry are shown in
TABLE 1. A table showing the antimicrobial resistance profiles of 224 CIP-CTX co-resistant Salmonella Indiana against a panel of 14 antimicrobial agents including resistant isolate numbers and rates.

| Antimicrobial drugs | Agent classes | Region/Province (number of isolates/%) |
|---------------------|---------------|--------------------------------------|
|                     |                | Beijing (n = 99)                      |
|                     |                | Jilin (n = 60)                        |
|                     |                | Jilang (n = 42)                       |
|                     |                | Shaanxi (n = 16)                      |
|                     |                | Guangdong (n = 7)                     |
|                     |                | Total (n = 224)                       |
| Carbenemens         | Impenem (IMP) | 0 (0.0)                              |
|                     | Meropenem (MEM)| 0 (0.0)                              |
| (Fluoro)Quinolones  | Nalidixic acid (NAL)| 99 (100.0)| 60 (100.0) | 42 (100.0) | 16 (100.0) | 7 (100.0) | 224 (100.0) |
|                     | Ciprofloxacin (CIP)| 99 (100.0)| 60 (100.0) | 42 (100.0) | 16 (100.0) | 7 (100.0) | 224 (100.0) |
| Cephalosporins      | Cefotaxime (CTX)| 99 (100.0)| 60 (100.0) | 42 (100.0) | 16 (100.0) | 7 (100.0) | 224 (100.0) |
|                     | Ceftazidime (CAZ)| 11 (11.1)| 27 (45.0) | 23 (54.8) | 7 (43.8) | 2 (28.6) | 70 (31.3) |
| Penicillins         | Ampicillin (AMP)| 98 (99.0)| 60 (100.0) | 42 (100.0) | 16 (100.0) | 7 (100.0) | 223 (99.6) |
| β-Lactam combination agents | Ampicillin-sulbactam (SAM)| 93 (93.9)| 57 (95.0) | 42 (100.0) | 16 (100.0) | 7 (100.0) | 215 (95.6) |
| Aminoglycosides     | Gentamicin (GEN)| 81 (81.8)| 58 (96.7) | 39 (92.9) | 16 (100.0) | 7 (100.0) | 201 (89.7) |
| Tetracyclines       | Tetracycline (TET)| 83 (83.8)| 40 (66.7) | 29 (69.1) | 16 (100.0) | 7 (100.0) | 175 (78.1) |
| Folate pathway      | Trimethoprim-sulfamethoxazole (SXT)| 88 (88.9)| 49 (81.7) | 38 (90.5) | 14 (87.5) | 7 (100.0) | 196 (87.5) |
| Phenicols           | Chloramphenicol (CHL)| 96 (97.0)| 60 (100.0) | 38 (90.5) | 16 (100.0) | 6 (85.7) | 216 (96.4) |
|                     | Florfenicol (FFC)**| 95 (97.0)| 58 (96.7) | 37 (88.1) | 16 (100.0) | 6 (85.7) | 212 (94.6) |
| Polymyxin           | Colistin (CTX)**| 0 (0.0)| 0 (0.0) | 1 (2.38)| 0 (0.0) | 0 (0.0) | 1 (0.4) |
| ESBLs               |                 | 92 (91.9)| 60 (100.0) | 42 (100.0) | 16 (100.0) | 7 (100.0) | 217 (96.9) |

*Interpretation according to the CLSI guidelines M31-A3, 2008.
*Interpretation according to EUCAST clinical breakpoints, 2018.
*Used as a feed additive in animal production.

Table 1 and Supplementary Table 1. All expressed resistance to at least four categories of antimicrobial compounds. The resistance rates were divided into three classes: (1) higher than 78%: CIP, CTX, NAL, AMP, CHL, SAM, FFC, GEN, SXT, and TET; (2) 31.3%: CAZ; (3) lower than 2%: CT, IMP, and MEM. No isolate was found to be resistant to carbapenem-type compounds (IMP and MEM). Minor resistance differences were observed between 8. Indiana isolated from different provinces, with the exception of CAZ and CT. Isolates from different regions showed a CAZ-resistant rate ranging from 11.1% (Beijing) to 54.8% (jiangsu), and one colistin-resistant strain recovered from jiangsu province, CFSA664, was identified and designated as a CIP-CTX-CT co-resistant isolate. Klebsiella pneumoniae ATCC™700,603 showed a 3 twofold concentration MIC value decrease for CTX tested in combination with clavulanate vs. when tested alone, the ESBL confirmation for all tested isolates was acceptable. All except seven isolates (3.1%, 7/224) from Beijing were confirmed as ESBL producers.

