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SALMONELLA ENTERICA SPECIES ISOLATED FROM LOCAL FOODSTUFF AND PATIENTS SUFFERING FROM FOODBORNE ILLNESS: SURVEILLANCE, ANTIMICROBIAL RESISTANCE AND MOLECULAR DETECTION

Zahraa A. AlShaheeb[1], Zaid A. Thabit[2]*, Asma G. Oraibi[3], Ahmed A. Baioumy[4], Tarek Gamal Abedelmaksoud[4]

[1] Iraqi Ministry of Health, Baghdad, Iraq
[2] Environmental Biotech Department, Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq
[3] Plant Biotechnology Department, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq
[4] Food Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt

Keywords: *foodborne pathogen, frozen chicken meat, azithromycin resistance gene mphA.*

Abstract

The aim of this study was to determine the prevalence of Salmonella enterica in raw chicken meat, eggs, and ready-to-eat foods containing poultry products and among patients suffering from diarrhea as a result of ingestion of this foodborne pathogen in Baghdad, Iraq. It assesses the antibiotics susceptibility, virulence and pathogenicity of S. enterica isolates. Thirteen Salmonella spp. isolates from foodstuff and seven from clinical patients were recovered from 80 and 20 samples, respectively. Isolates from foodstuff samples displayed the highest resistance to nalidixic acid (69.23%), followed by chloramphenicol (53.84%). Salmonella spp. isolated from clinical samples showed resistance to both azithromycin and cefotaxime at the same percentage level (71.42%). The results of antibiotic resistance gene amplification (gyrA, mphA) were analyzed and showed that these genes were present in 100% and 50% of phenotypically resistant isolates, respectively. Virulence genes invA, avrA, and sipB were found on average in 86% of food isolates, accounting for 69.2%, 92.3%, and 95%, respectively. In addition, the detection of these virulence genes among clinical isolates showed their presence at the same level (85.7%). Our study revealed that unhygienic chicken slaughterhouses and lack of food safety management are strong indicators of a high probability of the Salmonella presence in our food products in the Iraqi markets.

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Introduction

The World Health Organization (WHO) has stated that foodborne diseases remain a significant problem and can show severity among children, the elderly, and people with immunosuppression [1]. More than 250 different foodborne illnesses are caused by various microbial pathogens and toxins [2], poisonous chemicals, or bio-toxins. *Salmonella* infections are a critical epidemiological and economic problem worldwide [4,5]. For example, the European Food Safety Authority (EFSA) reports that *Salmonella* spp. is recognized to be the second most common cause of human diseases and food poisoning associated with contaminated food [6]. *Salmonella* is a genus of gram-negative bacteria that belongs to the *Enterobacteriaceae* family and can infect various animal hosts [7]. Moreover, *Salmonella* can survive under harsh conditions for about a year in frozen meat [8]. *Salmonella* exists in different environments such as soil, water systems, sewage, and the gut flora of several animals [9]. The members of the genus *Salmonella* are categorized on the basis epidemiology, host range, biochemical reactions, and structures of the O, H, and Vi antigens [10]. *Salmonella*, according to their DNA relativeness, can be classified into two species; *S. bongori*, which populates cold-blooded animals, and *S. enterica*, which is able to inhabit both cold and warm-blooded hosts [11]. Strains belonging to *S. enterica* subsp. *enterica* are responsible for almost 99% of *Salmonella* infections in people and warm-blooded animals [12].

Antimicrobial drugs are utilized to prevent microbial infectious diseases in both humans and animals [7] as well as in animal feed for prophylaxis, therapeutics, and growth promotion [13]. The antimicrobial resistance of *Salmonella*, particularly multidrug resistance (MDR), has become a significant worldwide problem [14]. For example, more recently, *S. enterica* in food-producing animals has shown high MDR resulting in a global problem due to the widespread use of antibiotic drugs [13]. The excessive use of the same antibiotic drugs as a treatment in clinical and veterinary medicine may lead to emergence of resistant strains that can easily be transmitted to the human population from animal products, which is a serious public health problem/concern [15–17]. In Iraq, there is currently a widespread lack of food safety aspects including domestic production of poultry and its products, as well as restaurants and this is leading to the increased risk of exposure to *Salmonella* infection. In Baghdad and central

Iraq, non-typhoidal *Salmonella* was indicated as the second cause of gastroenteritis in children after enteric viruses [18]. Non-typhoidal *Salmonella* was recovered from 10.3% of diarrheal stool samples from children under the age of 5 years in a recent study in southern Iraq [18]. To our knowledge, there have been no published studies on the molecular epidemiology of non-typhoidal *Salmonella* isolated from local hens in Iraq. Given the importance of this pathogen to worldwide health, the current study presents an evaluation of the molecular detection of virulence and antibiotic resistance genes of *Salmonella enterica* from Iraqi foodstuff. We focused on three virulence genes of the SPI-1 region involved in *Salmonella* pathogenicity (*avr*A, *inv*A, and *sip*B), which play a critical role in the initial invasion of the host organism and cause salmonellosis infection as previously mentioned in [19–21]. Therefore, the aim of this study was to determine the prevalence of *Salmonella enterica* in raw chicken meat, eggs, and ready-to-eat foods containing poultry products and in patients suffering from diarrhea as a result of ingestion of this foodborne pathogen in Baghdad (Iraq). It assesses the antibiotic susceptibility as well as virulence and pathogenicity.

Materials and methods

Sample collection

From October 2020 to May 2021, one hundred samples were collected in Baghdad, including 80 samples of Iraqi raw and ready-to-eat food from eight processing points (frozen whole 9-piece chicken = 10; frozen chicken breasts = 10; frozen chicken thighs = 10; eggshell = 10; cooked chicken shawarma = 10; cooked chicken tika = 10; ready-to-eat cake cream = 10; food appetizers containing chicken derivatives = 10) from local supermarkets, and 20 samples from clinically suspected patients with foodborne diseases from private medical laboratories.

Salmonella isolation

Bacteria were isolated according to the ISO 6579–1:2017(E) procedure [22]. An analytical unit of 25 grams from each food sample chopped finely and fecal samples from patients taken with sterilized cotton swabs were inoculated into sterile flasks with 225mL of Buffered Peptone Water (BPW) broth (Himedia, India). The flasks were incubated at 37 °C for 18 hours. An amount of 0.1mL from pre-enriched culture was transferred to 10ml of Rappaport Vassiliadis (RV) broth medium (Himedia, India), thoroughly mixed and incubated at 41 ± 0.5 °C for 24 hours. A loopful from the incubated selective enrichment broth culture was streaked on Xylose Lysine Deoxycholate (XLD) agar plate and *Salmonella-Shigella* (SS) agar (Himedia, India). Both agar plates were incubated at 37 °C for 24 hours.

