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Isolation and molecular detection of *Salmonella enterica* serovars Typhimurium from poultry processing environments at wet markets in Sulaymaniyah province, Iraq

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Abstract

Non-typhoidal *Salmonella* is a predominant etiological agent of foodborne infections globally. *Salmonella* serovars are present throughout the chicken's digestive tract, particularly in the ceca. This study, conducted from October 2024 to April 2025, employed cultural methods to isolate and identify *Salmonella* from samples collected at the broiler chicken wet market in Sulaymaniyah province, Iraq. The research additionally examined the prevalence of *S. enterica s*erovar Typhimurium in these samples by PCR. Two-hundred and ten samples were obtained from the cecum, cutting boards, knives, body swabs, workers' hands, and water. *Salmonella* was isolated from the samples and identified by culturing and molecular techniques. Consequently, 103 individuals (49.04%) tested positive for *Salmonella via* culture. The PCR results revealed that the contamination rate for *S. enterica* was 74 (71.84%); 47 (63.51%) were *S. Typhimurium*, and 33 (70.21%) of *S. Typhimurium* possessed the *hilA* gene. The highest prevalence was observed in chopping boards, with 91.67% for *S. enterica*, whereas the maximum rate for *S. Typhimurium* was recorded in water, at 100%. The phylogenetic tree indicates that the Iraqi isolate with accession number of PV250092 belongs to the principal group of isolates, exhibiting a bootstrap support value of 100%, signifying a robust genetic association with them. To conclude, this research demonstrated a notable prevalence of *Salmonella* in the broiler chicken processing environment, particularly a high incidence of *S. Typhimurium* isolates. The placement of the Iraqi isolate within the global isolate clade suggests a recent common ancestor with other isolates, indicating global transmission pathways.

Introduction

Salmonella is a widespread zoonotic food-associated pathogen posing a serious worldwide public health concern.[1] Poultry may harbor *Salmonella* in their gastrointestinal tracts, being excreted *via* feces, resulting in environmental contamination. Meat and shell eggs are the primary sources of epidemics. *Salmonella* serovars can reside in nearly all regions of the chicken intestinal system, especially in the ceca. Infected chickens can asymptomatically shed the disease, transmitting it to healthy birds and flocks, contaminating corpses during slaughter, and infecting eggs by fecal exposure or trans-ovarial transmission.[2] *Salmonella* consists of two species, including *S. bongori* and *S. enterica.* Among these*, S. enterica* poses a greater threat, as it has more than 2,600 different serovars, according to the recent studies. While not all of these serovars are harmful to humans, some are. This species is responsible for typhoid fever and gastroenteritis. The *S. enterica* is a rod-shaped, Gram-negative bacterium that can survive facultatively anaerobic, and it is a part of *Enterobacteriaceae* family.[3,4] Being taxonomically split into six sub-species with a great degree of sequence similarity, around the world, non-typhoidal *Salmonella* is a major cause of foodborne illnesses.[5] The research indicates that human clinical cases linked to the chicken meat are predominantly attributed to *S. enterica* serovars Enteritidis and Typhimurium, accounting for 9.90 and 10.40% of cases, respectively.[6] *Salmonella Typhimurium* is widely present in the environment and can pose significant risks to both humans and animals over time. As a result, the food industry is under serious threat from this bacterium, being notorious for causing contamination in dairy, beef, pork, and poultry products.[7]

The complex pathogenicity of *Salmonella* is brought about by several structural and functional factors, such as

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capsules, adhesion factors, flagella-like *Salmonella* pathogenicity islands, and type 3 secretion systems.[8] *Salmonella*'s high virulence genes include *invA*, *agfA*, *hilA*, *sivH*, and *sopE*.[9] The *hilA* gene is a *Salmonella* pathogenicity island -I transcriptional regulator. The gene displays a considerable ability to link bacterial invasion gene expression to various environmental and regulatory signals.[10] The *iroB* gene is from the *iroA* gene group (*i.e.*, iroBCDEN), encoding the biosynthesis and export of *Salmonella* enterobactin and is regulated by ferric uptake regulator and situated within a highly significant DNA locus required to differentiate *S. enterica* subsp. *enterica*.[11] Further, the presence of *S. Typhimurium* and *S. Enteritidis* serovars was determined using the spy and sdfI genes, respectively.[12] It has been shown that *spy* is necessary for maintaining protein homeostasis in the periplasm under a variety of envelope stress conditions.[13]

