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PREVALENCE AND WHOLE-GENOME ANALYSIS OF MULTIDRUG-RESISTANT *SALMONELLA* ISOLATED FROM CHICKEN CARCASSES IN HANOI

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SUMMARY

Salmonella enterica is one of the most dangerous food-borne pathogens posing a significant global concern especially to travelers returning from developing countries. Given that chicken is the main reservoir for *Salmonella*, the emergence and spread of multi-drug resistant *Salmonella* from chicken have not been fully described in Vietnam. The present study aimed to evaluate the phenotypic and genotypic antimicrobial resistances of *Salmonella* from chicken carcasses. Among 104 raw chickens collected from 5 districts in Hanoi city, 65 samples were contaminated with *Salmonella* of which the highest contamination rate was found in Thanh Xuan. A total of 63/65 (96.9%) of *Salmonella* isolates were resistant to at least one antibiotic and 61/65 (93.9%) of the isolates were found to be multidrug resistant. Whole-genome sequencing was employed to analyze 4 strains with high (12_S2 and 61_S18) and low (19_S4 and 8_S1) antimicrobial resistance patterns. Genomic analysis indicated the presence of 27 genes conferring antibiotic resistance. Genotypes were highly correlated to observed phenotypes in 4 strains. Importantly, extended-spectrum β-lactamase *bla*$_{\text{CTX-M-55}}$ and colistin resistance *mcr-3* were reported in isolates of *Salmonella enterica* serovar Typhimurium. This is the first report showing the prevalence and genome sequences of *Salmonella* from chicken carcasses collected in Hanoi, Vietnam. The results represented herein provided the basis to understand the dynamics of antibiotic resistance of *Salmonella* in Vietnam and to spot antimicrobial resistance determinants for early diagnosis.

Keywords: bla$_{\text{CTX-M-55}}$; chicken carcasses; multidrug resistance; *mcr-3*; Salmonella

INTRODUCTION

Salmonella is listed as a dangerous foodborne pathogen by the World Health Organization (WHO). In the US salmonellosis caused annually an estimation of 8,000 foodborne poisoning cases and 40 deaths [1]. In the period from 2016 to 2020, the European Food Safety Authority (EFSA) report indicated up to 100,000 cases of illness related to *Salmonella* infections annually with 700 to 1500 foodborne outbreaks that occurred in the whole continent in the same period (EFSA & ECDC, 2021). Among the *Salmonella* genus, *Salmonella enterica* is the common factor that causes salmonellosis. *Salmonella enterica* consists of 6 subspecies, which compose of more than 2600 serovars. *S. enterica* subsp. *enterica* is responsible for more

than 99% of human salmonellosis globally. There have been 1531 serotypes identified for *S. enterica* subsp. *enterica*, among which *S. enterica* subsp. Typhimurium and *S. enterica* subsp. Enteritidis stand out as the two dominant serovars since they were isolated in most infected cases [2].

The statistical data of foodborne illness outbreaks indicated that 95% of cases of salmonellosis were caused by contaminated food consumption [3]. According to the Centers for Disease Control and Prevention (CDC) report, poultry and eggs are the major sources of *Salmonella* infection, taking up more than 50% of the contaminated food in the US [4]. In Vietnam, the data for food safety monitoring showed that 48.7% of collected chicken samples in cities across Vietnam [5]and 49.62% of chicken samples in Ho Chi Minh city [6] were contaminated with *Salmonella*.

In Vietnam, antibiotics have been commonly used as the main solution for microbial infection control. However, inappropriate usage of antibiotics in agricultural and veterinary practice has led to the rise of antimicrobial resistance (AMR) bacteria and transferable genetic loci. Consequently, multidrug resistant (MDR) *Salmonella* infection in humans has spread and become a threat to public health [7, 8]. Previous studies reported that the persistence and dissemination of multiple resistant *Salmonella* serovars in the environment are due to the excessive application of antibiotics on land [9]. A recent study of the endemic *Salmonella* distribution in raw meat obtained from traditional markets in Ho Chi Minh city revealed that *Salmonella* isolates were resistant to multiple antibiotics, including tetracycline, ampicillin, chloramphenicol, streptomycin, and trimethoprim-sulfamethoxazole combination. Among these isolates, 37.89% were resistant to at least one antibiotic, 22.98% were resistant to two to five antibiotics and 8.70% were resistant to more than 6 antibiotics [6]. In addition to a high prevalence of *Salmonella* in broiler farms environment, 66.85% of isolated *Salmonella* exhibited resistance to 2-9 antibiotics including chloramphenicol, tetracycline, ampicillin, sulfamethoxazole/trimethoprim, and 62 multiple resistance patterns were observed in the Mekong Delta, Vietnam [10].