Antimicrobial resistance patterns of 224 isolates

The antimicrobial resistance patterns are shown in Table 2 and Supplementary Table 1 for all 224 CIP-CTX co-resistant 8. Indiana isolates tested. There were 2(0.89%), 1(0.45%), 12 (5.36%), 81 (36.16%), 127 (56.70%), and 1(0.45%) isolates resistant to 4, 5, 6, 7, 8, and 9 classes of antimicrobial agent tested, respectively. All 224 isolates were defined as MDR (resistant to three or more classes of antimicrobial drugs) and 209 (93.3%) of them were identified as high-level MDR (resistant to seven or more classes of antimicrobials). Among which, only one isolate was identified to be co-resistant to nine in ten classes of all drugs tested, with the resistant pattern of GEN-CHL-CIP-NAL-AMP-SAM-TET-CTX-SXT-FFC-CT. In total, 26 different AMR profiles were recorded among 224 S. Indiana isolates. The most prevalent MDR profiles identified were GEN-CHL-CIP-NAL-AMP-SAM-TET-CTX-SXT-FFC (86/224, 38.39%), followed by GEN-CHL-CIP-NAL-AMP-SAM-CAZ-CTX-SXT-FFC (38/224, 16.96%). The isolates from different regions showed some MDR difference, for instance, the isolates showed co-resistance to 4, 5, and 6 classes of drugs only recovered from Beijing, Jilin, and Jiangsu provinces, while the isolates recovered from Shaanxi (n = 16) and Guangdong (n = 7) provinces showed co-resistance to 7, 8, and 9 classes of drugs.

CIP and CTX resistance-encoding genes

Antimicrobial resistance genes detected for the 224 CIP-CTX co-resistant S. Indiana were listed in Table 3 and Supplementary Table 1. Five types of amino acid substitution were identified in QRDRs. Among the isolates studied, 164 (73.2%), 164/224 possessed the dominant amino acid substitution in GyrA
TABLE 2 The antimicrobial resistance pattern of 224 CIP-CTX co-resistant S. Indiana isolates.