Salmonella identification

Suspected colonies with *Salmonella* morphology from each plate were identified biochemically using the VITEK-2 system (bioMérieux, France). In addition, isolates were investigated by the conventional method in the Iraqi National Centre for *Salmonella* at the Central Public Health Laboratories in Baghdad using the serological test (Anti-*Salmonella* H test) (Sifin/ Germany) kits that were designed for the use in examining the H-antigens of *Salmonella* strains via slide agglutination in Baghdad's Central Public Health Laboratory (CPHL). Bacteria from 16–20-hour-old subculture (nutrient) agar were applied to a clean microscope slide and mixed well with a drop of 25µl of anti-*Salmonella* H reagent (test serum), then slowly stirred with a sanitized stick. The slide was put on a dark surface and visible agglutination was observed in the case of the positive reaction, while a negative result was seen as a homogeneous milky turbid suspension. On the same slide, the positive and negative controls were tested in the same way. Typical *Salmonella* phenotypes were further confirmed by single-step PCR for the 16S rRNA gene of *S. enterica* [23].

Genomic DNA extraction

Extraction of DNA was carried out as recommended by the manufacturer of the HiPer® Bacterial Genomic DNA Extraction Teaching Kit (Solution Based), India.

16S rRNA gene direct sequencing

Conventional PCR was carried out to analyze all the genes of this study, the confirmatory 16S rRNA gene (621bp) was amplified by using GoTaq® G2 Green Master Mix (Promega, USA). PCR conditions in this study and primer design were used as shown in Tables 1 and 2. Purified PCR products (45µL) from the identified 16S rRNA gene target were forwarded to Macrogen comp. (Korea) for DNA sequencing. Then, using the BioEdit and Mega7 software, consensus sequences were created by aligning the forward and reverse DNA sequences for each sample. In addition, the final sequence from each sample was

Table 1. PCR conditions of this study

Gene name / Step conditions		16S rRNA	*inv*A	*avr*A	*sip*B	*mph*A	*gyr*A
1 CYCLE	Initial denaturation	95 °C, 5 min					94 °C, 5 min
30 CYCLE	Denaturation	94 °C, 40 sec					94 °C, 30 sec
	Annealing	60 °C	56 °C	58 °C	60 °C	58 °C	58 °C, 30 sec
		40 secs					
	Extension	72 °C, 40 sec					72 °C, 30 sec
1 CYCLE	Final extension	72 °C, 5 min					72 °C, 10 min
—	Holding	4 °C, 10 min					4 °C, 10 min

further analyzed by searching for similar matches in the NCBI gene bank database. This was achieved by employing the BLAST website tool, the BLAST search to assess the similarity.

Table 2. Primers used in PCR amplification

Gene name	Primer sequences (5′→3′)	Amplicon size (bp)	References
16S rRNA	F primer: GGAACTGAGACACGGTCCAG R primer: CCAGGTAAGGTTCTTCGCGT	671	[23]
*inv*A	F primer: TTGTTACGGCTATTTTGACCA R primer: CTGACTGCTACCTTGCTGATG	521	[24]
*avr*A	F primer: CCTGTATTGTTGAGCGTCTGG R primer: AGAAGAGCTTCGTTGAATGTCC	425	[25]
*sip*B	F primer: GGACGCCGCCCGGGAAAAACTCTC R primer: ACACTCCCGTCGCCGCCTTCACAA	875	[26]
*mph*A	F primer: GTGAGGAGGAGCTTCGCGAG R primer: TGCCGCAGGACTCGGAGGTC	403	[27]
*gyr*A	F primer: TGGGCAATGACTGGAACA R primer: GGTTGTGCGGCGGGATA	396	[28]

Antimicrobial susceptibility test

The disk diffusion method described by the Clinical and Laboratory Standards Institute (CLSI) [29] was chosen to test antibiotic resistance of *Salmonella* isolates. Ten antimicrobial agents (Bioanalysis, Turkey) were selected from several family groups according to [29], and tested against *Salmonella* isolates: phenicols (chloramphenicol, 30µg), aminoglycosides (gentamicin, 10µg), quinolones (nalidixic acid, 30µg), carbapenems (meropenem and imipenem, 10µg), folate pathway antagonists (trimethoprim-sulfamethoxazole, 30µg), macrolides (azithromycin, 15µg), cephalosporins (ceftazidime and cefotaxime, 30µg), and fosfomycins (fosfomycin, 200µg). *Escherichia coli* ATCC25922 was used as a quality control strain. The isolates were described as susceptible, intermediate, or resistant according to the CLSI [29] guidelines. An isolate was defined as multi-drug resistant (MDR) when showing resistance to three or more different classes of antimicrobials [30].

Detection of virulence and antimicrobial resistance genes

The virulence genes *inv*A (521bp), *avr*A (425bp), *sip*B (875bp), and the quinolone resistance gene *gyr*A (396bp), macrolide azithromycin resistance gene *mph*A (403bp) were analyzed in isolates that showed resistance to these two antimicrobials only. Bioneer's master mix (Bioneer's AccuPower PCR PreMix, Korea) was used for amplification. PCR conditions performed in this study and primer design were used as shown in Table 1 and Table 2.

Gel electrophoresis

The agarose powder (1 percent% w/v) was dissolved in 1X TBE buffer. The mixture was microwaved and allowed to cool at 50 °C before adding 8µl of RedSafe™ (iNtRON/Korea) (0.5 g/ml) to the agarose solution and pouring it onto the tray. After the gel hardened, the comb was removed.

Results

Occurrence of Salmonella spp.