Although human and animal infections are linked to each other through the environment reservoirs, the transmission of antibiotics resistant *Salmonella* from animals and other environmental sources to humans is not fully understood. There have been different approaches to determine the subsequent transmission of antibiotic-resistant *Salmonella* in humans, animals, and environments such as pulsed-field gel electrophoresis (PFGE) [11], and multi-locus sequence-based typing (MLST) [12]. However, limitations of these methods lie in insufficient discriminatory power to separate closely related *Salmonella* isolates in outbreak investigations and to differentiate between the intraserovar isolates from different hosts. The use of whole genome sequencing (WGS) has shown a major impact on the study of molecular epidemiology of AR pathogens [13]. A WGS study in Denmark reported that single nucleotide polymorphisms (SNP), pangenome, and nucleotide difference trees were used superior to the classical typing method and were used to evaluate the association of the isolates to specific outbreaks of *S.* Typhimurium [14]. To date, only one report utilizing WGS for genomic investigation of foodborne pathogens in Vietnam [15]. Thus, this study aims to assess the prevalence of *Salmonella* contamination in chicken and to analyze the antibiotic-resistant genes, genotypes, and MLST of MDR *Salmonella* using WGS.

MATERIALS AND METHODS

Sample collection

A total of 104 raw chicken samples were collected in retail markets in 5 districts of Hanoi city including Ba Dinh (n=16), Cau Giay (n=32), Dong Da (n=17), Hoang Mai (n=20), and Thanh Xuan (n=19) during 2019. Each chicken sample (a whole chicken) was individually placed in a sterilized plastic bag. All samples were preserved in sample transport containers filled with dry ice

and sent to the laboratory within the same day for analysis.

Salmonella isolation

Isolation of *Salmonella* was performed according to the United States Department of Agriculture (USDA) standard methods for whole chicken samples [16]. In brief, each sample (a whole chicken) was aseptically placed in a sterile plastic bag, and 400 mL of buffered peptone water (BPW; Difco) was then poured into the plastic bag. The sample was shaken and rinsed throughout. Next, a portion of 30 mL of the rinsed fluid was added to 30 mL of BPW, then pre-enriched at 37°C for 18 - 24 h. Afterwards, 0.5 ± 0.05 mL and 0.1 ± 0.02 mL of the pre-enrichment culture were respectively transferred into 10 mL tetrathionate broth (TT; BD) and 10 mL modified Rappaport-Vassiliadis (mRV; BD), then incubated at 42 ± 0.5°C for 24 h. A loop of the enriched culture was streaked onto xylose–lysine–desoxycholate agar (XLD; BD) and Brilliant green sulfa agar (BGS; BD), which were subsequently incubated at 37°C for 24 h. Typical colonies were selected and cultivated on Tryptic Soy Agar (TSA; BD) for further investigations.

Selective colonies were subjected to biochemical tests such as polyvalent antisera O and H antigen for *Salmonella* identification (BD). In addition, *Salmonella* isolates were confirmed using Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI TOF) Mass Spectrometry (MS) on Vitek MS system (BioMerieux). For further studies, *Salmonella* strains were stored in 15% (v/v) glycerol at -80°C. *Salmonella* ATCC 14028, *Salmonella* ATCC 13076, and *Escherichia coli* ATCC 8389 were used as the quality control standard.

Antibiotic susceptibility test

All isolated strains were tested for antibiotic susceptibility using the disk agar diffusion method according to the laboratory protocol of the WHO Global Foodborne Infection Network. The antibiotic resistance results were interpreted as susceptible, intermediate, or resistant based on the standard breakpoints recommended by Clinical and Laboratory Standards Institute (CLSI) standard M100 (CLSI 2022).

