Jundishapur J Microbiol. 2018 October; 11(10):e69504. doi: 10.5812/jjm.69504.
Published online 2018 September 18. **Research Article**

Molecular Detection, Virulence Genes, Biofilm Formation, and Antibiotic Resistance of *Salmonella enterica* Serotype *enteritidis* Isolated from Poultry and Clinical Samples

Reza Khaltabadi Farahani [1, 2], Parastoo Ehsani [1], Mina Ebrahimi-Rad [3] and Azad Khaledi [4, 5, *]

[1] Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran
[2] Department of Molecular, Central Veterinary Laboratory, Iranian Veterinary Organization, Tehran, Iran
[3] Department of Biochemistry, Pasteur Institute of Iran, Tehran, Iran
[4] Infectious Diseases Research Center, Kashan University of Medical Sciences, Kashan, Iran
[5] Department of Microbiology and Immunology, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran

[*] *Corresponding author*: Department of Microbiology and Immunology, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran. Tel: +98-9127804713, Fax: +98-3615575057, E-mail: azadkh99@gmail.com

Received 2018 April 17; **Revised** 2018 September 02; **Accepted** 2018 September 05.

Abstract

Background: *Salmonella* spp. is one of the most important zoonotic pathogens transmitting among human and animals. Due to the similarity of antibiotic classes used to treat animals and humans, there is a high risk for emerging the multi-drug resistant (MDR) strains.
Objectives: The current study aimed at evaluating molecular detection, virulence genes, biofilm formation, and antibiotic resistance of *Salmonella enterica* serotype *enteritidis* recovered from poultry and clinical isolates.
Methods: A total of 282 isolates were recovered from chicken meat, live poultry feces, eggs, and human feces in Iran. The presence of virulent factors in the isolates was confirmed using biochemical and microbiological tests. The presence of *Salmonella* genus was determined using antiserum. Triplex polymerase chain reaction (PCR) was performed to detect *Salmonella* spp., serogroup D and the discriminate *S. enteritidis* from other species. Kirby-Bauer disk diffusion method was applied to perform the susceptibility testing. Quantification of biofilm formation was determined in 96-well microtiter plates as recommended by the defined protocol. The data were then analyzed with SPSS using consensus tables and Chi-square test.
Results: Based on the results, all the isolates were positive for *invA*, *sdiA*, *hilA*, and *ratA*. Moreover, *spvC* had the lowest prevalence (37.6%). Of all strains, 67% were MDR, 51.7% of which were recovered from humans. Furthermore, 34.5% of isolates were strong biofilm producers. There was a significant correlation between the strong biofilm formation and the antibiotic resistance to colistin, ceftazidime, chloramphenicol, gentamicin, trimethoprim, penicillin, and trimethoprim-sulfamethoxazole.
Conclusions: The results of the current study showed a significant correlation between the strong biofilm formation and the antibiotic resistance to some antibiotics.

Keywords: Antibiotic Resistance, Biofilm Formation, *Salmonella enteritidis*

1. Background

Salmonella spp. is one of the most important foodborne pathogens and a major agent of diarrheal complications worldwide. As estimated, it annually accounts for around 1 million cases of salmonellosis and over 450 deaths in the United States (1). It is a zoonotic microorganism (2). Commonly, the main sources of *Salmonella* infections are contaminated food products, poultry, pigs, contaminated drinking water, ruminants, and direct contact with infected animals (3). *Salmonell enteritidis* is commonly associated with poultry and poultry derivatives (4). The most common typical symptoms of illness caused by *S. enteritidis* are fever, vomiting, diarrhea, and abdominal cramps after the consumption of contaminated food products and water (5).

In the US, amongst different species, 36% of *Salmonella* outbreaks resulted from *S. enterica* serovar *enteritidis* (6). *Salmonell enteritidis* pathogenicity is attributed to its capability to invade non-phagocytic cells and survive and grow inside macrophages and dendritic cells (7). The capability of *Salmonella* spp. to cause disease can be attributed to various virulence genes found in the either chromosome or large virulence-associated plasmids (8). Different virulence genes of *Salmonella*, such as *viz*, *sef*, *pef*, *spv*, and *inv*

are recognized to be involved in adhesion to and invading the host cells. Other virulence genes such as *mgtC* are associated with the survival of the host cells, and some other genes such as *viz*, *sop*, *stn*, *pip* A, B, and D have a significant role in real demonstration of pathogenic routes (9, 10).