Among 100 collected samples, 20 isolates (20%) were culture positive for *Salmonella* spp. Within the food sample groups, *Salmonella* spp. was isolated from 16.25% of samples (13 out of 80 food samples), all of which were collected from raw poultry meat and egg groups, whilst the other food groups were *Salmonella* free. Raw frozen chicken breasts had the highest level of contamination with *Salmonella* spp. (60%) followed by raw frozen chicken thighs (40%), raw frozen 9-piece chicken (20%), and eggshell (10%). As regards clinical diarrheal patients, *Salmonella* spp. was isolated from 7 (35%) out of 20 samples. The *S. enterica* isolates from food products identified in this study were *S. enterica* serovar Typhimurium (11 isolates, 84.6%) isolated from raw frozen 9-piece chicken (2 isolates, 15.3%), raw frozen chicken breasts (5 isolates, 38.4%), and raw frozen chicken thighs (4 isolates, 30.7%), *S. enterica* subsp. *enterica* (one isolate from eggs, 7.6%), and *S. enterica* subsp. *diarizonae* (one isolate from raw frozen chicken breasts, 7.6%). *S. enterica* serovar Typhi (4 isolates, 57.1%), and *S. enterica* serovar Typhimurium (3 isolates, 42.8%) were isolated from human diarrheal patients as shown in Table 3. The percentage of each serovar identified in this study is presented in diagrams (Figure 1A and Figure 1B).

Table 3. Identified isolates and their sources

Isolates No.	Source of isolates	Type of species
1	Whole raw frozen 9- piece chicken	*S. enterica* serovar Typhimurium
2		*S. enterica* serovar Typhimurium
3	Raw frozen chicken breasts	*S. enterica* subsp. *diarizonae*
4		*S. enterica* serovar Typhimurium
5		*S. enterica* serovar Typhimurium
6		*S. enterica* serovar Typhimurium
7		*S. enterica* serovar Typhimurium
8		*S. enterica* serovar Typhimurium
9	Raw frozen chicken thighs	*S. enterica* serovar Typhimurium
10		*S. enterica* serovar Typhimurium
11		*S. enterica* serovar Typhimurium
12		*S. enterica* serovar Typhimurium
13	Egg shell	*S. enterica* subsp. *enterica*
14	Diarrheal human patients	*S. enterica* serovar Typhi
15		*S. enterica* serovar Typhi
16		*S. enterica* serovar Typhi
17		*S. enterica* serovar Typhi
18		*S. enterica* serovar Typhimurium
19		*S. enterica* serovar Typhimurium
20		*S. enterica* serovar Typhimurium

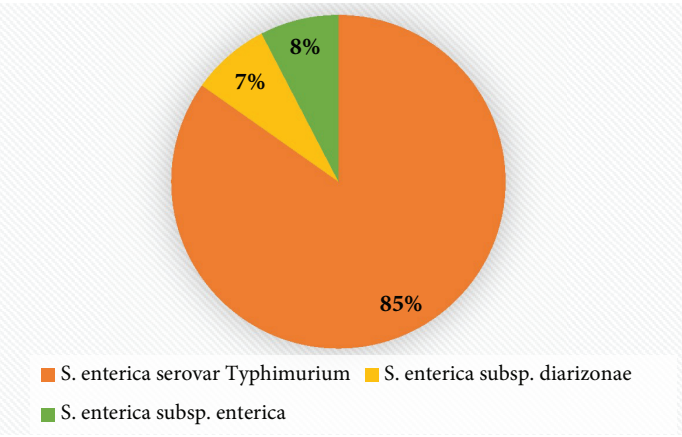

Figure 1A. Percentage of *S. enterica* serovars identified in raw food samples

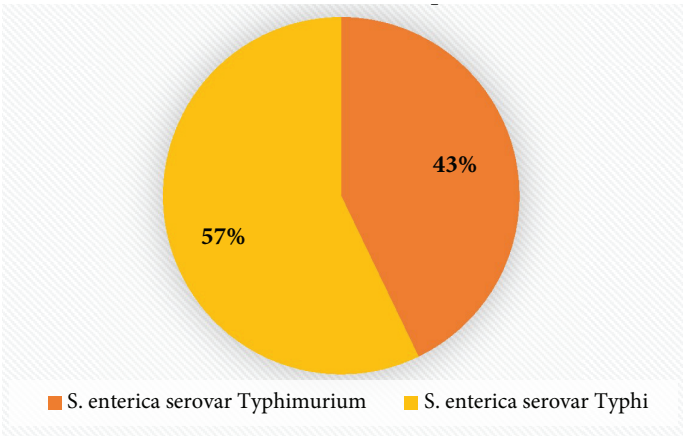

Figure 1B. Percentage of *S. enterica* serovars identified in clinical human diarrheal samples

As shown in Figures 1A and 1B, the most frequently identified serovars were *S. enterica* serovar Typhimurium (85%) and *S. enterica* serovar Typhi (57%) in raw food and clinical samples, respectively. The confirmatory molecular analysis indicated that all isolates (100%) had the 16S rRNA gene of *Salmonella*.

Sequencing analysis results

Several changes in nucleotide sequences were observed in isolates A4, A7, A13, A16, A17, and A19. There was approximately a maximum of 14 differences in base pairs as shown in Figure 2. With the presence of such polymorphisms, differences between isolates belonging to different subspecies and serovars were found.

During the further analysis for bacterial classification and detection of similarity in Figure 2, four clusters of bacterial strains were identified with reference strains. Two of them included AB680591.1 and MZ773245.1, and the other two included isolates A1 and A10. Each of them contained bacterial isolates with the highest similarity and the lowest genetic distance.

Differentiation of the reference strains from those of other serovars and subspecies was carried out as illustrated in Figure 3. It shows that isolates A11, AA2, A1, A8, A20, A12, A5, MZ773245.1, A4, A7, A17, A16, A13, A19, A6, A9, A10, A15, and A18 are more related to strain AB680591.1

Table 4. Alignment of partial 16S rRNA gene sequences of *Salmonella* spp. bacteria under consideration with the sequence of the NCBI database

Isolate no.	Result of strain sequencing	Similarity	e-value
A1	*S. enterica* **serovar Typhimurium**	100%	0.0
A2		100%	
A3		100%	
A4		99.53%	
A5		100%	
A6		100%	
A7	*S. enterica* **subsp.** *enterica*	100%	
A8	*S. enterica* **serovar Typhimurium**	99.50%	
A9	*S. enterica* **serovar Typhi**	100%	
A10	*S. enterica* **serovar Typhimurium**	99.84%	
A11	*S. enterica* **serovar Typhi**	100%	
A12		100%	
A13		99.05%	
A14	*S. enterica* **serovar Typhimurium**	100%	
A15		100%	
A16		98.73%	
A17		98.69%	
A18		100%	
A19		100%	
A20	*S. enterica* **subsp**. *enterica*	100%	