Antibiotic compounds were used in the form of antibiotic disc (Roseto degli Abruzzi, TE) with cefuroxime (CXM, 30 µg), ceftriaxone (CRO, 30 µg), cefoxitin (FOX, 30 µg), cefazoline (CZ, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), cefotaxime + clavulanic acid (CTL, 30 + 10 µg), ceftazidime (CAZ, 30 µg), ceftazidime + clavulanic acid (CAL, 30 + 10 µg), cefotaxime (CTX, 30 µg), cefotaxime 30 µg + cloxacillin (CTC), ceftazidime (CAZ, 30 µg), ceftazidime + cloxacillin (CAC, 30 + 10 µg), meropenem (MRP, 10 µg), imipenem (IMI, 10 µg) were placed on the surface of the inoculated plates followed by incubation at 37°C for 16-18 h. *Escherichia coli* ATCC 25922 was used as the quality control standard. *Salmonella* strains resistant to more than three classes and more than one antibiotic in a single class were considered an MDR strain.

Genomic DNA extraction, whole genome sequencing and *de novo* assembly

02 extended spectrum β-lactam (ESBL)-producing and 02 non-ESBL-producing isolated strains were selected for whole genome sequencing (WGS). Genomic DNA was extracted from 1 mL of overnight culture grown in Brain Heart Infusion broth (BHI; BD) using a PureLink™ Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's protocol. Genomic DNA was sequenced using the Illumina MiSeq platform (Illumina) to generate paired-end 2- by 25- or 2- by 300-bp reads.

Quality control was performed by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Read trimming was carried out using Trimmomatic removing the sequencing adaptor [18]. *De novo* assembly was performed using SPAdes 3.15 [19].

Annotation and determination of antimicrobial resistant genes

Genome features were analyzed using 2

pipelines including Prokka [20] and Rapid Annotation using Subsystems Technology (RAST) [21]. Serotyping and multilocus sequence typing (MLST) were analyzed through MLST Tseeman tool (https://github.com/tseemann/mlst). The AMR gene screening was conducted based on the ResFinder databases [22], the Comprehensive Antibiotic Resistance Database (CARD) [23], and Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) [24]. AMR genes were only selected in cases of >50% coverage and >75% nucleotide identity. The serotype was identified though submitting raw data to to the *Salmonella In Silico* Typing Resource (SISTR) (https://lfz.corefacility.ca/sistr-app/) and SeqSero (github.com/denglab/ SeqSero2).

RESULTS

Prevalence of *Salmonella* spp.

In total, *Salmonella* was present in 65 out of 104 samples (62.5%) (Table 1). The highest infection rate was recorded in Thanh Xuan (75.0%, n=15) and Cau Giay (70.0%, n=21). The lowest level was observed in Dong Da (47.1%, n=8).

Table 1. Prevalence of *Salmonella* following the districts of samples.

Locations	No. of samples	No. of positive samples
Ba Dinh	16	8 (50.0%)
Cau Giay	32	21 (70.0%)
Dong Da	17	8 (47.1%)
Thanh Xuan	20	15 (75.0%)
Hoang Mai	19	13 (68.4%)
Total	104	65 (62.5%)

Antibiotic resistance profiles of *Salmonella* isolates

To determine antimicrobial-resistant profiles, 65 *Salmonella* strains were tested for their β-lactam antibiotic sensitivity. Out of 65 strains, 63 strains were resistant to at least one of 15 tested antibiotics. The most common resistances were to ampicillin (92.3%, n=60), cefazolin (92.3%, n=60), followed by cefuroxime (84.6%, n=55), cefotaxime (83.1%, n=54), ceftriaxone (83.1%, n=54), and ceftazidime (47.7%, n=31) (Fig. 1). These strains were most susceptible to cefoxitin (98.5%, n=64), ceftazidime (32.3%, n=21), ceftriaxone and cefotaxime (15.4%, n=10), and cefuroxime (13.8%, n=10). Of note, 54 isolates were able to synthesize β-lactamase enzyme AmpC and ESBL, accounting for 83.3%.

Phenotypic antibiotic resistance profiles of *Salmonella* strains showed the presence of 8 resistance patterns of *Salmonella* isolates to 11 groups of antibiotics. No isolate was sensitive to all tested antimicrobial preparations. Surprisingly, 61 of 65 isolates (93.9%) were considered MDR strains based on CLSI guidelines. The predominant resistance patterns in MDR strains were CZ-AMP and CXM-CZ-AMP as revealed by isolates 8_S1 and 19_S4, respectively (Table 2). Further investigation led to the identification of 2 highly resistant *Salmonella* isolates including 12_S2 and 61_S18 that showed CXM-CRO-CZ-CTX-CAZ-AMP phenotype, produced ESBL, and AmpC enzyme.

