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Whole-genome sequencing-based characterization of *Salmonella enterica* Serovar Enteritidis and Kentucky isolated from laying hens in northwest of Iran, 2022–2023

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Abstract

Background The transmission of *Salmonella* spp. to human through the consumption of contaminated food products of animal origin, mainly poultry is a significant global public health concern. The emerging multidrug resistant (MDR) clones of non-typhoidal *Salmonella* (NTS) serovars, have spread rapidly worldwide both in humans and in the food chain. In this study NTS strains were isolated from diseased laying hens in Iran and were further studied by whole-genome sequencing (WGS) to investigate the prevalent serovars, multilocus sequence types, antimicrobial resistance and virulence genes.

Results Out of eight isolated *Salmonella* spp. six were identified as *S.* Enteritidis serovar ST11 (n = 5) or ST5824 (n = 1), and two isolates were recognized as *S.* Kentucky serotype ST198 lineages. The aminoglycoside resistance gene *aac(6′)-Iaa* was the most frequently detected gene being present in all serovars, but it did not confer phenotypic resistance to corresponding agents (tobramycin and amikacin). All *S.* Enteritidis isolates carried a single GyrA D87N/Y substitution. Other identified antimicrobial resistance genes (ARGs) including *tetA*, *floR*, *sul1*, *dfrA1*, *aph(3′)-Ia* and double *gyrA* and *parC* mutations conferring high-level ciprofloxacin resistance (CIP^R) (MIC ≥ 16mg/L) were only found in *S.* Kentucky isolates. The comparison of phenotypic and genotypic antimicrobial resistance (AMR) profiles revealed inconsistent results for some antibiotics. A total of 11 different *Salmonella* Pathogenicity Islands (SPIs) including SPIs-1, to 5, 9, 10, 13, 14, C63PI, CS54 and several virulence genes related to type III secretion system, adhesins, iron and magnesium uptake, serum and antimicrobial peptide resistance were detected among the isolates.

Conclusions Our study reports emergence of a highly MDR- CIP^R *S.* Kentucky ST198 clone form poultry associated sources in Iran. The presence of numerous virulence determinants, SPIs and ARGs in the examined NTS isolates poses a significant risk for food safety. The inconsistencies between the genotypic and phenotypic AMR profiles indicate that WGS data alone may not be always sufficient for guiding therapeutic strategies.

Keywords *Salmonella* Kentucky, ST198, *Salmonella* Enteritidis, Whole-genome sequencing, MLST, Antimicrobial resistance genes

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Introduction

Foodborne diseases are a widespread and serious public health issue worldwide. The majority of the food-related illnesses arise from pathogens that enter the human food chain at some point from farm-to-fork [1]. *Salmonella enterica* is one of the most common causes of food-borne zoonotic disease and is a major global public health concern. Over 2600 serotypes of *Salmonella* have been described that can be further divided into typhoidal and non-typhoidal *Salmonella* (NTS) serotypes based on the associated disease syndrome [2]. Typhoidal serovars, such as *Salmonella* Typhi and *Salmonella* Paratyphi are restricted to humans causing typhoid and paratyphoid fever (enteric fever). In contrast, NTS serovars have a broad host range infecting both human and animals. Poultry is the primary reservoir for various NTS serotypes among food-producing animals with contaminated poultry products, such as meat and eggs serving as the main sources of human salmonellosis [3]. Additionally, NTS transmission can occur through the consumption of fruits and raw vegetables contaminated by manure [4], or contact with pets including dogs, cats, rodents, reptiles, or amphibians [2]. The most epidemiologically significant NTS serotypes globally include *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) [5]. These serotypes typically, cause mild, self-limiting gastroenteritis, characterized by diarrhea, abdominal cramps and vomiting in immunocompetent adults. However, severe invasive disease with complicated extra-intestinal manifestations can occur in infants, the elderly, and immunocompromised individuals, including those with HIV [6]. *Salmonella enterica* serovar Kentucky (*S.* Kentucky) is one of the most prominent NTS serovars isolated form poultry carcasses in the USA [7]. However, the *S.* Kentucky sequence type 198 (ST198), often associated with high-level fluoroquinolone resistance, has emerged as a global human pathogen in several countries [8–10] and gained significant epidemiological importance. From a public health perspective, this particular serovar has the potential to become the most prominent *Salmonella* serotype in human salmonellosis infections [11].