In Sulaymaniyah province, Iraq, wet markets are quite common. These markets are usually not very clean, and water is constantly sprayed on the floors to keep them clean and maintain humidity. In traditional wet markets, it is a common practice to dress and process poultry outdoors. In addition to not wearing personal protective equipment, the chicken vendor is dressing the chicken with dirty utensils, like knives and cutting boards. Often, the entire dressed carcass is cleaned or washed using the same water.[14] Based on the significance of Salmonellosis in food at wet markets, in this research, we applied cultural techniques to isolate and identify *S. enterica* serovar Typhimurium from samples taken from a Sulaymaniyah province poultry wet markets using PCR.

Materials and Methods

Sampling. Samples were collected from poultry processing environments within wet markets in various regions of Sulaymaniyah province, in Iraq's Kurdistan region. The specific locations are illustrated in Figure 1, covering the period from October 2024 to April 2025. Samples were collected using sterile cotton swabs submerged in 10.00 mL of buffered peptone water (Liofilchim, Roseto degli Abruzzi, Italy). Using an insulated cool box, the samples were collected aseptically and quickly delivered to the Research Center at Sulaimani University's Veterinary Medicine College. The sample sources comprised chopping boards (n = 29), knives (n = 32), ceca from post-slaughter broilers (n = 75), body swabs (n = 27), workers' hands (n = 10), cages (n = 30), and the water used for washing the slaughtered broilers (n = 7). The samples were cultured on the same day they were obtained. This study has been examined and sanctioned by the Ethics and Research Registration Committee of the College of Veterinary Medicine at the University of Sulaimani, Sulaymaniyah, Iraq, with the approval number of VMUS.EC.Doc2-2025.

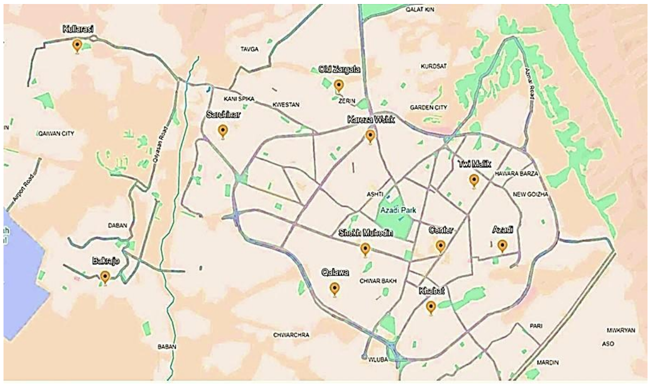

Fig. 1. Map of Sulaymaniyah province, Iraq, with the location of different wet markets pinned on the map.

Microbiological analysis. Sterile tubes with 10.00 mL of buffer peptone water (Liofilchim) were used to collect all samples, being then incubated for 24 hr at 37.00 ˚C. One milliliter of this pre-enrichment homogenate was added to 10.00 mL of tetrathionate broth (Liofilchim), and the mixture was incubated for 24 hr at a temperature between 37.00 and 42.00 ˚C. A sample of the tetrathionate broth was then plated onto xylose-lysine-deoxycholate agar (Liofilchim) after being initially plated onto MacConkey agar. These plates were incubated for roughly 24 to 48 hr at 37.00 ˚C. Biochemical tests, particularly the triple sugar iron and urease tests, were then used to analyze colonies showed traits typical of *Salmonella*.[15]

Extracting genomic DNA. Genomic DNA extraction was performed by boiling. The isolate was incubated at 37.00 ˚C for the entire night after being cultivated on nutrient agar (Liofilchim). Fresh colonies from the overnight culture were suspended again in 100 µL of nuclease-free water. After 50 min of steam heat treatment in a water bath at 99.00 ˚C, the suspension was chilled on ice for 5 min. After centrifuging the debris for 5 min at 15,000 rpm, the supernatant was used as a DNA template for PCR. To measure the DNA's concentration and purity, a Nanodrop spectrophotometer (Blue-Ray Biotech, New Taipei City, Taiwan) was used.[16,17]

Molecular detection of *S. enterica* serovars. Three primer pairs were utilized to conduct the PCR. The primer *iroB* (606 bp) was utilized to identify *S. enterica* spp.[18] The *spy* primer (401 bp) was employed to identify *S.* Typhimurium.[9] Additionally, the *hilA* primer (312 bp) was employed for the virulence gene (*hilA*).[12] Table 1 provides information regarding the target gene, primer sequences, annealing temperature, and amplified segment. The primers were manufactured by Macrogen (Seoul, South Korea).