| Antimicrobial resistance pattern | Regions | Number (rate/%) | Co-resistant drug classes | Number (rate/%) |
|--------------------------------|---------|----------------|--------------------------|----------------|
| CIP-NAL-AMP-SAM-CTX            | Jiangsu (1) | 1 (0.45) | 4 | 2 (0.89) |
| CIP-NAL-AMP-TET-CTX            | Beijing (1) | 1 (0.45) | | |
| CHL-CIP-NAL-AMP-CAZ-CTX-SXT-FFC| Jilin (1) | 1 (0.45) | 5 | 1 (0.45) |
| CHL-CIP-NAL-AMP-SAM-CAZ-CTX-SXT-FFC | Jilin (1) | 1 (0.45) | 6 | 12 (5.36) |
| GEN-CHL-CIP-NAL-AMP-CTX-SXT-FFC| Beijing (1) | 1 (0.45) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-CTX-FFC| Beijing (4) | 4 (1.79) | | |
| GEN-CHL-CIP-NAL-AMP-TET-CTX-FFC| Beijing (1) | 1 (0.45) | | |
| GEN-CHL-CIP-NAL-AMP-TET-CAZ-CTX-SXT-FFC | Beijing (1) | 1 (0.45) | | |
| GEN-CIP-NAL-AMP-CTX-SXT-FFC    | Beijing (1) | 1 (0.45) | | |
| GEN-CIP-NAL-AMP-SAM-TET-CTX    | Jiangsu (1) | 1 (0.45) | | |
| CHL-CIP-NAL-AMP-SAM-TE-TCT-SXT-FFC | Beijing (16), Jiangsu (2) | 18 (8.04) | 7 | 81 (36.16) |
| GEN-CHL-CIP-NAL-AMP-SAM-CAZ-CTX-SXT-FFC | Jilin (8), Jiangsu (7) | 15 (6.70) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-CTX-SXT | Jiangsu (1) | 1 (0.45) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-CTX-SXT-FCC | Beijing (11), Jilin (8), Jiangsu (3) | 22 (9.82) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-TET-CAZ-CTX-SXT-FFC | Jilin (2) | 2 (0.89) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-TET-CAZ-CTX-FCC | Jilin (8), Jilng (1) | 9 (4.02) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-TET-CAZ-CTX-FFC | Beijing (5), Jilng (1), Jilng (1), Shannxi (2) | 9 (4.02) | | |
| GEN-CHL-CIP-NAL-AMP-CTX-SXT-FFC | Beijing (1) | 1 (0.45) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-CTX-SXT-FCC | Jiangsu (1) | 1 (0.45) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-CTX-SXT-FFC | Beijing (1), Guangdong (1), Jiangsu (1) | 3 (1.34) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-TET-CTX-SXT | Beijing (1) | 1 (0.45) | 8 | 127 (56.70) |
| GEN-CHL-CIP-NAL-AMP-SAM-TET-CAZ-CTX-SXT | Beijing (1), Jiangsu (1) | 2 (0.89) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-TET-CAZ-CTX-SXT-FCC | Beijing (8), Guangdong (2), Jiangsu (14), Jilng (7), Shannxi (7) | 38 (16.96) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-TET-CT-SXT-FFC | Beijing (46), Guangdong (4), Jiangsu (7), Jilng (22), Shannxi (7) | 86 (38.39) | | |
| GEN-CHL-CIP-NAL-AMP-SAM-TET-CTX-SXT-FFC-CT | Jiangsu (1) | 1 (0.45) | 9 | 1 (0.45) |

(S83F and D87N) and ParC (T57S and S80R), along with other substitutions in GyrA (S83F and D87G) and ParC (T57S and S80R) being observed in 56 isolates (25.0%, 56/224). These two allelic types accounted for 98.2% (220/224) of all CIP-CTX co-resistant S. Indiana isolates. Two isolates were identified with amino acid substitutions in GyrA (D87G) and ParC (T57S), while one isolate showed a substitution type of GyrA (S83F) and ParC (T57S). One isolate containing an amino acid substitution in GyrA (D87G) and ParC (T57S) was found. No amino acid change was observed for GyrB and ParE.

PMQR-Encoding genes, especially qepA, oqxAB, and aac (6’)-Ib-cr, were identified in all 224 S. Indiana tested, while qnrS and qepA genes were also detected in 25 (11.2%) and 5 (2.2%) isolates, respectively (Table 3 and Supplementary Table 1). One hundred and ninety-five isolates were harboring only the qepA and aac (6’)-Ib-cr genes without any qnr or qepA genes, which represented 87.1% (195/224) of this collection. In addition to the qepA and aac (6’)-Ib-cr genes, there were 24 and 4 isolates carrying qnrS gene and qepA gene separately, and one isolate was observed to carry both qnrS and qepA genes, which also possessed two point mutations in QRDRs including amino acid substitutions in GyrA (S83F) and ParC (T57S). The PMQR genes qnrA, qnrB, qnrC, and qnrD were not detected.

Among 217 ESBL positive CIP-CTX co-resistant S. Indiana, we identified five subtypes of bla<sub>CTX-M</sub> gene, including bla<sub>CTX-M-10</sub> (n = 165), bla<sub>CTX-M-14</sub> (n = 19), bla<sub>CTX-M-27</sub> (n = 11), bla<sub>CTX-M-23</sub> (n = 9), and bla<sub>CTX-M-79</sub> (n = 8), while five isolates were bla<sub>CTX-M</sub> group negative. Of these, isolates with the bla<sub>CTX-M</sub> gene ranked the top with a rate of 76.0% (165/217) overall. The QRDRs, PMQR, and ESBLs genotypes of 224 CIP-CTX co-resistant S. Indiana are shown in Table 3 and Supplementary Table 1.