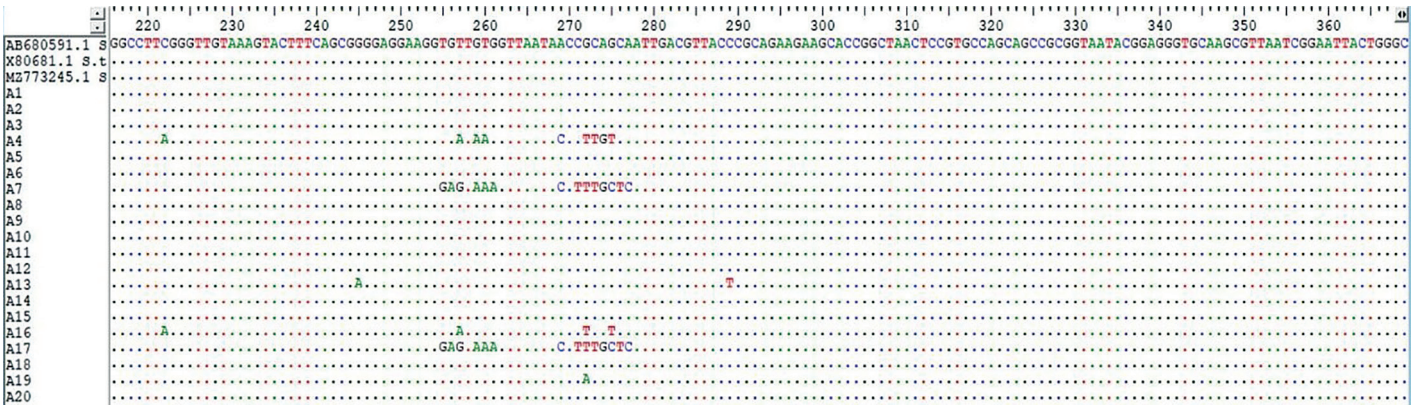

Figure 2. 16S rRNA gene sequence alignment of *S. enterica* isolates with the related reference sequence of the 16S rRNA gene by BioEdit software. Ref= Reference sequences of the 16S rRNA gene of *S. enterica* strains AB680591.1 S, X80681.1 S. t, and MZ773245.1 S (wild type). The black sign "A" denotes the names of isolates given in Table 4

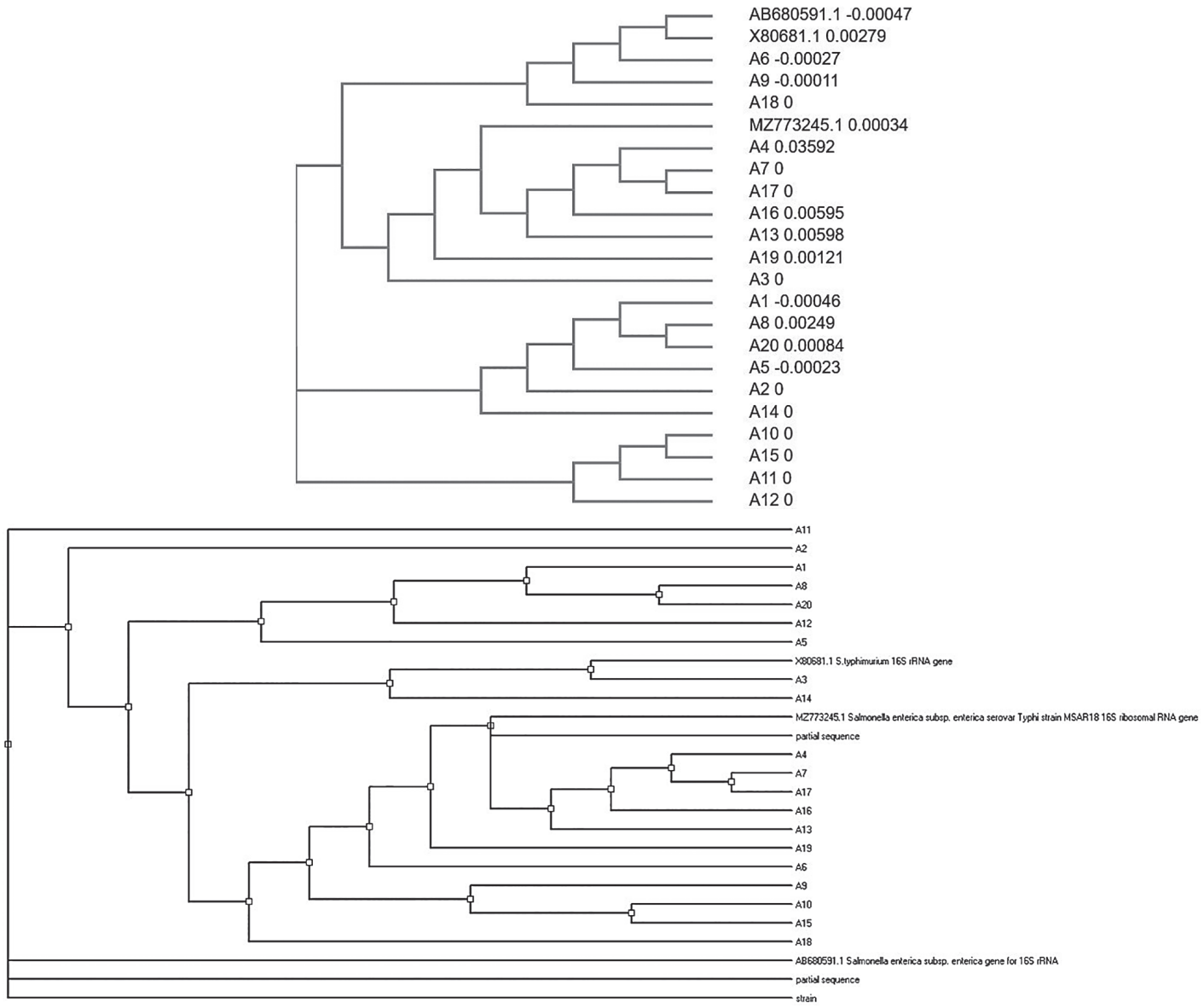

Figure 3. Phylogenetic tree of 16S rRNA gene sequences alignment of *S. enterica* spp. isolates with the related reference sequence of the 16S rRNA gene

and belong to different *S. enterica* serovars while isolates A14 and A13 belonging to *S. enterica* serovar Typhimurium have high similarity to X80681.1 as a common ancestor.

Additionally, the obtained data presented in Table 4 demonstrate the similarity (98–100%) of the isolated bacteria by the 16S rRNA gene, and show that the expected value (e-value) for all *Salmonella* spp. isolates was zero.