Polymerase chain reaction amplification. For gene amplification, the Addbio Taq Master mix (PCR; Daejeon, South Korea) was employed. The 0.20 mL PCR tube was selected for this procedure. Each tube contained 1.00 µL (10.00 pmol) of both forward and reverse primers, 5.00 µL of DNA, and 10.00 µL of master mix. To achieve a total volume of 20.00 µL, an extra 3.00 µL of diethylpyrocarbonate (GetNet Bio, Daejeon, Korea) treated water was added. The cycling parameters for each primer were standardized in a single plex PCR using a thermocycler (Prime, Staffordshire, UK). The specific cycling parameters are outlined in Table 2. Afterward, the PCR results were evaluated by loading 7.00 µL of the PCR product onto a 1.00% agarose gel (TransGene, Beijing, China) in 1.00 X Tris/borate/ethylenediaminetetra-acetic acid buffer (Addbio). The gel was stained with 10.00 µL of Safe dye (Addbio), and electrophoresis was carried out for 60 min at 120 volts.

Sequencing of *S. Typhimurium* gene and phylogenetic tree examination. A commercial PCR purification kit was used to purify the PCR products, and Sanger sequencing was then used to sequence them, provided by Macrogen. The resultant sequences were trimmed and assembled utilizing BioEdit (version 7.2.5; Ibis Therapeutics, Carlsbad, USA). Using the Clustal W Multiple Sequence Alignment Program (UCD, Dublin, Ireland) technique built into the MEGA Software (version 11.0; Biodesign Institute, Tempe, USA), sequence alignments were carried out. Using bootstrap analysis with1,000 replicates and the Maximum Likelihood method based on Tamura-Nei model, the phylogenetic tree was built,[19] with values of 100% recorded at all nodes in the tree.

Results

Detection of *Salmonella* by cultural methods. A total of 210 samples were gathered from 24 wet markets specializing in broiler chickens within the Sulaymaniyah province. These samples were cultured on two media, including xylose-lysine-deoxycholate and MacConkey agars. Samples exhibiting black-centered colonies with red hues on xylose-lysine-deoxycholate agar and colorless colonies on MacConkey agar were positive for *Salmonella*. Out of the 210 samples tested, 103 (49.04%) displayed suspicious colonies on xylose-lysine-deoxycholate agar. In this investigation, biological assays were employed to verify the presence of *Salmonella*, characterized by an alkaline (red) slant, an acidic (yellow) butt, and some blackening at the base. Furthermore, the urea agar test indicated that the bacteria were urease negative, as there was no color change in the media. Table 3 illustrates that the cages of broiler chickens had the highest percentage of bacteria (53.34%).

Detection of *Salmonella* by molecular methods. The amplification of the *S. enterica*-specific gene *iroB* showed that 71.84% (74 out of 103) of the isolates were serovars of *S. enterica,* with the chopping board having the highest rate, 91.67%. All 74 *Salmonella* isolates underwent additional screening to check for the presence of *S. Typhimurium* serovars by targeting the *spy* gene. Forty-seven (63.51%) isolates were confirmed as *S. Typhimurium*.

Table 1. Primers used for molecular identification by PCR.

Genes	Primer sequence (5′-3′)	Annealing temperature	Length (bp)	References
iroB	F: TGCGTATTCTGTTTGTCGGTCC R: TACGTTCCCACCATTCTTCCC	55.00 °C	606	18
spy	F: TTGTTCACTTTTTACCCCTGAA R: CCCTGACAGCCG TTAGATATT	57.00 °C	401	9
hilA	F: GACAGAGCTGGACCACAATAAGACA R: GAGCGTAATTCATCGCCTAAAC	55.00 °C	312	12

Table 2. Cycling conditions for all genes used in the study.

Genes	PCR program
iroB	Initial denaturation at 94.00 °C for 5 min, then 30 cycles of: Denaturation at 94.00°C for 40 sec, annealing at 55.00 °C for 40 sec, extension at 72.00 °C for 40 sec, and final extension at 72.00 °C for 10 min.
spy	Initial denaturation at 95.00 °C for 2 min, then 30 cycles of: Denaturation at 95.00 °C for 1 min, annealing at 57.00 °C for 1 min, extension at 72.00 °C for 2 min, and final extension at 72.00 °C for 5 min.
hilA	Following a single cycle of denaturation at 94.00 °C for 5 min, there are 30 cycles, including denaturation at 94.00 °C for 5 min, annealing at 55.00 °C for 45 sec, and extension at 72.00 °C for 1 min. The final extension is performed at 72.00 °C for 10 min.