Antibiotics used in poultry production for therapeutic, prophylactic, or growth promotion purposes drives the selection and emergence of antibiotic resistant bacteria which acquire resistance through chromosomal mutations and/or the horizontal transmission of antimicrobial resistance genes (ARGs). These resistant bacteria can be transmitted to humans through direct contact with contaminated poultry or consumption of their products [12]. Recently, resistance to clinically important antibiotics such as cephalosporins, quinolones and tetracyclines has been observed among *Salmonella* serovars [13–16] particularly Kentucky ST198 lineage [9]. The emergence and spread of multidrug-resistant (MDR) *Salmonella* serovars especially in poultry products pose serious public health risks, due to the limited range of treatment options. Therefore, it is crucial to continuously monitor the population structure and antimicrobial resistance profiles of *Salmonella* serovars across different geographical regions to prevent further spread and infection. While traditional antigen-based serotyping remains the first step in the epidemiological analysis of *Salmonella* infection in humans and animals, it lacks sufficient discriminatory power to assess the genetic relatedness of similar serovars, and may produce false results due to autoagglutination or loss of antigen expression [17]. In contrast, whole-genome sequencing (WGS) has been widely applied in epidemiological investigations for source tracking as well as virulence and antimicrobial resistance gene profiling of *Salmonella* serovars [18, 19]. In Iran, *S. enterica* significantly contributes to food born infections [20, 21]. However, the genomic features of *Salmonella* spp. in Iran remain largely unknown. Considering the poultry products as the major source of NTS transmission to humans and also lack of WGS-based studies assessing population structures of NTS in Iran, we aimed in this study to explore the genetic diversity, virulence genes, and genotypic as well as phenotypic antimicrobial resistance of *S. enterica* serovars recovered from laying hens.

Materials and methods

Sample collection and bacterial isolation

A total of 108 chicken carcasses showing gastrointestinal lesions indicative of salmonellosis were examined at the poultry disease clinic of the northwest of Iran between December 2022 and June 2023. Liver, gallbladder, spleen, oviduct and intestinal samples, each weighing approximately 2-3 g were aseptically collected and pre-enriched separately in 10 mL of buffered peptone water (BPW) then incubated at 37 °C ± 1 °C for 18–24 h. One milliliter of incubated BPW suspension was transferred to 10 mL Sodium Selenite broth (SSB) and incubated at 37 °C overnight. Approximately 10 µL of enriched SSB medium was then streaked onto xylose lysine deoxycholate (XLD) agar and incubated at 37 °C overnight. The plates were then examined for the presence of typical *Salmonella* colonies, which were subsequently purified on MacConkey agar or Eosin methylene blue (EMB) agar. A single non-lactose fermenting *Salmonella* colony was picked from EBM agar and inoculated into triple sugar iron (TSI) agar, Lysine Iron Agar (LIA), urea agar, Simmons citrate agar, Sulfide-Indole-Motility (SIM) media (Condalab, Madrid, Spain) for further biochemical identification. Colonies that exhibited characteristic *Salmonella* reactions- such

as an alkaline slant with acid butt on TSI, an alkaline slant and butt on LIA, positive for H_2S production, motility and citrate utilization, and negative urease and indole production- were preserved at − 80 °C in brain–heart infusion broth supplemented with 15% (vol/vol) glycerol.

Molecular Identification of *Salmonella* spp.

The presumptive *Salmonella* colonies were identified by detection of *invA* gene using polymerase chain reaction (PCR) and the specific primers (invA-F:5′-GTGAAA TTATCGCCACGTTCGGGCAA-3′and invA-R (5′-TCA TCGCACCGTCAAAGGAACC-3′) [22]. In brief, the DNA of isolated *Salmonella* were extracted using boiling method as described previously [23]. Briefly, pure colonies of each isolate were aseptically homogenized in 200 µL of TE buffer. The suspensions were boiled at 100 °C for 10 min and centrifuged at 13,000 rpm for 20 min. Thereafter, the supernatant was aseptically transferred to a new microcentrifuge tube and was directly used as a template for PCR reaction.

PCR was performed in 25 µL reaction volume under the following condition: 1 cycle of 95 °C for 10 min, 30 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s and a final extension of 72 °C for 10 min. The amplified DNA fragments were separated by agarose (1.5%) gel electrophoresis and visualized under UV light. Positive samples were further studied by WGS.

Enterobacterial repetitive intergenic consensus (ERIC)-PCR

ERIC- fingerprinting was used to detect the genetic relationship between *Salmonella* isolates. To this end, total bacterial DNA samples were isolated using the boiling lysate protocol as described above. DNA quantity (ng/µL) and quality were measured in a spectrophotometer. ERIC-PCR was performed using the primers ERIC-1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′AAGTAAGTGACTGGGGTGAGCG-3′) [24] under the following condition: one cycle at 95 °C for 6 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 10 min [25]. PCR amplicons were resolved on 2% agarose containing Safe DNA Gel Stain by electrophoresis and were visualized under the UV light.