Whole genome sequencing and genome characteristics

To understand genotypic resistance to antimicrobials, strains 12_S2, 19_S4, 61_S18, and 8_S1 were sequenced by using the Illumina platform. Total reads yielded from 539,366 to 672,108 reads, leading to the identification of 385 – 518 contigs after *de novo* assembly (Table 3). *Salmonella* genome size ranged from 4,688,801 to 4,954,070 bp with an overall genomic size of 4,793,668 bp.

The GC content was around 52%. Genome annotation indicated the presence of 4,400-4,592 coding sequences. The raw data of 12_S2, 19_S4, 61_S18, and 8_S1 was deposited at GenBank (NCBI) under SRA accession numbers: SAMN30227442, SAMN30227443, SAMN30247552, and SAMN30246726. The draft genome sequences were available in GenBank under the BioProject accession number PRJNA868167, PRJNA868167, PRJNA868477, and PRJNA868473 (Table 3).

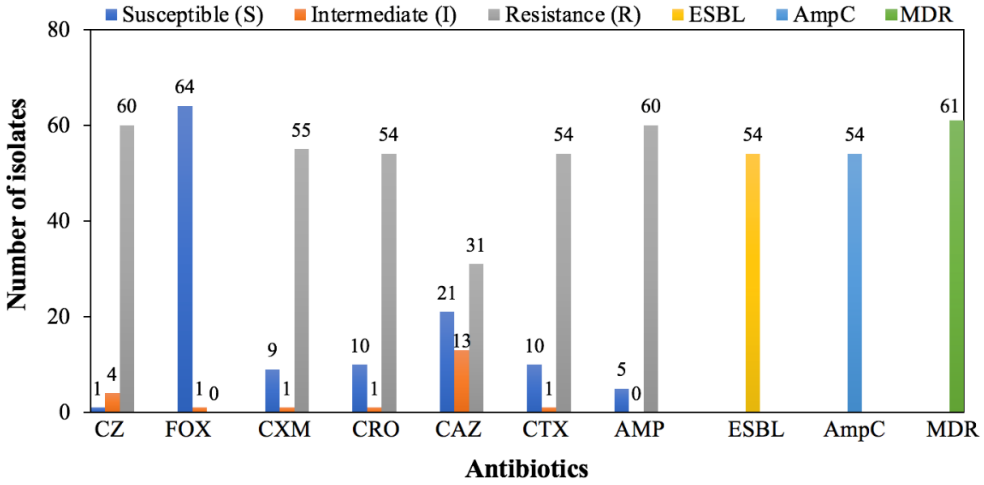

Figure 1. Antimicrobial resistance of *Salmonella* isolated from chicken carcasses. Cefazolin (CZ), cefoxitin (FOX), cefuroxime (CXM), ceftriaxone (CRO), ceftazidime (CAZ), cefotaxime (CTX), ampicillin (AMP), extended-spectrum β-lactam (ESBL), AmpC, multi-drug resistance (MDR).

Table 2. Representative resistance patterns displayed by *Salmonella* isolates.

Isolate	Resistance pattern	Location
12_S2	CXM-CRO-CZ-CTX-CAZ-AMP; ESBL and AmpC	Cau Giay
19_S4	CXM-CZ-AMP	Cau Giay
61_S18	CXM-CRO-CZ-CTX-CAZ-AMP; ESBL and AmpC	Cau Giay
8_S1	CZ-AMP	Dong Da

Note: cefazolin (CZ), cefuroxime (CXM), ceftriaxone (CRO), ceftazidime (CAZ), cefotaxime (CTX), ampicillin (AMP), Extended spectrum β-lactam (ESBL), AmpC, Multi Drug Resistance (MDR).

Table 3. Genomic characteristics of *Salmonella* isolates.