It seems that selection pressure resulted from the use of antimicrobial drugs promotes growth or as prophylaxis in food-producing animals likely acts as the best selective pressure to develop resistant *S. enteritidis* (11). Surely, animals carrying resistant microorganisms may act as reservoirs; consequently, resistant strains may be spread from animals to humans via the food chain (12). And due to the similarity of the antibiotic classes used to treat the animals and humans, there is a high risk for antibiotic resistance and forming the multi-drug resistant (MDR) isolates (13). Regarding drug resistance, the role of antimicrobial drugs misuse and overuse in the emergence of antimicrobial resistance cannot be ignored (14).

Biofilm formation is one of the characteristics of these microorganism that intensifies the antibiotic resistance dilemma (15). Nowadays, the biofilm formation on equipment and tools is considered as a source of pathogenic bacteria that increases the risk of food product contamination in food processing systems (16). Biofilms protect bacteria from being damaged by environmental agents such as disinfectants and antibiotics; therefore, the bacteria can develop antibiotic resistance that is difficult to eliminate (17). Due to the high prevalence of resistant isolates recovered from poultry and domestic animals, and the possibility of their transmission through the food chain to humans, it seems to be necessary to evaluate the antibiotic resistance and determine the relationship between biofilm formation and antimicrobial resistance.

2. Objectives

The current study aimed at evaluating molecular detection, virulence genes, biofilm formation, and antibiotic resistance of *S. enterica* serotype *enteritidis* recovered from clinical and poultry strains.

3. Methods

3.1. Ethics Statement

The Ethics Committee of Pasteur Institute of Iran approved the current study protocol (IR.pII.REC.1394.83).

3.2. Isolates

The current study was conducted on 282 isolates including 165 isolates recovered from chicken meat, 30 isolates from live poultry feces, 31 isolates from eggs, and 36 isolates from human feces. The isolates were collected from five provinces including Tehran (n = 261), Mazandaran (n = 2), East Azerbaijan (n = 15), Qom (n = 1), and Sistan and Baluchistan (n = 3). The isolates were cultured on the TSI (triple sugar iron) agar (Himedia, India) and transferred to the Central Veterinary Laboratory of Iran Veterinary Organization (CVL-IVO) under standard conditions. Initially, several colonies were picked up from TSI, inoculated into a nutrient broth, and placed in an incubator at 37°C overnight. Afterward, the bacterial isolates were identified through culturing on xylose lysine deoxycholate (XLD) agar and Hektoen enteric (HE) agar (Himedia, India); the procedure was conducted according to ISO 6579 and Italian National Reference Centers (IZSVE) protocols. Then, *Salmonella* genus was determined using antiserum. Agglutination in polyvalent antisera and biochemical tests were used for final genus/species-level identification of *Salmonella* spp. (18). Finally, Luria-Bertani (LB) broth (Himedia, India) was used to culture the confirmed isolates.

3.3. Detection of Salmonella spp. Using Triplex Polymerase Chain Reaction and Differentiation of S. enterica Serotype enteritidis from Other Salmonella spp.

3.3.1. Extraction of DNA

High pure PCR template preparation kit (Roche Company, REF: 11796828001) was used to extract DNA. Two hundred microliters of broth containing the cultured isolates were used, as recommended by the manufacturer's manual.

3.3.2. Triplex PCR Performance

In order to perform PCR assay, three primer pairs were used. The *invA* primer (199 bp) with sequences of F-*invA*: AAA CGT TGA AAA ACT GAG GA, and R-*invA*: TCG TCA TTC CAT TAC CTA CC (19) was used to detect *Salmonella* genus. A *tyv* primer (614 bp) with sequences of F-*tyv*: GAGGAAGGGAAATGAAGCTTTT, and R- *tyv*: TAGCAAACTGTCTCCCACCATAC (20) was used to detect serogroup D. Finally, both of the two primers were used to confirm the findings in each culture and determine the serogroup with antiserum (Mast). Furthermore, *sdf* primer (299 bp) with sequences of F-*sdf*: AAA TGT GTT TTA TCT GAT GCA AGA GG and R- *sdf*: GTT CGT TCT TCT GGT ACT TAC GAT GAC was used to detect the serovar (20). The strains positive for all the three genes were considered as S. enterica serotype *enteritidis*.