Whole genome sequencing and data analysis

DNA samples were extracted from bacterial colonies using 195 µL of lysis buffer from the Maxwell® RSC Pathogen Total Nucleic Acid Kit, combined with 5 µL of lysozyme solution (10 mg/mL). The samples were incubated for one hour at 37 °C with shaking at 550 rpm. After incubation, genomic DNA was purified using the Maxwell® RSC Pathogen Total Nucleic Acid Kit (AS1890) on the Maxwell® RSC 48 Instrument, an automated system for nucleic acid isolation. The quality of the extracted DNA was assessed with the Qubit® 3.0 Fluorometer. High-quality DNA samples were then used for library preparation with the Nextera XT DNA Library Prep Kit (Illumina, CA, USA), following the manufacturer's instructions.

Short-read sequence fragments of 150-bp were produced by paired-end sequencing on an Illumina NextSeq 500 platform. The sequenced raw reads were trimmed with Trimmomatic and reads shorter than 20 bp were discarded. The high quality cleaned read files were then ensured using FASTQC, before further analysis.

Online tools from the "Center for Genomic Epidemiology" (http://genomicepidemiology.org/services/, accessed on 2 July 2024) were used to extract information about MLST sequence types, acquired antimicrobial resistance genes and chromosomal mutations associated with antimicrobial resistance (ResFinder v4.5.0 [26]), *Salmonella* pathogenicity island (SPIFinder v2.0,) [27] and plasmid replicons (PlasmidFinder v2.0.1) [28]; Moreover, Virulence Factor Database (VFDB) [29] was used to detect virulence factors.

A core genome phylogeny, based on 1000 single-copy core genes, was inferred for all isolates in this study, along with 77 isolates from different serovars and countries (Supplementary Table 1), as previously described [30]. We employed the general time-reversible model of nucleotide substitution with a gamma distribution to account for rate heterogeneity, performing 1000 alternative runs on different starting trees. Support values were calculated using 100 iterations of "Rapid" bootstrapping. The best-scoring maximum-likelihood topology was annotated and colored using iTOL online tool [31]. The FASTQ sequences of all strains were deposited in the NCBI sequence read archive with BioProject number PRJNA1152350.

Phenotypic antimicrobial susceptibility testing (AST)

Testing the susceptibility of isolates to colistin, and ciprofloxacin was performed using the reference broth dilution method. The Kirby–Bauer disc diffusion method was used for testing susceptibility to other antibiotics including ampicillin (10 µg), ceftriaxone (30 µg), gentamicin (10 µg), amikacin (30 µg), kanamycin (30 µg), tobramycin (10 µg), tetracycline (30 µg), nalidixic acid (30 µg), trimethoprim-sulfamethoxazole (1.25 µg/23.75 µg) and chloramphenicol (30 µg) (Liofilchem, Roseto degli Abruzzi, Italy). The susceptibility testing results were interpreted according to CLSI (M100-ED34, 2024) [32] guidelines for all antibiotics except colistin for which breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, V14.0) were used. *Escherichia coli* ATCC 25922 was used as a

Table 1 Genomic features of *Salmonella enterica* subsp. *enterica* serovars isolated from poultry

Strain no	Serovar	Isolation source	ST (MLST)	Antimicrobial resistance genes						Plasmid profile	SPIs
				Aminoglycoside	Tetracycline	Phenicol	Sulfonamide	Trimethoprim	Quinolone		
S9	*S.* Kentucky	Liver	198	*aac(6′)-Iaa*	*tet(A)*	*floR*			GyrA (S83F, D87N) ParC (T57S, S80I)	No hit found	C63PI, SPI-1,2,3,5,9
S10	*S.* Enteritidis	Liver	11	*aac(6′)-Iaa*					GyrA D87Y	IncFIB(S)-IncFII(S)	C63PI, CS54, SPI-1,2,3,5,9,10,13,14
S11	*S.* Kentucky	Liver	198	*aac(6′)-Iaa, aph(3′)-Ia*	*tet(A)*	*floR*	*Sul1*	*dfrA1*	GyrA (S83F, D87N) Par C (T57S, S80I)	IncI1-I(α)	C63PI, SPI-1,2,3,5,9
S12	*S.* Enteritidis	Liver	8524	*aac(6′)-Iaa*					GyrA D87N	IncFIB(S)-IncFII(S)	C63PI, CS54, SPI-1,2,3,4,5,9,10,13,14
S13	*S.* Enteritidis	Liver	11	*aac(6′)-Iaa*					GyrA D87Y	IncFIB(S)	C63PI, CS54, SPI-1,2,3,4,5,9,10,13,14
S14	*S.* Enteritidis	Liver	11	*aac(6′)-Iaa*					GyrA D87Y	IncFIB(S)-IncFII(S)	C63PI, CS54, SPI-1,2,3,4,5,9,10,13,14
S15	*S.* Enteritidis	gallbladder	11	*aac(6′)-Iaa*					GyrA D87Y	IncFIB(S)-IncFII(S)	C63PI, CS54, SPI-1,2,3,4,5,9,10,13,14
S16	*S.* Enteritidis	Liver	11	*aac(6′)-Iaa*					GyrA D87N	IncFIB(S)	C63PI, CS54, SPI-1,2,3,4,5,9,10,13,14