Screening for mcr gene-positive isolate

The complete collection was tested for the presence of the mcr-1–mcr-10 genes and only one S. Indiana (0.45%, 1/224) denoted as CSFA664 was found to be positive for mcr-1 without any other mcr genes being detected, and this isolate was also detected as the only isolate exhibiting CTX-CIP-CT co-resistance. This isolate was recovered from a chilled, packaged chicken sample collected from a supermarket in Yangzhou city in Jiangsu province in 2011.
TABLE 3 A table recording the QRDRs, PMQR, and ESBLs genotypes of 224 CIP-CTX co-resistant S. Indiana.

| QRDRs amino acid substitutions | Genes | β-Lactamase genes | Total number |
|--------------------------------|-------|--------------------|--------------|
| Gyra                           | ParC  | ParE               | **BLACS**    |
| S83E, D87N                     | T57S  | S80R               | N/A (n = 1)  |
| S83E, D87G                     | T57S  | S80R               | N/A (n = 1)  |
| D87G                           | T57S  |                    | N/A (n = 1)  |
| S83F                           | T57S  |                    | N/A (n = 1)  |
| S83L, D87N                     |       |                    | N/A (n = 1)  |

QRDRs: quinolone resistance determinant regions.
PMQR: plasmid-mediated quinolone resistance.
ESBLs: extended-spectrum β-lactamases.
No substitution detected.
InDels: Lots of nucleotide insertions and deletions were observed in parC gene.
Four in seven were ESBL negative.
One in two was ESBL negative.

Antimicrobial susceptibility testing profile for **mcr** gene-positive isolate

The MIC values and resistance phenotypes against an extended panel of 27 antimicrobial compounds for this isolate were listed in Table 4. The isolate showed an MIC value of 4 mg/L which was a relevant low level of colistin resistance (2–8 mg/L) but was susceptible to ceftriaxone, cefepime, three carbapenem drugs, aztreonam, and tigecycline, while expressing an intermediate resistant phenotype to cefotaxime, nitrofurantoin, and polymyxin B. Further, this isolate was confirmed as an ESBL positive strain and demonstrated an MDR phenotype against ten classes (16 kinds) of antimicrobial compounds.

Genome features of **Salmonella Indiana** CFSA664

Based on the genome data, CFSA664 was confirmed as S. Indiana and identified as the MLST type of ST17 by the SISTR platform. Sequencing provided 88,121 reads in total with a mean read length of 8,319 bp and coverage of 85.69x. There were 4,760 coding genes and 176 pseudogenes along with 120 RNA genes (22 rRNAs, 86 tRNAs and 12 ncRNAs) within this genome from the prediction of NCBI PGAP annotation, and it consisted of a single circular chromosome (4,733,813 bp, 45.4% GC content). The replicon type of plasmid pCFSA664-1 included IncHI2, IncHI2A, IncN, and RepA-pKPC-CATV1321, presenting a multiple Inc. type in the same plasmid; plasmids pCFSA664-2 and pCFSA664-3 were denoted as IncP1 and IncI2 replicon types, respectively. The virulence factors (VF) of CFSA664 predicted from VFDB database were shown in Supplementary Table 2.