Each PCR reaction contained 2.5 μL of 10X PCR buffer (Qiagen, Germany), 3 μL of DNA template, 1.5 μL of $MgCl_2$ (25 mM) (Qiagen, Germany), 1.2 μL of dNTP (10 Mm) (Qiagen, Germany), 1.5 μL of each of the forward and reverse

primers of *invA* (10 μM) (MWG Company, Germany), 0.75 μL of each of the forward and reverse primers of *Sdf* (10 μM) (MWG Company, Germany), 0.75 μL of each of the forward and reverse primers of *tyv* (10 mM) (MWG Company, Germany), 0.2 μL of hot start Taq DNA polymerase [(5 U/μL) (Qiagen, Germany)], and 10.5 μL of distilled water in a total volume of 25 μL. The following steps were taken to carry out PCR reaction: initial denaturation (95°C for 10 minutes), denaturation (94°C for 60 seconds, 30 cycles), annealing step (60°C for 90 seconds), extension step (72°C for 90 seconds), and final extension (72°C for 10 minutes, one cycle). The PCR products were analyzed on a 1.5% (w/v) agarose gel electrophoresis comprising SYBR® Safe (Qiagen, Germany) using the standard protocol. DNA ladder 100 bp (Qiagen, Germany) was used as a marker.

3.4. Antibiotic Resistance Testing

All *S. enterica* serotype *enteritidis* isolates were studied via Kirby-Bauer disk diffusion method to evaluate their resistance to antibiotic disks. The criteria proposed by the National Committee for Clinical Laboratory Standards was used to determine susceptibility rates (21). Antibiotic disks used in the current study were purchased from MAST, UK. The results of the tests were categorized into three classes of resistant, intermediate, and sensitive. *Escherichia coli* ATCC 35218 and ATCC 25922 were used as reference strains for quality control. The antibiotics used in the current study were as follows: nalidixic acid (30 μg), amoxicillin (30 μg), amoxicillin + clavulanic acid (20/10 μg), cefoxitin (30 μg), colistin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), cefalotin (30 μg), enrofloxacin (5 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefepime (10 μg), kanamycin (30 μg), ampicillin (10 μg), neomycin (10 μg), ertapenem (10 μg), streptomycin (10μg), chloramphenicol (30 μg), trimethoprim + sulfamethoxazole (23.75/1.75 μg), tetracycline (30 μg), imipenem (10 μg), and meropenem (10 μg).

3.5. Primer Design, PCR Amplification Conditions, and Detection of Virulence Genes

As mentioned earlier, *sdiA*, *spvC*, *ratA*, *lpfC*, *sitC*, *sifA*, *sopB*, *spiA*, *sipA*, *invA*, and *hilA* virulence genes primer sequences were expected to form the desired products by mPCR technique on the DNA extracted from *S. enterica* serotype *enteritidis* isolates (22). The specificity of the primers was approved by the Blast program of GenBank database. The primers were designed using Gene Runner software and synthesized by MWG Company, Germany. The sequences of virulence genes primers are presented in Table 1.

PCR assay was carried out in a DNA Thermocycler (Model CP2-003, Corbett, Australia) as follows: one cycle of 95°C for 5 minutes, followed by 35 cycles of denaturation (95°C for one minute), annealing step [(*sdiA* and *spvC* (53°C), *ratA* (56°C), *lpfC* (64°C), *sitC*, *sifA*, *sopB*, and *spiA* (60°C), *hilA* (52°C), and *invA* (58°C)] (incubation time for all genes in the annealing step were 40 seconds), and elongation step at 72°C for 30 seconds and 10 minutes of final elongation at 72°C. The total PCR reaction (25 μL) included 2.5 μL of 10X Buffer, 14.7 μL of distilled water, 1 μL of dNTP (10 mM), 1.5 μL of $MgCl_2$ (25 mM), 0.3 of hot start Taq DNA polymerase (5 U/μL), 1 μL (10 μM) of each of the forward and revers primers, and 3 μL of the template DNA. Electrophoresis was performed on 1.5% agarose gel subjected to 140 V for one hour to visualize PCR product bands later analyzed using GeneTools and SnapGene software.