MLST Multilocus sequence typing, *ST* Sequence type, *SPI Salmonella* pathogenicity island

quality-control strain for antimicrobial susceptibility testing. The phenotypic AST results were compared with those obtained by WGS and the agreement between two approaches was assessed with Cohen's kappa statistical analysis using IBM SPSS (V26).

Results

Bacterial identification and MLST

Among the 108 samples studied, 8 presumptive *Salmonella* isolates were recovered on selective media. Seven isolates were obtained from liver and one from gallbladder. The recovered *Salmonella* serovars exhibited typical biochemical test results and were positive for *invA* gene by PCR. WGS identified six isolates as *Salmonella enterica* serovar Enteritidis (*S*. Enteritidis), with five belonging to ST11 and one to ST5824. The remaining two isolates were recognized as *S*. Kentucky serotype ST198 lineage (Table 1). Although the isolates were obtained from 8 different farms, ERIC-PCR typing (Fig. 1) and WGS-based phylogenetic tree analysis (Fig. 2) revealed a high degree of similarity among serovars within the same sequence types (STs).

Genotypic and phenotypic AST results

According to ResFinder database which identifies ARGs in bacterial whole-genome sequences, all eight *Salmonella* spp. isolates in our study carried at least one ARG. The aminoglycoside resistance gene *aac(6′)-Iaa* was the most frequently detected gene in all isolates (n = 8, 100%). The remaining ARGs were detected exclusively in *S*. Kentucky isolates and included tetracycline resistance gene *tetA* (n = 2), florfenicol/chloramphenicol resistance gene *floR* (n = 2), folate pathway inhibitor resistance genes, *sul1*, *dfrA1* (n = 1) and aminoglycoside [3] phosphotransferase *aph(3′)-Ia* (n = 1). A single mutation in quinolone resistance-determining regions (QRDRs) of GyrA (D87N/Y) was detected in all serovars, with *S*. Kentucky serovars showing additional mutations at *gyrA* and *parC* (Table 1). In total, 96 phenotypic AST results (n = 16, broth dilution and n = 80, disc diffusion) were obtained for 12 antibiotics tested in 8 isolates. Comparison of phenotypic and WGS-based AMR profiles yielded a concordance of 82.3% (n = 79 tests) with discordant results (17.7%, n = 17) being mainly related to phenotypically susceptible isolates harboring ARGs (kappa = 0.599, SE = 0.081, P value < 0.001). The discordances were mainly observed for amikacin and tobramycin, followed by tetracycline which were responsible for 94% and 5.8% of all detected discordances respectively. This issue was due to phenotypic susceptibility of all eight *aac(6′)-Iaa* positive isolates and a *tetA* positive isolate to amikacin/tobramycin and tetracycline respectively (Table 2). Based on phenotypic and genotypic AST results, all *S*. Enteritidis isolates were characterized with susceptibility to colistin, ampicillin, ceftriaxone, gentamicin, kanamycin, tetracycline, chloramphenicol and sulfonamides but resistance to nalidixic acid and non-susceptibility (intermediate) to ciprofloxacin (MIC = 0.12–0.25 mg/L) due to single GyrA D87Y/N substitution (phenotypic ciprofloxacin susceptibility testing results interpreted as intermediate were categorized as resistant here for comparison to WGS-based AST results). Despite displaying a different AMR profile as shown in Table 2, both *S*. Kentucky ST198 isolates in our study exhibited high-level resistance to ciprofloxacin (MIC ≥ 16 mg/L) which could be attributed to double mutations at *gyrA* (S83F, D87N) and *parC* (T57S, S80I) genes.

Plasmid profiles, *Salmonella* pathogenicity islands and virulence genes

The search for plasmids using PlasmidFinder revealed a high prevalence of IncFIB(S) in all 6.