Antimicrobial susceptibility test

Antimicrobial susceptibility testing of *Salmonella enterica* in this study showed that all isolates tested except one were resistant to one antibiotic at least as shown in Table 5.

In general, the most effective antimicrobials against *Salmonella* isolated from food samples were imipenem (100%), cefotaxime, meropenem and ceftazidime (69.2% each). The highest resistance of all 13 isolates was recorded for nalidixic acid (69.23%), followed by chloramphenicol (53.84%), gentamicin (46.15%), trimethoprim-sulfamethoxazole (46.1%), fosfomycin (46.1%), and azithromycin (38.46%), whilst resistance to both cefotaxime and meropenem was 30.7% as shown in Table 6.

Different *Salmonella* serovars/subspecies tested in this study showed different levels of resistance. It was revealed that 63.63% (7/11) of isolates of *S. enterica* serovar Typhimurium recovered from food were resistant to nalidixic acid and chloramphenicol. Lower resistance was recorded for trimethoprim-sulfamethoxazole and gentamicin, which had the same percentage of 45.45% (6/11), followed by meropenem, cefotaxime, and azithromycin with the same proportion of 36.36% (4/11). Imipenem and ceftazidime were found to be effective antimicrobials against food isolates of *S. enterica* serovar Typhimurium (100% and 76.93%, respectively).

All food isolates (100%) of *S. enterica* subsp. *enterica* (n = 1) and *S. enterica* subsp. *diarizonae* (n = 1) were susceptible to meropenem, ceftazidime, cefotaxime, and chloramphenicol. Only *S. enterica* subsp. *diarizonae* showed high resistance (100%) to azithromycin, trimethoprim-sulfamethoxazole, gentamicin, nalidixic acid, and fosfo-

Table 5. Antibiotic resistance of each isolate to ten antibiotics chosen according to CLSI [29]

sample	Name of serovar/subspecies	NA	SXT	C	CTX	AZM	MEM	IPM	CN	CAZ	FOS	
1	*S. enterica* serovar Typhimurium	S	S	S	R	S	R	S	R	R	S	MDR
2	*S. enterica* serovar Typhimurium	S	S	S	R	S	S	S	S	S	S	—
3	*S. enterica* serovar Typhimurium	S	S	S	S	S	S	S	S	S	S	—
4	*S. enterica* serovar Typhimurium	S	S	R	S	S	S	S	S	S	R	—
5	*S. enterica* serovar Typhimurium	R	R	R	R	R	R	S	R	R	R	MDR
6	*S. enterica* serovar Typhimurium	R	R	S	S	S	R	S	S	S	S	MDR
7	*S. enterica* subsp. *diarizonae*	R	R	R	S	R	S	S	R	S	R	MDR
8	*S. enterica* serovar Typhimurium	R	S	S	S	S	S	S	S	S	S	—
9	*S. enterica* serovar Typhimurium	R	R	R	S	S	S	S	S	S	R	MDR
10	*S. enterica* serovar Typhimurium	R	R	R	R	R	R	S	R	R	S	MDR
11	*S. enterica* serovar Typhimurium	R	S	R	S	R	S	S	R	S	S	MDR
12	*S. enterica* serovar Typhimurium	R	R	R	S	R	S	S	R	S	R	MDR
13	*S. enterica* subsp. *enterica*	R	S	S	S	S	S	S	S	S	S	—
14	*S. enterica* serovar Typhi	S	R	S	R	S	S	S	R	R	R	MDR
15	*S. enterica* serovar Typhi	R	S	I	R	R	S	S	S	S	S	MDR
16	*S. enterica* serovar Typhi	R	S	R	R	S	S	S	S	S	S	MDR
17	*S. enterica* serovar Typhi	I	R	I	S	R	S	R	R	S	S	MDR
18	*S. enterica* serovar Typhimurium	R	S	S	R	R	S	S	I	S	R	MDR
19	*S. enterica* serovar Typhimurium	S	R	R	S	R	R	S	S	R	S	MDR
20	*S. enterica* serovar Typhimurium	R	S	S	R	R	S	S	I	R	S	MDR

NA: nalidixic acid; SXT: trimethoprim/sulfamethoxazole; C: chloramphenicol, CTX: cefotaxime; AZM: azithromycin; MEM: meropenem; IPM: imipenem; CN: gentamicin; CAZ: ceftazidime; FOS: fosfomycin; S: susceptible; I: intermediate; R: resistant; MDR: multidrug-resistant

mycin. *S. enterica* subsp. *enterica* showed high levels of resistance (100%) to nalidixic acid and high levels of susceptibility (100%) to gentamicin.

Regarding human isolates, the results obtained indicate that in general isolates (*S. enterica* serovar Typhi (n = 4) and *S. enterica* serovar Typhimurium (n = 3)) recovered from the diarrheal patient samples were resistant to azithromycin and cefotaxime showing the same percentage (71.42%; 5/7), while imipenem and meropenem were the ultimate effective antibiotics with the same proportion of susceptible isolates (85.7%; 6/7) as shown in Table7.

As for *Salmonella* serovars, *S. enterica* serovar Typhimurium (n= 3) isolates were completely susceptible (100%) to imipenem, followed by meropenem, fosfomycin, cefotaxime and chloramphenicol with the same proportion (66.67%, 2/3). However, they showed resistance to trimethoprim-sulfamethoxazole, nalidixic acid, and ceftazidime with the same proportion (66.67%, 2/3). The resistance to gentamicin was intermediate (66.67%, 2/3).

S. enterica serovar Typhi (n = 4) isolates exhibited intermediate resistance to chloramphenicol (50%; 2/4). Moreover, they showed resistance to trimethoprim-sulfonamide, nalidixic acid, azithromycin, and gentamicin, all of which had the same ratio (50%; n = 2/4). The highest susceptibility (100%) was observed for meropenem followed by ceftazidime, fosfomycin, and imipenem with the same proportion of 75% (3/4). *S. enterica* serovar Typhi was resistant (75%; 3/4) to cefotaxime.

All isolates except for five isolates from food samples (one from eggshell and four from frozen raw chicken breasts) exhibited multidrug resistance to ≥ 3 antibiotics as shown in Table 5.