3.6. Biofilm Formation

For quantification of biofilm formation 96-well microtiter plates were used; biofilm formation was measured in line with a previous study (27). Firstly, 230 μL of the nutrient broth medium (Himedia, India) was poured into wells of a sterile 96-well flat-bottomed polystyrene microplate. Three wells were allocated to each sample and the negative control (wells contained broth only). About 20 μL of bacterial culture (grown overnight) was poured into each well of the microplates. Afterward, incubation of the plates was performed overnight at 35°C. The content of the microplates was discarded; the wells were washed three times using 300 μL of PBS. Then, 250 μL of methanol was added to each well to fix the microorganisms attached to the surface of microplates. After 15 minutes, the content of microplates was taken out and air dried at 25°C.

The microplates were stained using Gram staining with 250 μL of Crystal violet per well for 5 minutes. To wash the extra stain, microplates were put under running tap water. Finally, microplates were air dried, and about 250 μL of 33% (v/v) glacial acetic acid was poured into each well to wash the bound dye. An automated Multiskan ELX808 reader (Labsystems, USA) at 570 nm and software (Gene5) were used to measure the optical density (OD) of wells. Taking into account the optical density (OD) of each well, isolates were categorized into four groups: these groups were isolates with a weak, moderate, or without the potential to produce the biofilm. The other group included isolates with a potential of producing a strong biofilm. In brief, optical density cut-off value (ODc) was equal to the average OD of negative control plus 3 x standard deviation (SD) of the negative control. The tested isolates were categorized as follows: $OD \leq ODc$: non-adherent; $ODc < OD \leq (2 \times ODc)$: weak biofilm producers; $2 \times ODc < O.D \leq 4 \times ODc$:

Table 1. The Sequences of Virulence Genes Primers Used in the Current Study

Target Gene, Primer Name	Sequences (5' to 3')	Annealing Temperature (°C)	PCR Product Size (bp)	Reference
sdiA		53	274	(23)
sdiA-F	AATATCGCTTCGTACCAC			
sdiA-R	GTAGGTAAACGAGGAGCAG			
spvC		53	571	(23)
spvC-F	ACTCCTTGCACAACCAAATGCGGA			
spvC-R	TGTCTCTGCATTTCGCCACCATCA			
ratA		56	243	(24)
ratA-F	GACGTCGCTGCCGTCGTACC			
ratA-R	TACAGCGAACATGCGGGCGG			
lpfC		64	641	(3)
lpfC-F	GCC CCG CCT GAA GCC TGT GTT GC			
lpfC-R	AGG TCG CCG CTG TTT GAG GTT GGA TA			
sitC		60	768	(3)
sitC-F	CAG TAT GCT CAA CGC GAT GTC TCC			
sitC-R	CGG GGC GAA AAT AAA GGC TGT GAT GAA C			
sifA		60	449	(3)
sifA-F	TTT GCC GAA CGC GCC CCC CAC AGC			
sifA-R	GTT GCC TTT TCT TGC GCT TTC CAC CCA TCT			
sopB		60	220	(3)
sopB-F	CGG ACC GGC CAG CAA CAA AAC AAG AAG			
sopB-R	TAG TGA TGC CCG TTA TGC GTG AGT GTA TT			
spiA		60	550	(3)
spiA-F	CCAGGGGTCGTTAGTGTATTGCGTGAGATG			
spiA-R	CGCGTAACAAAGAACCCGTAGTGATGGATT			
sipA		60	875	(3)
sip A-F	GGACGCCGCCCGGGAAAAACTCTC			
sip A-R	ACACTCCCGTCGCCGCCTTCACAA			
hilA		52	497	(25)
hilA2-up	CTG CCG CAG TGT TAA GGA TA			
hilA2-down	CTG TCG CCT TAA TCG CAT GT			
invA		58	796	(26)
invA-F	CGG TGG TTT TAA GCG TAC TCT T			
invA-R	CGA ATA TGC TCC ACA AGG TTA			
invA		60	199	(26)
	AAA CGT TGA AAA ACT GAG GA			
	TCG TCA TTC CAT TAC CTA CC			

moderate biofilm producers; and 4 × ODc < OD: strong biofilm producers. The obtained results are presented as means and standard deviation (SD).

3.7. Statistical Analysis of Data

The data were then analyzed with SPSS version 16.0 (Chicago, Illinois, USA). Consensus tables and Chi-square test were used to evaluate the correlation between biofilm formation potential and antibiotic resistance. P values < 0.05 were considered statistically significant.