Table 3. Percentage of positive samples collected from different sources in poultry processing environment using cultural methods.

Type of samples	Number of samples tested	Number of samples tested positive by culture (%)
Cecum	75	39 (52.00)
Cages	30	16 (53.34)
Body swabs	27	12 (44.45)
Knives	32	14 (43.75)
Chopping boards	29	15 (51.72)
Workers' hands	10	4 (40.00)
Water	7	3 (42.85)
Total	210	103 (49.04)

Forty-seven of *S. Typhimurium* isolates being confirmed positive by PCR, underwent additional screening for the virulence gene, *hilA*. Thirty-three (70.21%) isolated *S. Typhimurium* possessed the *hilA* gene (Table 4). Agarose gel electrophoresis of amplified products of *S. enterica*, *S. Typhimurium*, and the *hilA* gene is shown in Figure 2.

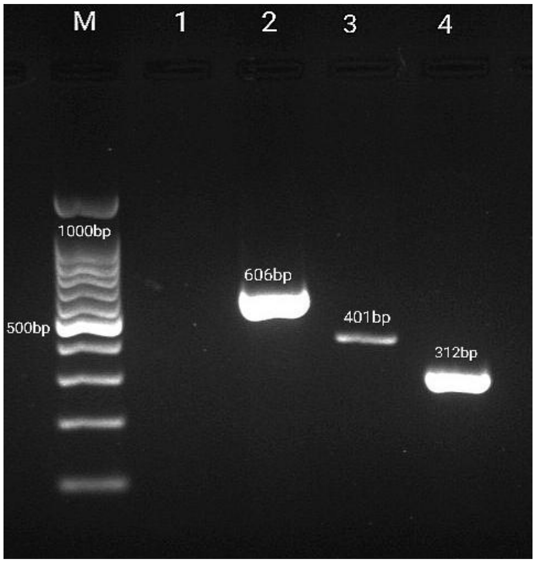

Fig. 2. The target DNA from *Salmonella* is selectively amplified through PCR, utilizing specific primers. Lane M: 100 bp DNA size marker; Lane 1: Negative control (PCR reaction mix) lacking DNA; Lane 2: *iroB* gene of *Salmonella enterica* at 606 bp; Lane 3: *spy* gene at 401 bp; Lane 4: *hilA* gene at 312 bp.

Sequence analysis. The analysis of the *spy* gene sequence in *S. Typhimurium* showed an extremely high percentage of nucleotide similarity at 99.90% compared to the reference sequences found in the NCBI GenBank. The representative isolates have been submitted to the GenBank, assigned the accession number of PV250092 for *S. Typhimurium*.The phylogenetic tree depicted in Figure 3, being based on the partial *spy* gene sequences, effectively illustrates the close genetic relationships among all examined *S. enterica* subsp. *enterica* serovar Typhimurium isolates. Notably, the Iraqi isolate (PV250092) is positioned within the main cluster of isolates, exhibiting a bootstrap support score of 100%, indicating a strong phylogenetic connection. Also, all other isolates formed a monophyletic group characterized by high bootstrap values, suggesting minimal sequence divergence within this serovar.

Discussion

Salmonella is a bacterium linked to chickens causing illness worldwide. Consequently, it is crucial to control *Salmonella* during the conversion of broilers into poultry meat**.** The sanitation and disinfection of a poultry processing facility, along with its machinery, represent a critical control strategy utilized by poultry integrators. The presence of *Salmonella* on sanitized equipment and the associated risks to food safety have not been comprehensively assessed.[20] The slaughterhouse has been

Table 4. Number and prevalence (%) of *Salmonella enterica*, *Salmonella Typhimurium*, and the *hilA* virulence gene in poultry processing environment using conventional PCR.