This study illustrates a high proportion (77.7%) of food isolates of *S. enterica* serovar Typhimurium (n = 7) consid-

Table 6. Percentage of food isolates of *Salmonella* spp. resistant, intermediate and susceptible to antibiotics

Antibiotics	Food Isolates No.	Resistant (%)	Intermediate (%)	Susceptible (%)
Nalidixic acid (NA)	13	69.2%	0%	30.7%
Trimethoprim/ Sulfamethoxazole (SXT)	13	46.1%	0%	53.8%
Chloramphenicol (C)	13	53.8%	0%	46.1%
Cefotaxime (CTX)	13	30.7%	0%	69.2%
Azithromycin (AZM)	13	38.4%	0%	61.5%
Meropenem (MEM)	13	30.7%	0%	69.2%
Imipenem (IPM)	13	0%	0%	100%
Gentamicin (CN)	13	46.1%	0%	53.8%
Ceftazidime (CAZ)	13	30.7%	0%	69.2%
Fosfomycin (FOS)	13	46.1%	0%	53.8%

Table 7. Percentage of clinical *Salmonella* spp. isolates resistant, intermediate and susceptible to antibiotics

Antibiotics	Clinical Isolates No.	Resistant (%)	Intermediate (%)	Susceptible (%)
Nalidixic acid (NA)	7	57.1%	14.2%	28.5%
Trimethoprim/ Sulfamethoxazole (SXT)	7	42.8%	0%	57.1%
Chloramphenicol (CAM)	7	28.5%	28.5%	42.8%
Cefotaxime (CTX)	7	71.4%	0%	28.5%
Azithromycin (AZM)	7	71.4%	0%	28.5%
Meropenem (MEM)	7	14.2%	0%	85.7%
Imipenem (IPM)	7	14.2%	0%	85.7%
Gentamicin (GEN)	7	28.5%	28.5%	42.8%
Ceftazidime (CAZ)	7	42.8%	0%	57.1%
Fosfomycin (FOS)	7	28.5%	0%	71.4%

ered multidrug resistant (MDR). Two isolates were resistant to three antibiotic groups, two isolates were resistant to four antibiotic groups, one isolate was resistant to six antibiotic groups and two isolates were resistant to eight antibiotic groups as shown in Table 8. All (100%) clinical isolates of *S. enterica* serovar Typhimurium (n = 3) were MDR: one isolate was resistant to three antibiotic groups, one isolate was resistant to four antibiotic groups and one isolate was resistant to five antibiotic groups as shown in Table 8.

Table 8. MDR of food and clinical isolates of *S. enterica* serovar Typhimurium

No. of antimicrobial groups	Food isolates (n= 7)		Clinical diarrheal isolates (n= 3)	
	Antimicrobial resistance patterns	No. of isolates (%)	Antimicrobial resistance patterns	No. of isolates (%)
Three	Quinolones (NA), Sulfa drug)SXT(, Carbapenem (MEM). Cephems (CTX, CAZ), Carbapenem (MEM), Gentamycin (CN).	2 (28.56%)	Quinolones (NA), Cephems (CTX, CAZ), Macrolides (AZM).	1 (14.28%)
Four	Quinolones (NA), Sulfa drug)SXT(. Chloramphenicol (C), Fosfomycin (FOS). Quinolones (NA), Chloramphinical (C), Macrolides (AZM), Gentamycin (CN).	2 (28.56%)	Quinolones (NA), Chloramphenicol (C), Macrolides (AZM), Fosfomycin (FOS).	1 (14.28%)
Five	—	—	Cephems (CAZ), Carbapenem (MEM), Chloramphenicol (C), Macrolides (AZM), Sulfa drug (SXT(.	1 (14.28%)
Six	Quinolones (NA), Chloramphenicol (C), Fosfomycin (FOS) Carbapenem (IPM), Macrolides (AZM), Sulfa drug)SXT(.	1 (14.28%)	—	—
Eight	Cephems (CAZ, CTX), Carbapenem (MEM), Chloramphenicol (C), Macrolides (AZM), Sulfa drug (SXT(, Gentamycin (CN), Quinolones (NA), Fosfomycin (FOS). Cephems (CAZ, CTX), Carbapenem (MEM), Chloramphenicol (C), Macrolides (AZM), Sulfa drug (SXT), Gentamycin (CN), Quinolones (NA), Fosfomycin (FOS).	2 (28.56%)	—	—

Antibiotic resistance gene detection in S. enterica isolates

The fluoroquinolone resistance gene (*gyr*A) was associated with all fluoroquinolone (nalidixic acid) resistant isolates (16/16). All nine resistant isolates recovered from food (*S. enterica* serovar Typhimurium, n = 7; *S. enterica* subsp. *enterica,* n = 1; *S. enterica* subsp. *diarizonae,* n = 1) and all seven resistant clinical isolates (*S. enterica* serovar Typhi, n = 4; *S. enterica* serovar Typhimurium, n = 3) showed the presence of the *gyr*A gene. The azithromycin resistance gene (*mph*A*)* was associated with about half of the macrolide (azithromycin) resistant isolates (n = 6/10). The *mph*A gene was detected in four resistant isolates recovered from food (*S. enterica* serovar Typhimurium, 3/4; and *S. enterica* subsp. *diarizonae*; 1/1) with the proportion equaled 75% and 100%, respectively. Among resistant clinical isolates, 2 out of 5 isolates showed the presence of the *mph*A gene (*S. enterica* serovar Typhi,1/2; *S. enterica* serovar Typhimurium; 1/3), which accounted for 50% and 33.33%, respectively.

Virulence gene detection in S. enterica isolates

The *inv*A gene was revealed in 75% (15/20) of all *Salmonella* isolates. Among food isolates, *inv*A was found in 9 out of 13 (69.2%) *S. enterica* isolates. Detection of this gene gave negative results in four isolates, including three isolates from raw frozen chicken breasts and one isolate from raw whole 9-piece chicken. All these four negative isolates belonged to *S. enterica* serovar Typhimurium. So, the proportion of *inv*A gene detection was 63.6% (7/11) for *S. enterica* serovar Typhimurium isolates, whilst it was 100% for both *S. enterica* subsp. *enterica* (1/1) and *S. enterica* subsp. *diarizonae* (1/1). The *avr*A virulence gene was detected in 90% (18/20) of all *Salmonella* isolates. Among food isolates, *avr*A was detected in 12 out of 13 isolates (92.3%). Only one isolate of *S. enterica* subsp. *enterica* from eggshell showed a negative result of *avr*A gene detection. *S. enterica* serovar Typhimurium and *S. enterica* subsp. *diarizonae* showed a positive result of *avr*A gene detection in all isolates (100%) from local raw poultry meat. The *sip*B virulence gene was found in 95% (19/20) of all *Salmonella* isolates. Regarding local raw poultry meat and eggshell, the *sipB* gene was detected in all (100%) isolates of *S. enterica* serovar Typhimurium, *S. enterica* subsp. *diarizonae*, and *S. enterica* subsp. *enterica*.