Resistome of **Salmonella Indiana** CFSA664

As shown in Table 4, two point mutations were identified in the gyrA and parC genes on the chromosome of S. Indiana CFSA664, and these gave rise to amino acid substitutions of GyrA (S83F, D87N) and ParC (T57S, S80R), mediating the quinolone resistance phenotype detected. A total of 26 antimicrobial resistance-encoding genes were identified in the genome of S. Indiana CFSA664, including two copies of the sul2 gene on the same plasmid pCFSA664-1. No AMR gene was found on the IncP1-type plasmid pCFSA664-2. The aac(6’)-Ib-cr and the mcr-1 gene were the only resistance genes localized to the chromosome and the IncI2 plasmid pCFSA664-3, respectively. All of the other 24 plasmid-mediated antimicrobial resistance-coding genes, including three ESBL genes (**BLACS**), and Tn3 family transposase, were located on the same plasmid pCFSA664-1. Twenty-one of these 24 genes were mapped within an ~56-kbp locus (230,792–31,620 bp, Figure 1) that contained more than 22 mobile genetic elements (MGE) including insertion sequence (IS) elements and transposon CDSs, such as IS3, IS4, IS5, IS6, IS91, and Tn3 family transposase, and also IS6-, IS69-, and Tn3-like element (IS26, IS1006, ISV5a3, and Tn4313) family transposase. Additionally, 16 ORFs and 11 hypothetical proteins were also included in this region. BLASTn comparisons indicated that a high similarity (100% coverage value and 99.99% nucleotide identity) of this region was observed mapping to a plasmid pD90-1 (GenBank no. CP022451.1) from an S. Indiana D90, which was isolated from a...
whole chicken carcass collected in a poultry slaughterhouse in 2012 in Henan, China and carrying four different Inc. type plasmids (HI2/HI2A/N/Q1-, I2-, N/X1-and unknown-Inc types). In terms of the remaining three of the 24 resistance genes [mph(A), aph(3′)-IIa, and tet(A)], they were separated and positioned at ~84-, 88- and 203-kbp, respectively on plasmid pCFSA664-1, with the number of transposase CDSs varying from 1 to 4 (Figure 1). Other than these AMR genes observed in plasmid pCFSA664-1, a ter gene operon (terABCDFWXYZ), required for mediating resistance to potassium tellurite, was situated in front of tet(A) gene, positioning at 180–200-kbp (Figure 1).

### Structure of mcr-1-carrying plasmid pCFSA664-3

A total of thirteen plasmids, which were collected from Enterobacteriaceae (E. coli, n = 8; Salmonella, n = 4; Klebsiella, n =1) isolates from GenBank and had an almost identical backbone structure, were used for structural comparative analysis with plasmid pCFSA664-3 (Figure 2). Plasmid pCFSA664-3 could be differentiated from these other plasmids by an insertion sequence element IS3 transposase, a phage tail protein, and hicA-coding protein. In terms of the mcr-1 gene region, all plasmids

| Antibiotic class | Antibiotic agent (abbreviation) | MIC (mg/L) | R/I/S* | Chromosome | Plasmid |
|------------------|---------------------------------|-----------|-------|------------|--------|
| Penicillins      | Ampicillin (AMP)                | ≥32       | R     |            |        |
| β-Lactam combination agents | Ampicillin/sulbactam (SAM) | ≥32/16    | R     |            |        |
| Cephalosporins   | Cefotaxime (CTX)                | ≥16       | R     |            |        |
|                  | Cefotaxime+clavulanate (CTX + CLA) | 0.12/4   | –     |            |        |
|                  | Cefazidime (CAZ)                | ≥16       | R     |            |        |
|                  | Cefazidime+clavulanate (CAZ + CLA) | 0.5/4    | –     |            |        |
|                  | Cephaplatin (KF)                | ≥32       | R     |            |        |
|                  | Cefoxitin (FOX)                 | 16        | I     |            |        |
|                  | Ceftiamine (CRO)                | 0.25      | S     |            |        |
|                  | Cefepine (FEP)                  | 2         | S     |            |        |
| Carbapenems      | Imipenem (IMP)                  | 0.12      | S     |            |        |
|                  | Meropenem (MEM)                 | 0.03      | S     |            |        |
|                  | Ertapenem(ETP)                  | 0.5       | S     |            |        |
| Monobactams      | Aztreonam (ATM)                 | 0.12      | S     |            |        |
| Aminoglycosides  | Gentamicin (GEN)                | ≥16       | R     | aac(6′)-Iaa |        |
|                  | Amikacin (AK)                   | ≥64       | R     |            |        |
| Tetracyclines    | Tetracycline (TET)              | ≥16       | R     | tet(A)     |        |
|                  | Tigecycline (TGC)               | 0.25      | S     |            |        |
| (Fluoro)Quinolones | Nalidixic (NAL)               | ≥32       | R     | GyrA (S83F, D87N) |        |
|                  | Ciprofloxacine (CIP)            | ≥8        | R     |            |        |
| Folate pathway inhibitors | Trimethoprim/sulfamethoxazole (SXT) | ≥8/152 | R | sull, sul2, dfrA17 |
|                  | Trimethoprim (TMP)              | ≥16       | R     |            |        |
| Phenicsols       | Chloramphenicol (CHL)           | ≥32       | R     | floR, catB3 |        |
| Nitrofurans      | Nitrofurantoin (NIT)            | 64        | I     |            |        |
| Polymyxins       | Polymyxin E (Colistin, CT) a,b   | 4         | R     | mcr-1 a,b |        |
|                  | Polymyxin B                     | 4         | I     |            |        |
| Fosfomycins      | Fosfomycin (FOS)                | ≥32/16    | R     | fomA3      |        |
| Rifampicin c     | –                                | –         | –     | arr-3      |        |
| Macrolide d      | –                                | –         | –     | mph(A)     |        |
| Disinfectant resistance e | –                                | –         | –     | oqxAB, qacEΔ1 |      |