S. Enteritidis serovars followed by IncFII(S) which was co-harbored by IncFIB(S) in four.

S. Enteritidis isolates. The IncI1-I(α) plasmid was harbored in a *S*. Kentucky serovar (Table 1). According to SPIFinder analysis, we identified 11 different *Salmonella* Pathogenicity Islands (SPIs) among studied isolates. All isolates including Kentucky and Enteritidis serovars contained SPI-1, SPI-2, SPI-3, SPI-5, SPI-9 and C63PI. The SPI-10, SPI-13, and SPI-14 and CS54 were present in all (n = 6), and SPI-4 was detected in five (83.3%) *S*. Enteritidis isolates respectively (Table 1). A detailed search for virulence factors using VFBD, revealed existence of about 123–135 virulence genes in each strain. The identified virulence genes were mainly related to Type III secretion system (T3SS) and its secreted proteins (such as *ssaC*, *ssaG*, *ssaH*, *ssaI*, *ssaJ*, *ssaK*, *ssaL*, *ssaM*, *ssaP*, *ssaQ*, *ssaR*, *ssaS*, *ssaT*, *ssaU*, *ssaV*, *spaP*, *spaQ*, *spaR*, *spaS*, *sipD*, *sipC*,*prgH*, *prgK*, *prgI*, *prgJ*, *invG*, *InvA*, *InvC*, *invE*, *spiC sptp*, *sopA*, *sopB*, *sopD*, *sopE2*, *sipA*, *sipB*), magnesium uptake *(mgtB/C)*, iron uptake (*entA*, *entB*, *ent*C, *entE*, *fepC*, *fepD*, *fepG*, *iroN*)), adherence (fimbrial *fimC*, *fimD*, *fimI*, *FimH*, *fimF*, *lpfA*, *lpfB*, *lpfE*, *lpfC*, *lfpD*, *csgA*, *csgB*, *csgC*, *csgF*, *csgE*, *csgG*, non-fimbrial adhesins *sinH*, *ratB*, *misL*, *shdA*,), resistance to antimicrobial peptides (*mig-14*) which were found in all isolates regardless of the serovar. However, *spvRABCD* and fimbrial *pefABCD* operons, as well as *rck* gene coding for serum resistance protein were only detected in *S*. Enteritidis isolates (Supplementary Table 2).

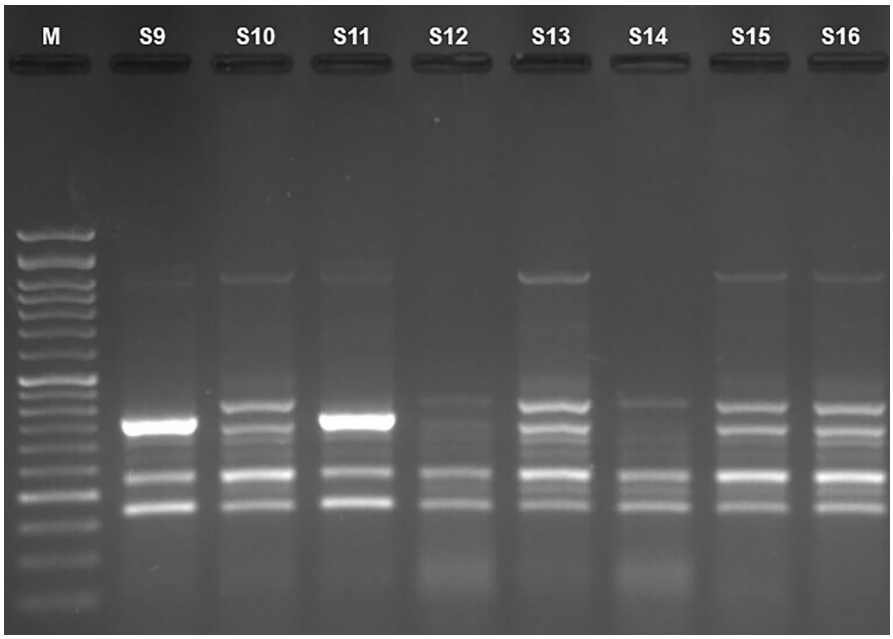

Figure. 1 DNA fingerprints generated by ERIC-PCR analysis of 8 *Salmonella* spp. recovered from laying hens on 2% agarose gel. Each lane is labeled with sample numbers S9 to S16; M, 50 bp DNA Ladder (SMOBio, Taiwan),