Type of samples	No. *Salmonella* isolates	*S. enterica* (%)	*S. Typhimurium* (%)	*hilA* gene (%)
Cecum	39	35 (89.74)	19 (54.28)	14 (73.68)
Cages	16	10 (62.50)	7 (70.00)	5 (71.42)
Chopping boards	12	11 (91.67)	8 (72.72)	6 (75.00)
Knives	14	5 (35.71)	4 (80.00)	3 (75.00)
Body swabs	15	8 (53.34)	5 (62.50)	3 (60.00)
Workers' hands	4	3 (75.00)	2 (66.67)	1 (50.00)
Water	3	2 (66.67)	2 (100)	1 (50.00)
Total	103	74 (71.84)	47 (63.51)	33 (70.21)

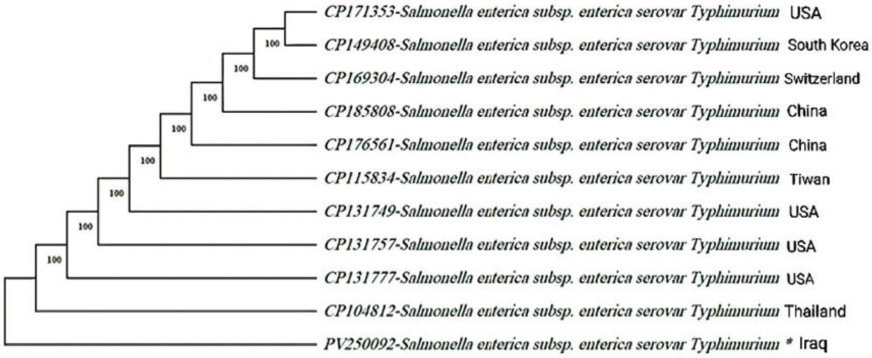

Fig. 3. Phylogenetic tree of *Salmonella Typhimurium* from Iraq (*). The phylogenetic tree was the Maximum Similarity approach following the Tamura-Nei model of MEGA Software (version 11.0; Biodesign Institute, Tempe, USA), and a bootstrap analysis was performed based on 1,000 re-examples. The input data consist of a partial DNA sequence of the coupled partial *spy* gene.

identified as a potential source of *Salmonella* contamination in poultry meat. During the slaughtering process, carcasses may become contaminated with bacteria from the animals digestive systems. Cross-contamination may arise through slaughter machinery, transport boxes, and water.[21]

This research investigated the occurrence of *Salmonella*, specifically *S. enterica* serovar Typhimurium, in samples collected from the poultry processing environment, suggesting contamination by *Salmonella*. The data indicated that out of 103 samples, 49.04%, being almost half of the total 210 samples, tested positive for *Salmonella* using culture methods, with the highest percentage found in cages at 53.34%, highlighting contamination in this area due to the chicken feces. These findings are comparable to another study conducted in Malaysia's wet markets and small-scale poultry processing settings, reported a *Salmonella* prevalence ranging from 38.30 to 50.00% in poultry carcasses sourced from these locations. Conversely, some studies report a lower prevalence of *Salmonella* in these environments. For instance, a study in India analyzed 450 samples, found that only 14.50% were positive for *Salmonella*. Additionally, another study in Kuwait showed a total prevalence of 5.40% in samples collected from poultry farms and processing plants.[15,22] The difference in results could be due to the hygienic practices and sanitation levels, sample size and study design, sampling environment, and type of samples. Iranian research reveals similar but inconsistent patterns of *Salmonella* prevalence in poultry. A retail study of chicken meat and giblets in Iran reported an average contamination level of approximately 19.80%, indicating a moderate burden of *Salmonella* at the retail level. A separate study reported that *Salmonella* was identified in 9.00% of 400 poultry meat samples collected using the culture method, with quail meat having a prevalence of 11.25% compared to the other poultry types.[23,24] The variation in *Salmonella* prevalence between studies and different kinds of poultry may be due to the differences in sample sources, geographic conditions, handling procedures, and laboratory detection methods. The overall findings support the conclusions of this study, indicating that poultry meat is a significant reservoir of *Salmonella* contamination in Iran and its surrounding areas.