| Resistance genes or point mutation | | |
|-----------------------------------|--------------------------|
| chromosome                        | plasmid                 |

*R, resistant; I, intermediate; S, susceptible; R/I/S according to the CLSI guidelines M100-S28, 201.
*1R/I/S according to the CLSI guidelines M31-A3, 2008.
*Used as a feed additive in animal production.
*R/I/S according to EUCAST clinical breakpoints, 2018.
*aac(6′)-Iaa gene only got one copy but mediated two resistance mechanisms.
*bqxAB and oqxAB genes only got one copy but mediated two resistance mechanisms.
*two sul2 genes were detected on the same plasmid but different position.
*mcr-1 gene was located on plasmid pCFSA664-3 while other plasmid-mediated antimicrobial resistance-coding genes localized at pCFSA664-1.
*iNo AST data but with antimicrobial-resistant genes predicted from genome data.

### TABLE 4 Antimicrobial susceptibility of CIP-CAZ-CT co-resistant S. Indiana CFSA664 to a panel of antimicrobial agents.
demonstrated a high degree of sequence conservation. In plasmid pCFSA664-3, the sequences relevant to shufflon C appeared to be disrupted by IS3 transposase, a feature which was missing in all other plasmids. Plasmid pHNSHP45 (Accession number: KP347127), belonging to the first isolate reported to contain an mcr-1 gene (Escherichia coli strain SHP45), was devoid of three more sequences for encoding DNA primase, DnaJ, and XRE transcriptional regulator. Several conjugal transfer proteins were observed in these plasmids, and the mcr-1 gene was the only acquired gene conferring resistance to a known antimicrobial agent. A zinc transporter gene was also located near the mcr-1 and relaxase locus.

**Genetic environment context of mcr-1 gene**

To better understand the genetic environment of the mcr-1 locus of plasmid pCFSA664-3, sequences in different plasmids, belonging to three replicon types, were extracted and compared...
(Figure 3). This analysis revealed that the mcr-1 genes in three IncI2 type plasmids (pCFSA664-3, pHNSHP45, and pCFSA244-2) were found to be located between a PAP2 family protein-encoding gene (arrowed in yellow) and a relaxase-encoding gene (arrowed in green). In the case of plasmid pHNSHP45, an IS30 family element ISApI was followed by its relaxase-encoding gene downstream. In
infections both in the clinical setting and in food-producing animals. At the same time, this feature does not link with the original statistical description for general S. Indiana isolates (Gong et al., 2017). A resistant rate of 31.3% to CAZ, another important first-line third-generation cephalosporin, was observed. Only one isolate exhibited colistin resistance while none were resistant to imipenem and meropenem. Though compared with Beijing, Jilin, and Jiangsu, less CIP-CTX co-resistant S. Indiana isolates were obtained from Shaanxi and Guangdong provinces, they showed a distinct high level of multi-drug resistance to the compounds we tested.

**Transfer by conjugation of plasmid pCFSA664-3**

In this study, plasmid pCFSA664-3, the mcr-1-carrying plasmid in S. Indiana CFSA664, could transfer into E. coli J53 at relatively high frequencies (1.6 × 10⁻⁴) per recipient (30°C) and 2.2 × 10⁻⁴ per recipient (37°C). This was confirmed following the selection of 5 mcr-1-positive transconjugants (E. coli J53 + pCFSA664-3) by PCR, with MIC values of 4 mg/L to colistin, similar to that described previously for CFSA664.