4. Results

4.1. Identification of Salmonella enterica Serotype enteritidis

Genus, serogroup, and serovar were determined using triplex-PCR. The *invA*, *tyv*, and *sdf* genes were used to detect *Salmonella* genus, serogroup D, and serovar, respectively. All the tested isolates were positive for all the three genes and were identified as *S. enterica* serotype *enteritidis* (Figure 1).

Table 2. Antibiotic Resistance Frequency of *Salmonella enterica* Serotype *enteritidis* Isolated From Different Sources

Sample (%)	Antibiotic																				
	Fox	AM	Aug	C	IMI	MEM	ATM	CIP	CAZ	CAZ+ Clav	CRO	CPM	ETP	P	AM	FM	LVX	GM	K	CO	T	NA
Human	47.1	20.6	14.7	35.3	14.7	2.9	2.9	23.5	2.9	-	-	-	-	94.1	14.7	50	-	11.8	47.1	91.2	91.2	88.2
Chicken	7.1	-	-	42.9	-	-	-	64.3	7.1	-	7.1	7.1	-	100	-	92.9	7.1	42.9	50	50	14.3	78.6
Poultry	-	-	-	5.9	-	-	-	11.8	5.9	-	-	-	-	70.6	AM	17.6	-	-	-	-	-	94.1

Abbreviations: Fox, cefoxitine; A, amoxicillin; ATM, aztreonam; Aug, augmentin; C, colistin; CAZ, ceftazidime; Clav, clavelanic acid; CO, chloramphenicol; CPM, cefepime; IM, imipenem; MEM, meropenem; CIP, ciprofloxacin; CRO, ceftriaxone; EP, ertapenem; K, kanamycin; NA, nalidixic Acid; Ne, neomycin; P, penicillin; TMP/SMX, trimethoprim/sulfamethoxazole; T/S, trimethoprim-sulfamethoxazole.

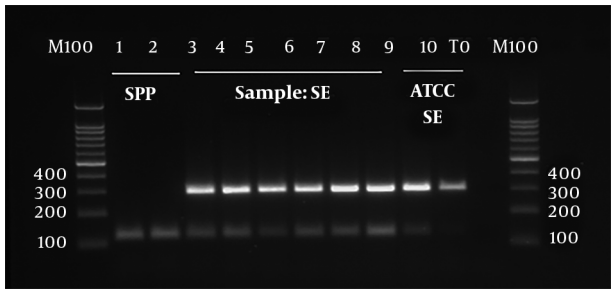

Figure 1. PCR amplification of virulence genes. Left to right: Lane M 100; ladder 100 bp; lanes 1 and 2: *Salmonella* spp.; lanes 3, 4, 5, 6, 7, 8 and 9 (Samples): *S. entritidis*; Lane 10: ATCC SE.

4.2. Results of the Analysis of Virulence Genes in Salmonella enterica Serotype enteritidis

Analysis of virulence genes showed that the prevalence of *invA*, *sdiA*, *hilA*, and *ratA* was 100% (n = 282). The prevalence of other genes was as follows: *sopB* (n = 281, 99.6%), *sitC* and *sifA* (n = 275, 97.9%), and *spiA* and *sipB* (n = 280, 99.3%). The lowest prevalence was observed in *spvC* (n =106, 37.6%) (Figure 2).

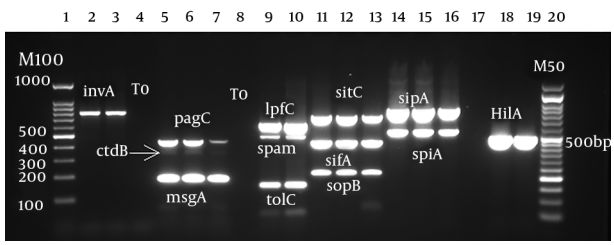

Figure 2. Virulence genes detection by multiplex PCR. Lane 1: marker 100 bp; lanes 2 and 3: invA (780 bp); lanes 4, 8, and 17: negative control; lanes 5, 6, and 7: pag (454 bp), cdtB (268 bp), and msgA (189 bp); lanes 9 and 10: ipfC (641 bp), span (504 bp), and tolC (161 bp); lanes 11, 12, and 13: sitC (768 bp), sifA (449 bp), and sopB (220 bp); lanes 14, 15, and 16: sipA (875 bp) and spiA (550 bp); lanes 18 and 19: hilA (497 bp), lane 20: marker 50 bp.