Strain	Contigs	Genome size (bp)	GC (%)	N50	CDSs	SRA accession no.	BioProject accession no.
12_S2	465	4,688,801	52.58	24,135	4334	SRR21052451	PRJNA868167
19_S4	483	4,954,070	52.14	24,679	4592	SRR21052450	PRJNA868167
61_S18	518	4,707,959	52.63	20,003	4400	SRR21051775	PRJNA868477
8_S1	385	4,823,844	52.29	28,113	4486	SRR21051816	PRJNA868473

Antibiotic resistance genes

The presence of AMR genes were identified by BLAST searching the assembled *Sal- monella* genomes against ResFinder, CARD), and ARG-

ANNOT databases. Indeed, 27 different AMR genes related to different antibiotic groups were identified in the genomes of 4 *Salmonella* strains (Fig. 2). These strains contained genes provoking resistance to drug classes including aminoglycoside (*aac(6')-Iaa*), multi-drug classes (*golS*, *mdsA*, *mdsB*, *mdsC*, *mdtK*, *sdiA*), and tetracyclin (*tetR*). The genes *aph(3)-Ib_5* and *aph(6)-Id_1* encoding for aminoglycoside phosphotransferases were only found in the 12_S2 and 61_S18 genomes, but not with 19_S4 and 8_S1. In addition, 61_S18 and 12_S2 carried β-lactamase $bla_{\text{CTX-M-55\_1}}$ (alternative name $bla_{\text{CTX-M-57}}$) found in the *Enterobacteriaceae* family that are known to confer resistance to the broad-spectrum of β-lactam antibiotic group, including amoxicillin, ampicillin, aztreonam, cefepime, cefotaxime, ceftazidime, ceftriaxone, piperacillin, and ticarcillin. In contrast, 19_S4 and 8_S1 were phenotypically susceptible to β-lactam despite carrying either $bla_{\text{TEM-1B\_1}}$ or $bla_{\text{TEM-1A\_1}}$ gene.