Discussion

The overuse and misuse of antibiotics in poultry farming significantly contribute to the development of resistant bacteria which can rapidly spread between animals and farms through the food chain. Consequently, food is a critical transmission pathway for antimicrobial resistant pathogens from animals to humans. According to national data from the Ministry of Health of Iran, NTS, is one of the leading causes of bacterial foodborne illness in the country resulting in a significant number of infections ranging from mild or asymptomatic to sever cases and even deaths. Indeed, a total of 832 *Salmonella*-related deaths were identified between 2013 and 2019 with 800 cases being associated with NTS and 32 being related to typhoid fever [21]. Although NTS is commonly isolated from different food-animal sources, poultry is the primary reservoir for several clinically important NTS serotypes [33]. The genomic background and AMR profiles of NTS serovars in Iran are largely unknown. In this study, we used WGS to analyze the genomic features of eight *Salmonella* spp. isolates belonging to Enteritidis and Kentucky serovars. The majority of *Salmonella* spp. isolated in our study were recognized as Enteritidis serovar predominantly assigning to ST11. This specific clone represents the most predominant MLST profile among *S.* Enteritidis isolates worldwide [34]. The severity of disease caused by *S.* Enteritidis and its AMR profile varies significantly across different geographic regions. *Salmonella* Infantis [15] and *S.* Typhimurium [35] from Turkey, *S.* Typhimurium from Pakistan [36] and *S.* Enteritidis from China [37] were reported to be the most common serovars isolated from animal sources or products. In this study, we report for the first time, the isolation of MDR-ciprofloxacin resistant $(CIP)^R$

S. Kentucky ST198 from animal sources from Iran. The serotype Kentucky is a foodborne pathogen (occurring commonly in laying hens) that causes gastroenteritis in humans, with its global spread largely attributed to contaminated poultry products [38]. While *S.* Typhimurium and *S.* Enteritidis are the main serovars involved in human salmonellosis with strong link to contaminated eggs and poultry meat, the association of serovar Kentucky with human illness has been documented in Europe, South-East Asia, China and Africa [9, 39]. This serovar, particularly ST198, is known for displaying resistance to several antibiotics commonly used to treat salmonellosis notably fluoroquinolones [9]. This globally emerging MDR clone with high-level ciprofloxacin resistance, has spread rapidly worldwide, from Africa, to the Middle East, Asia and Europe, affecting both humans and the food chain [40]. Phenotypic and genomic analysis of the AMR profile of Enteritidis and Kentucky serovars revealed a higher rate of AMR among *S.* Kentucky isolates specially, isolate S11, which showed an MDR phenotype displaying resistance to tetracycline, chloramphenicol, kanamycin, sulfamethoxazole/trimethoprim and nalidixic acid/ciprofloxacin (MIC = 32 mg/L) due to the presence of the *tetA*, *floR*, *aph(3′)-Ia*, *dfrA1* + *sul1*,

Figure. 2 Phylogenetic tree illustrating the core genome phylogeny of eight *Salmonella enteric* serovars obtained in this study (highlighted in red) and 77 serovars from diverse geographic regions. The tree was constructed from 1000 single-copy core genes shared across all study isolates and strains of human and animal origin from different geographic locations including Russia, India, Saudi Arabia, China, Taiwan, Turkey, USA, etc. (Supplementary Table 1). The maximum-likelihood method was employed, using the general time-reversible model with a gamma distribution to account for rate variation among sites. Support values were calculated from 100 "Rapid" bootstrap iterations, ensuring robust branch support. The best-scoring tree was visualized using the iTOL online tool, with color coding applied to distinguish study isolates and isolation type. The scale bar (0.001) indicates genetic distance, representing sequence divergence among isolates

and *gyrA* + *parC* double mutations respectively. Similarly, WGS analysis of *S.* Kentucky ST198 strains obtained from human clinical infections and poultry farms in north Africa between 2017 and 2020 revealed presence of at least ten ARGs among 92% of isolates and occurrence of mutations in QRDR of the *gyrA* and *parC* genes among all isolates [9]. On the other hand, *S.* Enteritidis isolates were phenotypically susceptible to all tested antibiotics except for quinolones. Nalidixic acid resistance and ciprofloxacin non-susceptibility observed in all *S.* Enteritidis isolates were linked to a single *gyrA* D87N/Y substitution, a mutation reported by several studies to be associated with this resistance phenotype [41, 42]. In contrast, multiple substitutions in both GyrA (positions S83, D87) and ParC (positions T57, S80) have been shown to result in high level fluoroquinolone resistance in *Salmonella* spp. [41]. In addition to an overall low antibiotic resistance rate in *S.* Enteritidis serovars, no resistance was detected against colistin and β-lactam antibiotics in any of studied NTS isolates. This is in contrast with results reported by Adiguzel et al. who reported ESBL positive and colistin resistant *mcr*-negative *Salmonella* spp. from Turkey [15]. Li et al. also reported high antibiotic resistance rate for ampicillin (95.2%), cefotaxime (81%) and tetracycline (61.9%) among NTS strains from commercial broiler carcasses in China [37]. Moreover, 24.29% of