All these virulence genes were detected in 85.7% (6/7) of clinical isolates. The proportion of clinical isolates of *S. enterica* serovar Typhi positive for these genes was 25% (1/4).

Discussion

To the best of our knowledge, this work is one of the first studies interested in the *Salmonella* prevalence in food and clinical samples in Iraq. The results of *Salmonella* isolates are approximate, with various studies done in many areas in Iraq between 2008 and 2017, with percentage of *Salmonella* isolation ranging from 1.07% to 16%. The great-

est proportion was recorded in Al-Hawijah, while the lowest percentage was recorded in Mosul [31–37].

Different studies on *Salmonella* prevalence were carried out in several countries. In [38] performed in China, *Salmonella* spp. were isolated from 249 out of 664 (37.5%) samples, including 190 (36.7%) chicken, 48 (40.7%) duck and 11 (39.2%) pigeon samples. *Salmonella* prevalence of 13.4% was documented by Rabby [39] in poultry meat retailed in wet- and super-markets in Dhaka city, Bangladesh. Salih *et al.* [40] examined the distribution of *Salmonella* spp. in 121 specimens from diarrheal patients in Duhok, Iraq, and showed that 72 isolates (59.5%) belonged to *Salmonella* spp. Sadeq *et al.* [41] analyzed 40 chicken samples, and found that 14 isolates (35%) belonged to *S. enterica* serovar Typhimurium with the presence of the *inv*A gene in 11 (78.5%) out of 14 isolates of *S. enterica* serovar Typhimurium. These results are almost identical to the results of our study since we recorded the presence of seven food isolates of *S. enterica* serovar Typhimurium positive for the *inv*A gene with a proportion of 53%. A recent study in Babil, Iraq, was carried out by Obayes et al. [42] who collected samples from 120 children with diarrhea and revealed that 58 samples were positive for different *Salmonella* spp. The most common serovar of *Salmonella enterica* in their study was *Salmonella enterica* serovar Typhi (29.3%) and this result agrees with the result of our study, which indicates the presence in the clinical human diarrheal samples of four isolates (57.1%) of *S. enterica* serovar Typhi as the most common serovar.

The most common serovars transferred from animals to humans are *Salmonella* Enteritidis and *Salmonella* Typhimurium. Typhoid fever, paratyphoid fever, food poisoning and gastroenteritis are all disorders caused by *Salmonella* [43]. The 16S rRNA gene sequence is considered an important approach toward identification of bacterial genus, species, and sub-species, and was used in this study as a confirmatory detection test. It is unique for each bacterial organism and can be considered a unique identification gene for bacterial species.

In general, the reasons for *Salmonella* spp. contamination detected in this study are the lack of HACCP control and due diligence. The Iraqi *Central Organization for Standardization and Quality Control* (COSQC, document No. 2270) indicates that the percentage of *Salmonella* growth should be zero in chicken cuts (thighs, breasts, wings), and for this reason the problem of the absence of quality control, HACCP and food handling instructions have to be dissolved and they should be applied in Iraqi slaughterhouses along the poultry processing chain until reaching consumers to prevent an increase in foodborne diseases as much as possible.

Concerning nalidixic acid, other studies also documented *Salmonella* resistance to this antimicrobial agent, and the resistance increased significantly (94.1%) in 2021 compared with the 2018 report (77.3%) indicating more applications of nalidixic acid in both veterinary medicine and human medicine fields [44, 45]. As for other antibiotics, a study conducted in Bangladesh reported that 95% of the isolates were resistant to azithromycin [46], another study reported low resistance (8%) to chloramphenicol [47]. In [48] 12% of *Salmonella* isolates were resistant to azithromycin and 1% to chloramphenicol, whereas the present study recorded much higher resistance to azithromycin and chloramphenicol compared to previous studies.

In comparison with [49], the partial similarity was noticed, particularly, the susceptibility of the isolates to imipenem. Resistance to SXT was higher in the present study than in [49], which recorded 31.3%, while the study by Velez [50] displayed that *Salmonella* isolates showed higher resistance (100%) to gentamicin than in our study. Moreover, in the current study, nine isolates were resistant to cefotaxime (approximately 30.7%), which is higher than the result reported in China by [14] where 2.44% of *Salmonella* isolates were found to be resistant to cefotaxime.

On other hand, resistance to ceftazidime, meropenem, and fostomycin was in the same line with an Iraqi study [51]. Antibiotic-resistant bacteria can cause life-threatening infections in people and constitute a serious danger to public health and wellbeing. Furthermore, the use of antimicrobials in veterinary medicine may increase the emergence of resistant bacteria harmful to humans and posing a possible threat to public health from zoonotic pathogens, such as *Salmonella*. As a result, the high resistance/MDR in human isolates of *Salmonella* spp. to antibiotics may be caused not by the misuse of drugs and their wrong consumption, but rather this resistance transfers through consumption of foods (e. g., poultry meat) from food-producing animals that received antibiotics on farm to enhance and promote their quality and prevent the growth and transmission of microbes.

Bacteria may acquire resistance genes via mobile genetic elements such as plasmids, which provide the flexibility to a host bacterium and aid in the dissemination and dispersion of these genes among various bacterial populations [52]. In our study, the *gyr*A and *mph*A genes conferring resistance to nalidixic acid and azithromycin, respectively, were detected among isolates that showed the phenotypic resistance. Similarly, there were high percentages of the *gyr*A genes found in nalidixic acid resistant *Salmonella* Albany (92%), *Salmonella* Corvallis (75%), and *Salmonella* Kentucky (85%) isolated in chicken food chains in Cambodia [53].

Quinolones with a broad spectrum of activity have a greater ability to inhibit gyrase in gram-negative bacteria. According to the researchers, antibiotic resistance in *Salmonella* spp. is mostly caused by mutations within the quinolone resistance-determining regions (QRDRs) of the target enzymes DNA gyrase (*gyr*A and *gyr*B) and topoisomerase IV (*par*C and *par*E) [54].