**Discussion**

Based on the surveillance data relevant to the serotype distribution and antimicrobial resistance of Salmonella from chicken sourced in different regions of China in our previous study (Hu et al., 2018a), we re-characterized the isolates that were expressing resistance to both ciprofloxacin and cefotaxime, all of which were identified as serovar Indiana, suggesting that CIP-CTX co-resistant S. Indiana had a broad geographical distribution. High antimicrobial resistance rates (78% was the lowest) to most of the tested compounds were observed in the S. Indiana study collection. This is not surprising since these drugs are routinely used for the treatment of Salmonella infections both in the clinical setting and in food-producing animals. At the same time, this feature does not link with the original statistical description for general S. Indiana isolates (Gong et al., 2017). A resistant rate of 31.3% to CAZ, another important first-line third-generation cephalosporin, was observed. Only one isolate exhibited colistin resistance while none were resistant to imipenem and meropenem. Though compared with Beijing, Jilin, and Jiangsu, less CIP-CTX co-resistant S. Indiana isolates were obtained from Shaanxi and Guangdong provinces, they showed a distinct high level of multi-drug resistance to the compounds we tested.

**Double amino acid substitutions in GyrA** (S83F and D87N) and in ParC (S80R and T57S) were noted among all tested strains. In contrast, fewer isolates were identified to be positive for the other two PMQR genes (qnrS, n = 25; qepA, n = 3), especially for qepA. Our data showed various acquisition types for transferable PMQR genes among our isolates. Our findings highlighted the fact that PMQR confers low-level quinolone resistance, but previous studies indicated these markers could facilitate the subsequent emergence of high-level resistance via mutation(s) in one or more of the topoisomerase genes (Hernández et al., 2011).
Seven kinds of \( \text{bla}_{\text{CTX-M}} \) variants were identified among 407 S. Indiana ST17 isolates collected from foods, patients, and environments in 16 provinces of China from 2002 to 2018, and among these variants, \( \text{bla}_{\text{CTX-M-65}} (n = 55) \) was the most common one in ST17 isolates, followed by \( \text{bla}_{\text{CTX-M-25}} (n = 14), \text{bla}_{\text{CTX-M-14}} (n = 6), \text{bla}_{\text{CTX-M-27}} (n = 6), \text{bla}_{\text{CTX-M-122}} (n = 3), \text{bla}_{\text{CTX-M-15}} (n = 1) \) and \( \text{bla}_{\text{CTX-M-90}} (n = 1). \) Although \( \text{bla}_{\text{CTX-M-43}} \) was also the most common \( \text{bla}_{\text{CTX-M}} \) subgenotype identified among the S. Indiana isolates in this study, it remains to be clarified how this event emerged, and further research on the selection, dissemination, and maintenance of MGE carrying this gene may provide some clues, as \( \text{bla}_{\text{CTX-M-43}} \) is often located on conjugative plasmids, which might contribute to the rapid expansion of ceftriaxone resistance through conjugation among Enterobacteriaceae (Zhang et al., 2022).

Three \( mcr-1 \) gene positive (0.88%, 3/341) Salmonella were detected among 341 Salmonella isolated from 1,234 raw retail meat samples collected in Beijing, China, 2017 (Lyu et al., 2021). Another study, investigating Salmonella isolated from farms, downstream abattoirs, and markets of chickens in Sichuan province of China, showed a relevant higher percentage of \( mcr-1 \) gene (8.3%, 2/24; 14.3%, 4/28; 13.9%, 5/36; Ma et al., 2017). One \( S. \) *typhimurium* cultured from RTE prepared pork was found to contain the \( mcr-1 \) gene was identified among 30 Salmonella isolates (3.33%, 1/30) recovered from RTE foods collected from mainland China in 2014 (Wang et al., 2018). According to our latest data from China national food safety risk surveillance network, 18 in more than 5,100 foodborne Salmonella recovered from various kinds of foods collected in mainland China between 2011 and 2020 were detected as \( mcr-1 \) positive, corresponding to 9 serotypes and accounting for a positivity rate of 0.35% (data not published), which could be regarded as a low prevalence rate (Hu et al., 2021). In this study, only one \( mcr-1 \)-carrying isolate CFS664 was recovered among 224 CIP-CTX co-resistant S. Indiana (0.45%), expressing MIC values of 4 mg/L, which were consistent with \( mcr-1 \)-mediating low-level colistin resistance (2–8 mg/L; Zhang et al., 2019).