4.3. Results of Susceptibility Testing

Of all strains, 67% were MDR, of which 51.7% were recovered from humans and the remaining 15.3% from chicken meat. No species was isolated from live poultry. Only five isolates were resistant to one antibiotic; in addition, one strain was resistant to two antibiotics, and the other isolates were resistant to three or more antibiotics. The isolates recovered from human samples were mainly resistant to penicillin, followed by colistin, and trimethoprim-sulfamethoxazole with a prevalence of 94%, 91.2%, and 91.2%, respectively. Furthermore, there was no resistance to ciprofloxacin, levofloxacin, ertapenem, and cefepime. The isolates recovered from live poultry were mainly resistant to nalidixic acid ad penicillin, with a prevalence of 94.1% and 70.6%, respectively; however, there was no significant resistance to other antibiotics. Finally, the isolates recovered from chicken meat were mainly resistant to nitrofurantoin and penicillin, with a prevalence of 100%; however, they showed no resistance to ampicillin, amoxicillin, and meropenem (Figure 3).

4.4. Results of Biofilm Formation

According to the results of biofilm formation test, 98 (34.5%) isolates were strong biofilm producers; in addition, 168 (59.6%) and 16 (5.7%) isolates were moderate and weak biofilm producers, respectively. As shown in Table 3, there was a statistically significant correlation between strong biofilm formation and resistance to colistin (P = 0.001), ceftazidime (P = 0.04), penicillin (P = 0.001), gentamicin (P = 0.04), chloramphenicol (P = 0.001), trimethoprim (P = 0.001), and trimethoprim-sulfamethoxazole (P = 0.001).

5. Discussion

The results of virulence genes analysis showed that all the isolates (100%) were positive for *invA*, *sdiA*, *hilA*, and *ratA*. Moreover, the lowest prevalence (37.6%) was observed in *spvC*. In the current study, *S. enteritidis* isolated from poultry, chicken meat, and eggs had the virulence genes similar to the ones present in isolates recovered from human feces. The results indicated that isolates recovered from poultry, chicken meat, and eggs were human pathogens. This finding was also reported by Akiyama et al., since they stated

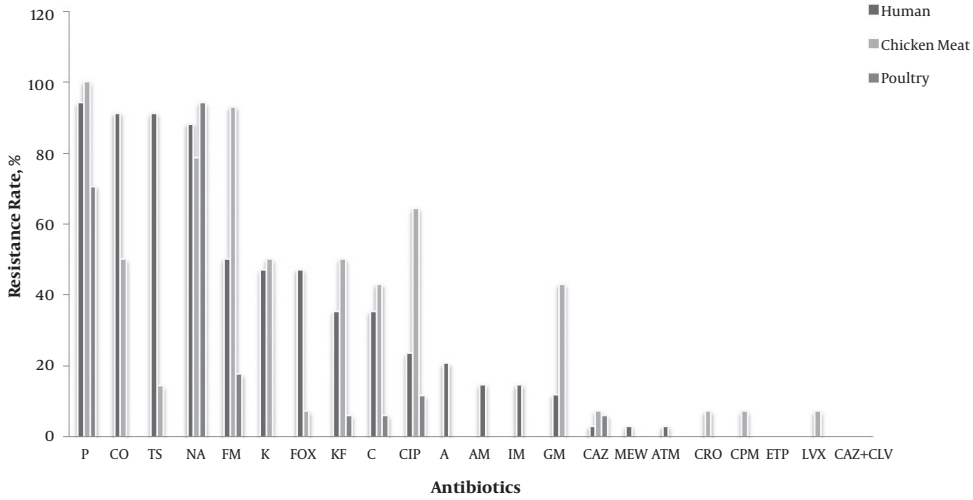

Figure 3. The total results of antibiotic resistance in live poultry, human, and chicken meat. A, Amoxicillin; C, colistin; CAZ, ceftazidime; CIP, ciprofloxacin; CO, chloramphenicol; CPM, cefepime; CRO, ceftriaxone; EP, ertapenem; FM, framycetin; Fox, cefoxitine; GM, gentamicin; IM, imipenem; K, kanamycin; KF, cefalotine; LVX, levofloxacin; MEM, meropenem; NA, nalidixic acid; NIT, nitrofurantoin; P, penicillin; TMP/SMX, trimethoprim/sulfamethoxazole; T/S, trimethoprim-sulfamethoxazole.