Wang et al. [28] identified the *mph*A gene in 15 out of 31 azithromycin-resistant *Salmonella* isolates. This is partially similar to the results of our study. Azithromycin is an

azalide antibacterial drug, which was shown to be equal to chloramphenicol, fluoroquinolones, and extended-spectrum cephalosporins for the treatment of uncomplicated typhoid fever [55]. The *mph*A gene is the key gene involved in *Salmonella* resistance to the macrolide azithromycin. It is typically found on plasmids and spreads rapidly, posing a significant threat to current *Salmonella* infection therapy [56].

Salmonella employs several virulence factors expressed at various phases of the disease process to develop a successful infection. A number of these parameters are linked to *Salmonella* Pathogenicity Islands (SPIs) on the *Salmonella* chromosomes [57]. The virulence *inv*A gene is involved in *Salmonella* pathogenicity. The *inv*A gene acts as a unique biomarker for *Salmonella* identification [58]. Previous studies [59–62] show that the *inv*A gene has been found in 100% of *Salmonella* strains, whilst, our study recorded a lower percentage (75%). Likewise, other authors, such as Mthembu *et al.* [21], revealed lower rates (54.4%; 106/195) and Somda *et al.* [63] showed the presence of the *inv*A gene in 91% (52/57) of non-typhoidal *Salmonella* isolates from human diarrhea, environment, and lettuce samples in Burkina Faso. Furthermore, Nikiema *et al.* [64] found that the *inv*A gene was present in 67% (61/91) of clinical isolates and 60% (9/15) of sandwich samples. In our study, 25% of *Salmonella* isolates did not harbor the *inv*A gene and hence would be unable to invade host cells. Salih and Yousif [65] conducted a study in Iraq to detect five virulence factors among four isolates of *S. enterica* serovar Typhimurium isolated previously from three puppies and one adult dog and reported that the *inv*A gene was detected in two isolates only. In the same line, another Iraqi study showed that the *inv*A gene was detected among eight *Salmonella* strains with a proportion of 50% [66]. Therefore, *Salmonella* might be virulent (*inv*A) or avirulent [67]. Furthermore, asymptomatic animals carrying either virulent or avirulent strains might be possible sources of transmission to humans through the food chain, as well as due to their close proximity to people and poor animal effluent management [21, 67]. All strains containing this *inv*A gene, which encodes a protein found in bacterial inner membrane and is crucial for invasion of host epithelium, are pathogenic [68,69]. Moreover, a key component of the pathogen's virulence phenotype is the virulence-associated effector protein AvrA of *Salmonella enterica*, which blocks the first line of defense of the host organism. AvrA expression increases the ability of the bacterium to invade the host [70,71]. Our study indicated that the *avr*A gene was revealed in 90% (18/20) of all *Salmonella* isolates. The other Iraqi researchers Jbar *et al.* [72] detected the *avr*A gene in 100% (30/30) of *Salmonella enterica* isolates. Similarly, an Egyptian study showed that all 6 (100%) *Salmonella* isolates carried the *avr*A gene [69]. This presence of the *avr*A gene in all isolates suggests a higher rate of gastroenteric illnesses in humans that may be transmitted from contaminated food. In addition, Hersh *et al.* [73] established the role of *sip*B in *Salmonella*-induced macrophage death, as well as the possible involvement of caspase-1 in this process. In our study, the *sip*B gene was found in 95% (19/20), while another Iraqi study [42] found that the *sip*B gene occurrence in *Salmonella* isolates was 18.9% (11/120).

Moreover, having regard to the serious roles of these three genes as illustrated briefly above and the outcome of our study, which shows the high occurrence of the *inv*A, *avr*A, and *sip*B genes (75%, 90% and 95%, respectively), it is safe to assume that the severity of infections that may occur in the Baghdad population will be increasing.

Differences in the presence of virulence genes in bacterial isolates from this investigation and prior studies might be attributed to geographic circumstances, dietary variables, and the migration of virulence genes through integrons and transposons. In addition to plasmids, [74] indicated that conjugation is a crucial method for the transmission of virulence genes in bacterial groups. These virulence genes, despite their high pathogenicity, are still in circulation, but the variables, as well as the mechanisms of the asymptomatic carriage, are poorly understood. Nonetheless, certain variables, such as a decrease in virulence gene expression or even the expression of bacterial components unique to a carrier, might be blamed. These pathogenicity island-encoded genes are critical in various phases of *Salmonella* pathogenesis [75,76].

Conclusions

This study shows that *Salmonella enterica* exists in local chicken meat and eggshell with high resistance to antibiotics, including multidrug resistance (75%). In addition, it demonstrates high proportions of *Salmonella enterica* isolates positive for virulence genes (*inv*A, *sip*B, *avr*A) responsible for initial invasion of the host cells.

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AUTHOR INFORMATION

Zahraa A. AlShaheeb, MSc, Assistant lecturer, Laboratory Scientist, Iraqi Ministry of Health. Baghdad 64074, Iraq. Tel.:+9647830602282, E-mail: zahraa.abdulkareem@ced.nahrainuniv.edu.iq
ORCID: https://orcid.org/0000–0002–0193–9218

Zaid A. Thabit, PhD, Assistant professor, Academic Stuff, Environmental Biotech Department, Biotechnology Research Center, Al-Nahrain University. Baghdad 64074, Iraq.
Tel.:+9647700326685, E-mail: zaid.alrawi@brc.nahrainuniv.edu.iq
ORCID: https://orcid.org/0000–0002–3431–7562
* corresponding author

Asma G. Oraibi, PhD, Professor, Academic Stuff, Plant Biotechnology Department, College of Biotechnology, Al-Nahrain University, Baghdad 64074, Iraq.
Tel.: +9647819905976, E-mail: asma.ghatea@nahrainuniv.edu.iq
ORCID: https://orcid.org/0000–0002–0533–1349

Ahmed A. Baioumy, PhD, Assistant Professor, Food Science Department, Faculty of Agriculture, Cairo University. 12613, 1 Gamaa Street, Giza, Egypt. Tel.: +2–0122–838–44–78, E-mail: ahmedadel35@agr.cu.edu.eg
ORCID: https://orcid.org/0000–0002–2909–603X

Tarek G. Abedelmaksoud, PhD, Assistant Professor, Food Science Department, Faculty of Agriculture, Cairo University. 12613, 1 Gamaa Street, Giza, Egypt. Tel.: +2–0110–144–12–80, E-mail: tareekgamal_88@agr.cu.edu.eg
ORCID: https://orcid.org/0000–0002–7012–6667

All authors bear responsibility for the work and presented data.

All authors made an equal contribution to the work.

The authors were equally involved in writing the manuscript and bear the equal responsibility for plagiarism.

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