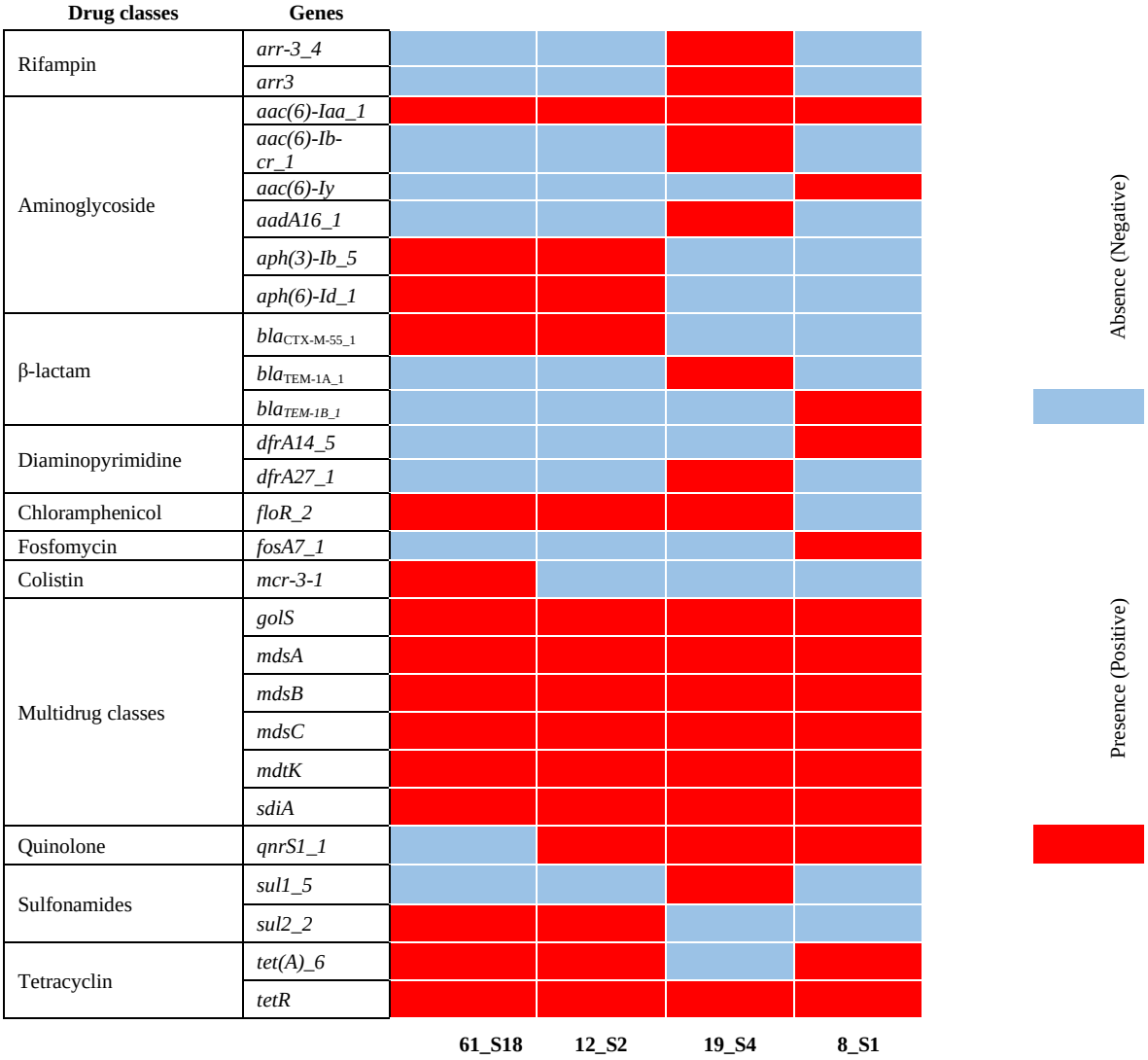

Figure 2. Distribution of antimicrobial resistance genes within 4 *Salmonella* genomes.

Table 4. Serotyping and MLST of *Salmonella* isolates.

Sample code	Serovar	Serogroup	H1	H2	O Antigen	MLST
8_S1	Agona	B	f,g,s	-	1,4,[5],12	13
12_S2	Typhimurium	-	l,v	1,6	3,{10}{15}	155
19_S4	Corvallis	C2-C3	z4,z23	-	8,2	1541
61_S18	Typhimurium	-	l,v	1,6	3,{10}{15}	155

Apart from that, quinolone resistance gene *qnrS1* was found in 3 (8_S1, 12_S2, and 19_S4*)* out of 4 genomes (Fig. 2). Three strains 12_S2, 19_S4, and 61_S18 were predicted to be resistant to chloramphenicol and sulfonamide due to the presence of chloramphenicol acetyltransferase *floR* and dihydropteroate synthase *sul*, respectively. Only 61_S18 harbored *mcr* gene conferring resistance to colistin which is a common antibiotic used to treat Gram-negative infection.

Identification of *Salmonella* serotypes

The raw sequencing reads of 4 strains were submitted to the *Salmonella In Silico* Typing Resource (SISTR) and SeqSero to predict the serotype. It revealed that strain 8_S1 was identified as *Salmonella enterica* serovar Agona, while 19_S4 was predicted to be *Salmonella enterica* serovar Corvallis (Table 4). The highly resistant strains 12_S2 and 61_S18 belonged to serovar Typhimurium. In support of these results, MLST analysis showed that sequence type (ST) 155 was observed in both *Salmonella enterica* serovar Typhimurium 12_S2 and 61_S18. Strains 8_S1 and 19_S4 were identified as ST13 and ST1541, respectively.

DISCUSSION

The rapid emergence and spread of AMR are particularly worrisome in *Salmonella* which is one of the most common causes of foodborne disease and an important cause of mortality worldwide. *Salmonella* frequently carries virulence factors and mobile elements, allowing them to accumulate and spread antibiotic resistance genes from other *Salmonella* strains and other species developed in the human digestive system [25]. Chickens are known to be the main reservoir for *Salmonella* spp. [26]. However, there is a lack of detective tools and information related to antibiotic resistance of *Salmonella* in Vietnam. The present study provided for the first time distribution and genome sequences of *Salmonella* from chicken carcasses collected in Hanoi, Vietnam.

Investigation of *Salmonella* spp. in raw chickens from Hanoi indicated that the overall rate of *Salmonella* was 62.5% and differed by location during 2019. In agreement with this result, the contamination rate of chicken meats with *Salmonella* was 63.6% in China during 2016-2017 and 65.7% in Thailand during 2014-2015 [27, 28]. Of note, a previous report in Vietnam showed that the overall prevalence of *Salmonella* isolates obtained from retail meats including chicken, pork, and beef was only 28%, among which the rate of chicken contaminated with *Salmonella* was 36% [29]. It proved that the contamination rate of *Salmonella* spp. in chicken samples had increased significantly. The different levels of contamination attribute to various factors such as environmental contamination, management systems, breed, sample size, and method of isolation [30, 31]. Thus, strict regulation on hygienic practices, processing, and circulation of meat is required to reduce salmonellosis outbreaks.