From the whole genome data, three plasmids (pCFS664-1–3) were detected in S. Indiana CFS664. We identified a 56-kbp locus similar to a *Salmonella* Genomic Island (SGI) located on plasmid pCFS664-1, with several different AMR genes and various insertion sequence elements or transposases. This SGI-like region might have undergone a series of unique events designed to capture and integrate resistance genes with the help of these ISs or transposases, resulting in stabilization and subsequent dissemination of these resistant genes. Three different types of \( \beta \)-lactamase were also observed at the same time in this SGI-like region, a feature that is rarely observed in a plasmid of S. Indiana. In terms of plasmid pCFS664-2 and the \( mcr-1 \)-carrying plasmid pCFS664-3, several Enterobacteriaceae plasmids submitted to NCBI were found to share the same backbone structure, highlighting the successful spread of these plasmids among S. Indiana and other genera in the Enterobacteriaceae family. Our results support the fact that plasmids could contribute to the gene movement and the rapid spread of the \( mcr-1 \) gene worldwide. This also indicated that the plasmids identified in this study might need further investigation for their transferability, the impact on bacterial cell fitness due to the maintenance of the conjugative plasmids, and how they are involved in the rapid spread of multiple antimicrobial-resistant determinants. Hence, surveillance for the emergence of these types of \( S. \) enterica serovar Indiana should be undertaken as a routine public health protection measure (Dong and Soong, 2021).

After all, there are some limitations of this study as below: (1) The *Salmonella* Indiana isolates in this retrospective study were recovered from chicken samples collected from 2010 to 2012 and in only six provinces of China, and isolates from newly collected samples from more regions of prolonged monitoring would give more accurate results; (2) Whole genome sequencing technology would help to state the genetic evolution analysis or traceability, and to explore the phylogenomic relationship and genetic diversity among this batch of Salmonella Indiana isolates; (3) Pathogenicity data for the severe MDR CFS664 would help with assessing the overall food safety or public health risk to humans in combination of the antimicrobial resistance data.

**Conclusion**

This study described the phenotypic AMR patterns and mechanisms for ciprofloxacin and cefotaxime co-resistant *Salmonella* Indiana of retail chicken origin from China. S. Indiana exhibited a highly drug-resistant phenotype in our study. One \( mcr-1 \)-carrying isolate with co-resistance to ciprofloxacin, cefotaxime, and colistin was identified. The complexity of the ESBL and MDR phenotype, AMR genes, and the transferability of the \( mcr-1 \)-harboring plasmid demonstrated subjective public health risk. Our study as we know, AMR One Health Strategies had already focused on the access and the use of antimicrobials in clinical for human medicine, but in food production, which is another major area of the use for antimicrobial compounds, has not yet systematically adapted to achieve the one health objectives (George, 2019), so continuous surveillance and the One Health strategy are needed to limit the emergence of S. Indiana across the food chain.

**Nucleotide accession numbers**

The genome data of chromosome and plasmid sequences of S. Indiana CFS664 were deposited in the NCBI nucleotide database under BioProject no. PRJNA498334 with Biosample no. of SAMN10292850. Related accession numbers were: CP033356, CP033353, CP033354, and CP033355.

**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/genbank/, CP033356, CP033353, CP033354 and CP033355.

Author contributions

YuH performed the literature search. YuH, YiH, FL, and SF designed the research. YuH, YiH, SN, CL (4th author), XG, WW, YD, CL (5th author), and JX performed the experiments and collected the data. YuH, YiH, SN, CL (4th author), WW, and JX analyzed and interpreted the data and finished the figures and tables. YuH, YiH, SN, and CL (4th author) wrote the manuscript. FL and SF reviewed and edited the manuscript. YuH, YiH, SN, CL (4th author), FL, and SF have accessed and verified the underlying data. All authors contributed to the article and approved the submitted version.

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We state that all authors had full access to the full data in the study and accept responsibility to submit for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.955827/full#supplementary-material

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