The PCR analysis revealed that 74 of the 103 isolates tested positive for *S. enterica*, identified by the *iroB* gene, a genetic marker for *S. enterica*, in 71.84% of the positive samples. Chopping boards had the highest prevalence of *S. enterica* among *Salmonella* isolates, accounting for 91.67%. The cecum exhibited a notable prevalence of 89.74%, underscoring the importance of both internal and external sources in hosting *S. enterica*. Conversely, knives exhibited a much reduced prevalence of *S. enterica* at merely 35.71%. This may result from the habitual washing procedures. This concurs with an earlier research comparing 200 South African chicken slaughterhouse samples with a 51.00% positive sample rate for *Salmonella*. The *iroB* gene was identified in 68.60% of South African chicken samples.[25] The rate of *S. Typhimurium* was 63.51% of the samples in *S. enterica* isolates. The highest rate of *S. Typhimurium* was found in water samples, with a percentage of 100%, being evident in Table 4. This indicates that this serovar is widely distributed in the environmental compartment, along with cutting tools and other surfaces being in contact with food and can potentially serve as reservoirs for its transmission. These results are consistent with those from research conducted in Pakistan, whereby 51.35% of 111 meat samples were *Salmonella* positive, with 45.40% being *S. Typhimurium*.[26] In contrast, in a study conducted in Iraq in 2023, 12.70% of 150 chicken meat samples were *Salmonella* positive, and of these, 36.80% consisted of all the isolates of *Salmonella*.[27]

Interestingly, 70.21% of the *S. Typhimurium* isolates tested positive for the *hilA* gene, which is a transcriptional regulator helping turn on genes making the important proteins and building blocks of the *Salmonella* pathogenicity island-1 type 3 secretion system. Disruption of *hilA* significantly impairs *Salmonella*'s capacity to colonize and penetrate the intestines.[28] There were very high percentages for chopping boards (75.00%), knives (75.00%), and cecum (73.68%). This means that these isolates have a lot of potential to be virulent, meaning that if they get into humans, they are more likely to get sick. On the other hand, *hilA* was found on the workers' hands and in water only half the time (50.00%). This could be because the environment or number of bacteria has changed. This proves that these very dangerous bacteria are very common in places where food is processed. This is also confirmed by a recent study on Egyptian commercial and backyard egg production systems, reported that 70.00% of *S. Typhimurium* isolates had the *hilA* gene.[29] The *hilA* was reported by some research to be most commonly linked with the pathogenicity of *Salmonella,* but it differs from study to study and even from environment to environment. For example, a Malaysian research identified that all isolates of *Salmonella* (100%) contained the *invA* gene, being another gene making the bacteria more deadly. The *hilA* was identified, but not across all serovars as it ought to be. It was present predominantly in some samples, signifying that not all *Salmonella* strains carry this gene. This diversity suggests that the presence of *hilA* can vary with the particular serovar and conditions in the environment in which it grows.[30] The high-resolution phylogenetic tree reveals that the *spy* gene is a good means of demonstrating how *S. Typhimurium* strains have also developed over time. The Iraqi isolate is in the middle of the global isolates;

therefore, it has a relation with strains from other parts of the world came down from the same common ancestor. This means there are most probably worldwide transmission routes, most probably due to the food or animal carriage, contaminated and moved across borders. This implies that there are probably pathways for disease to spread globally, most notably by translocation of infected food or animals. This provided a solid foundation upon which to explain phylogenetic relationships using the Tamura-Nei model and Maximum Likelihood method, both of which were supported by high bootstrap values.[19]The steady grouping of strains underscores the clonal nature of *S. Typhimurium*, thereby validating previous studies that have proved this serovar to exhibit limited genetic diversity in many parts of the globe. This phylogenetic study provides significant data for global surveillance of *Salmonella* and stresses the value of molecular techniques in monitoring the dissemination of disease-causing bacteria.[31]

Finally, this research has elucidated that *Salmonella* prevalence is greater in poultry processing settings in the Sulaymaniyah province, Iraq, highlighting the health hazards that wet markets pose to the public. The result showed that more than half of the strains were *S. Typhimurium*, and among these, a vast majority had the virulence gene *hilA*. The detection of the virulence gene *hilA* indicates that the bacteria can lead to serious infections if they get into humans. The close genetic relatedness of the Iraqi isolate with global *S. Typhimurium* strains suggests that there might be international transmission routes through the food trade. Consequently, we recommend that future studies include sampling during the summer months to provide a more comprehensive understanding of seasonal variation in *Salmonella* prevalence**.** Furthermore, future studies on *Salmonella* should encompass comprehensive genomic monitoring in poultry chains, including anti-microbial resistance profiling and other virulence factors, such as *invA* and *sopE*. Better hygiene practices, stricter enforcement of biosecurity measures, and the establishment of a continuous molecular monitoring system could be key factors in reducing the public health burden caused by salmonellosis.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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