Table 2 Phenotypic and genotypic WGS-based antimicrobial resistance profile of *Salmonella* spp

Strain no. -serovar	Phenotypic				Genotypic*	
	Broth dilution		Disc diffusion			
	CL MIC (mg/L)	CP MIC (mg/L)	S	R	S	R
S9- *S.* Kentucky	0.25 S	32 R	AM, CR, GM, KA, AK, TB, TE, SXT	CH, NA	AM, CR, GM, KA, CL, SXT	AK, TB, NA, CP, TE, CH
S10-*S.* Enteritidis	0.5 S	0.12 I	AM, CR, GM, KA, AK, TB, TE, CH, SXT	NA	AM, CR, GM, KA, TE, CH, CL, SXT	AK, TB, NA, CP
S11- *S.* Kentucky	0.25 S	16 R	AM, CR, GM, AK, TB	KA, NA, TE, CH, SXT	AM, CR, GM, CL	AK, TB, NA, KA, CP, TE, CH, SXT
S12-*S.* Enteritidis	0.5 S	0.25 I	AM, CR, GM, KA, AK, TB, TE, CH, SXT	NA	AM, CR, GM, KA, TE, CH, CL, SXT	AK, TB, NA, CP
S13-*S.* Enteritidis	0.25 S	0.12 I	AM, CR, GM, KA, AK, TB, TE, CH, SXT	NA	AM, CR, GM, KA, TE, CH, CL, SXT	AK, TB, NA, CP
S14-*S.* Enteritidis	1 S	0.12 I	AM, CR, GM, KA, AK, TB, TE, CH, SXT	NA	AM, CR, GM, KA, TE, CH, CL, SXT	AK, TB, NA, CP
S15-*S.* Enteritidis	1 S	0.25 I	AM, CR, GM, KA, AK, TB, TE, CH, SXT	NA	AM, CR, GM, KA, TE, CH, CL, SXT	AK, TB, NA, CP
S16-*S.* Enteritidis	0.25 S	0.25 I	AM, CR, GM, KA, AK, TB, TE, CH, SXT	NA	AM, CR, GM, KA, TE, CH, CL, SXT	AK, TB, NA, CP

CP ciprofloxacin, *CL* colistin, *AM* ampicillin, *CR* ceftriaxone, *GM* gentamicin, *AK* amikacin, *KA* kanamycin, *TB* tobramycin, *TE* tetracycline, *NA* nalidixic acid, *SXT* trimethoprim-sulfamethoxazole, *CH* chloramphenicol, *S* susceptible, *R* Resistant, *I* Intermediate (non- susceptible)

* WGS-predicted phenotype

NTS strains in a study from UK were characterized with MDR phenotype with resistance to ampicillin, streptomycin, sulphonamides and tetracyclines being the most common MDR profile [43]. We observed a correlation between phenotypic and genotypic AMR profiles based on WGS for most antibiotics tested, except for certain aminoglycosides and tetracycline for which false resistant results were obtained by WGS-based analysis. While the *aac(6′)-Iaa* was the most frequently detected gene, it did not confer resistance to any tested aminoglycosides and was found to be non-functional. Similarly, a *tetA* positive *S.* Kentucky serovar was found to be susceptible to tetracycline. Variants *of aac(6′)*, are reported to be transcriptionally silent in *Salmonella* spp. [44] with several studies reporting phenotypic aminoglycoside susceptibility among *Salmonella* serovars harboring *aac(6′)-Iaa* variants [43, 45]. Similarly, Guan et al. reported substantial inconsistencies between phenotypic and WGS-based AMR profiles for some *Salmonella* isolates which possessed specific resistance genes but showed phenotypic susceptibility to the corresponding agents or showed a resistance phenotype for some agents while did not contain corresponding resistance genes [46]. The results obtained by the phenotypic and genotypic AST of NTS population in a study from UK were highly correlated with only 2.18% discordant results [43]. The lack of correlation between the presence of ARG, and the corresponding phenotypic resistance has been reported in seafood-associated *Salmonella* strains from India [47]. These cryptic genes which are silent under certain conditions, may become active i*n vivo* or when transferred to a new host under the selective pressure of antibiotics [48]. Therefore, the mere presence of an acquired ARG in bacterial genome does not necessarily confer phenotypic resistance, necessitating the validation of genomic AMR results by phenotypic assays.