The present study also reported high AMR (96.9%) in *Salmonella* isolates recovered from chicken meat in Hanoi. Among them, 93.9% of strains are resistant to at least three antibiotic classes, which was around 1.5-fold higher than the prevalence of AMR recorded in 2018 (Nguyen et al., 2018)(Nguyen et al., 2018). In addition, lower levels were observed in Japan and India (Deekshit et al., 2012; Katoh et al., 2015).(Deekshit et al., 2012; Katoh et al., 2015).Similar to our study, a high

antibiotic resistance rate (90%) to at least one antibiotic (90%) was also found in Thailand and Cambodia [28]. The phenomenon could be due to the overuse of antibiotics for animals, and humans to prevent and treat infectious diseases caused by microorganisms in Vietnam.

The most important finding of this study was to employ whole genome sequencing to decipher AMR profiles of MDR *Salmonella* strains. As expected, in silico AMR gene predictions were highly relevant to phenotypic resistance in *S.* Agona 8_S1, Corvallis 19_S4, and Typhimurium 12_S2 and 61_S18. High resistance of *Salmonella* strains 12_S2 and 61_S18 to β-lactam antibiotics was attributed to the presence of $bla_{\text{CTX-M-55}}$ which is mainly located on the chromosome of *S.* Typhimurium. Typhimurium serotype is known as a new healthcare problem, leading to an increase in human diseases over the world [35]. Many studies showed that $bla_{\text{CTX-M-55}}$ and $bla_{\text{CMY-2}}$ are responsible for extended-spectrum cephalosporin resistance gene in *Salmonella* strains [36–38]. CTX-M-55 produced *Salmonella* was frequently detected in livestock meats and in the environment in Asia. In Vietnam, $bla_{\text{CTX-M-55}}$ and bla_{TEM} were only determined in extended-spectrum β-lactamase-producing *E. coli* isolated from Vietnam [39]. Despite having bla_{TEM}, *Salmonella* Agona 8_S1 and Corvallis 19_S4 were not resistant to cephalospotins indicating the role of $bla_{\text{CTX-M-55}}$ against third-generation cephalosporins. Hence, our study was the first report revealing the presence of $bla_{\text{CTX-M-55}}$ and bla_{TEM} located chromosomally in *Salmonella* strains from chicken carcasses.

Using whole-genome sequencing, *S.* Typhimurium 61_S18 was shown to contain the colistin resistance gene *mcr-3*. Given that colistin is a last-resort antibiotic in fighting MDR Gram-negative bacteria, the worldwide spread of plasmid-mediated colistin resistance is of major concern [40]. Originating from Asia, a number of *mcr-3*-positive isolates from human with *Salmonella* infection has rapidly spread in Denmark (Litrup et al., 2017). (Litrup et al., 2017). In Brazil, *S. enterica* isolated from food-producing animals showed high resistance to colistin although colistin is no longer used to treat infectious disease caused by *Salmonella* [42]. It could be due to genetic mobile elements such as *mcr*-like genes that can be horizontally transferred to other bacterial species of animal and human origin. More attention should be paid to the controlled use of colistin in poultry and agriculture to prevent the spread of colistin resistance.

CONCLUSION

Chicken meat is the main reservoir of *S. enterica* contamination. The present study showed a high prevalence of *Salmonella* spp. in chicken meats collected in Hanoi in 2019, indicating that *Salmonella* poses a high risk to both animals and humans. In line with that, the ability to resist antibiotics of subjected *Salmonella* isolates was recorded at a high rate. Whole genome sequencing accompanied with *in silico* analysis of AMR genes in *Salmonella* confirmed a high correlation between the phenotype and their corresponding genotype. Moreover, the presence of $bla_{\text{CTX-M-55}}$ and *mcr-3* was also determined in the chromosome of MDR *S.* Typhimurium. Thus, these findings highlighted the importance of whole-genome sequencing in monitoring AMR mechanisms in *Salmonella* from Vietnam and providing a future strategy to guide interventions against AMR increase.

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