Salmonella's ability to invade various phagocytic and non-phagocytic cells and cause diseases depends on the production of several virulence factors, which are commonly encoded by specific regions of genome known as SPIs. To date, 24 SPIs have been identified and characterized with SPIs-1 to 5 commonly found in all serotypes of *Salmonella* [49]. SPIFinder analysis of studied bacterial genomes detected 11 different SPIs with SPI-1 to 3, SPI-5, SPI-9, and C63PI shared by all NTS isolates, regardless of the serotype. However, SPI-4, 10, 13, 14, and CS54 were only found in *S.* Enteritidis isolates. *S.* Enteritidis ST11 isolated from human, food and farm samples in China were reported to carry a similar set of SPIs [50]. SPI-1 and SPI-2 located next to the $tRNA^{Val}$ gene and between the *fhlA* and *mutS* genes, respectively, encode two distinct T3SSs (*T3SS-1*, *T3SS-2*). This syringe-like export system allows the bacterium to deliver multiple effector proteins to the host cell cytosol facilitating bacterial invasion, intracellular survival and macrophage apoptosis [51, 52]. Centisome 63 pathogenicity island (C63PI) essential for iron uptake was consistently present in all isolates, underscoring its crucial role in bacterial survival. This

PI has been reported in most *Salmonella* strains [53, 54]. SPI-3, SPI-5 and SPI-9 have been implicated in mediating the survival of *Salmonella* in macrophages, enteropathogenicity, and adhesion to epithelial cell surfaces respectively [55]. The constant presence of SPIs 1–5, 9, and C63PI has been documented in several studied *salmonella* Kentucky ST198 genomes [53].

All *Salmonella* isolates were found to carry various virulence genes coding for fimbrial and non-fimbrial adherence factors, T3SS, SptP and Sop effectors, iron and magnesium uptake system, and antimicrobial peptide resistance determinants regardless of serovar. However, *spvRABCD* operon, a significant virulence factor for some *Salmonella* serotypes was detected only in *S.* Enteritidis isolates. The two effector proteins encoded by this operon, SpvB (an actin-ADP-ribosyltransferase, preventing actin polymerization) and SpvC (a phosphothreonine lyase) are translocated into the host cell by T3SS-2, enhancing the virulence of NTS serovars and facilitating extra-intestinal disease [56]. Additionally, the serum resistance gene *rcK*, conferring high resistance to the bactericidal activity of the complement system [57] and the *pefABCD* fimbrial operon involved in adhesion to the intestinal epithelium [58] were detected only in *S.* Enteritidis.

Conclusion

While some serotypes such as Enteritidis and Typhimurium have garnered significant attention in Iran, the genomic background and epidemiology of other serovars remain poorly understood. Our study represents the first report of MDR- CIP^R *S.* Kentucky ST198 isolation form poultry associated sources in this region. Given the potential of this emerging antimicrobial-resistant enteric pathogen to cause human infections, its isolation from a non-human source may pose a significant public health challenge. High-level ciprofloxacin resistance due to multiple *gyrA* + *parC* mutations along with chloramphenicol and tetracycline resistance genes were found exclusively in *S.* Kentucky serovars. The excessive use of antibiotics in the poultry industry may explain the emergence of this highly resistant clone from an animal source in the country. Our study also revealed the presence of numerous virulence determinants and SPIs in the examined isolates, posing a significant risk to food safety. The discrepancies observed between phenotypic and genotypic AMR profiles suggest that WGS data alone may not always be sufficient for guiding clinical therapeutic strategies. Our findings highlight the need for increased monitoring of genomic features, virulence, AMR and epidemiology of NTS from both human and animal sources to identify alternative disease control and prevention measures, improve food safety and prevent further dissemination of MDR *Salmonella* clones.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13099-025-00679-3.

Supplementary material 1: Table S1: Details of 77 publicly available *Salmonella enterica* serovars from different hosts and regions used for construction of phylogenetic tree.

Supplementary material 2: Table S2: Genes coding for virulence factors in the studied *Salmonella* Enteritidis and *Salmonella* Kentucky serovars identified using Virulence Factor Database (VFDB).

Author contributions

SV performed the experiments (bacterial isolation, identification, AST), MH conceived and designed the study, analyzed the data and wrote the manuscript, AF was involved in sample collection and bacterial isolation, KM performed WGS, AG and MO performed the WGS data analysis. DMC supervised the WGS and data analysis workflow. All the authors have read and agreed to the published version of the manuscript.

Funding

This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability

The FASTQ sequences of all strains were deposited in the NCBI sequence read archive with BioProject number PRJNA1152350.

Declarations

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of University of Tabriz (Approved IRB No. IR.TABRIZU.REC.1402.124).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 30 August 2024 Accepted: 2 January 2025
Published online: 16 January 2025

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