Table 3. Correlation Between Strong Biofilm Formation and Antibiotic Resistance

Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)	P Value
Fox	64.6 ± 0.07	9.2 ± 1.14	26.2 ± 3.29	0.075
A	81.5 ± 2.1	7.6 ± 0.11	10.7 ± 1.2	0.174
C	44.1 ± 0.111	26.1 ± 0.06	29.2 ± 0.111	0.02
IM	81.5 ± 4.5	10.8 ± 0.08	7.7 ± 1.3	0.174
MEM	98.4 ± 5.7	0 ± 0.01	1.6 ± 0.67	0.674
CIP	0 ± 0.01	70.7 ± 4.2	29.3 ± 2.34	0.138
CAZ	90.7 ± 4.56	4.6 ± 0.06	4.6 ± 0.06	0.04
CRO	98.4 ± 3.23	0 ± 0.001	1.6 ± 0.95	0.24
CPM	96.9 ± 3.1	1.5 ± 0.08	1.5 ± 0.21	0.214
P	9.2 ± 0.08	1.1 ± 0.5	89.2 ± 3.1	0.001
FM	32.3 ± 2.3	16.9 ± 3.32	50.7 ± 3.76	0.537
AM	76.9 ± 0.07	4.6 ± 0.9	18.4 ± 0.07	0.966
LVX	98.4 ± 5.6	0 ± 0.001	1.6 ± 1.1	0.24
GM	60 ± 3.7	24.6 ± 2.1	15.4 ± 1.3	0.04
NA	1.5 ± 02.1	10.7 ± 1.32	87.6 ± 3.33	0.602
TMP/SMX	49.2 ± 0.95	0 ± 0.01	50.8 ± 2.13	0.001
Co	41.5 ± 0.06	0 ± 0.002	58.5 ± 3.89	0.001
ATM	98.4 ± 4.1	0 ± 0.01	1.6 ± 0.11	0.674
EP	98.4 ± 1.14	0 ± 0.02	1.60 ± 1.11	0.674
T	49.2 ± 0.06	0 ± 0.002	50 ± 5.1	0.001

Abbreviations: Fox, cefoxitine; A, amoxicillin; C, colistin; CAZ, ceftazidime; CIP, ciprofloxacin; CO, chloramphenicol; CPM, cefepime; CRO, ciprofloxacin; EP, ertapenem; FM, framycetin; GM, gentamicin; IM, imipenem; MEM, meropenem; NA, nalidixic acid; P, penicillin; TMP/SMX, trimethoprim/sulfamethoxazole; T/S, trimethoprim-sulfamethoxazole.

that the isolates recovered from the environmental specimens carried the genes similar to those harbored by clinical strains, which contributed to human pathogenesis (28). In line with the current study findings, a study conducted by Mezal et al., showed that all isolates were positive for most of the virulence genes found in *S. enterica* (3).

In the current study, 67% of all the strains were MDR, of which 51.7% were recovered from humans and the re-

maining 15.3% were isolated from chicken meat and eggs; furthermore, no isolate was recovered from live poultry. Moreover, five strains were resistant to only one antibiotic, one strain was resistant to two antibiotics, and the remaining were resistant to three or more antibiotics. Nowadays, it is believed that animals and their products are the main sources of MDR *Salmonella* species; the resistance first emerges in animals, before transmitting to humans via the food chain (29). Surprisingly, no resistant strains were retrieved from poultry. On the other hand, the resistant strains were mainly isolated from human and partly from chicken and the eggs. This finding might be attributed to the correct administration of antibiotics for chickens. Our findings were inconsistent with those demonstrating that the increase of antimicrobial resistance amongst food-borne bacteria in the recent decades might be attributed to the selection pressure produced by the unsuitable or unrestricted use of antibiotics in veterinary medicine (30).

Overall, there was no resistance to ertapenem and ceftazidime-clavulanic acid; on the other hand, the resistance to penicillin and nalidixic acid was the most common type. Resistance to nalidixic acid in *S. enterica* serovar *enteritidis* recovered from different sources was high, and the prevalence rates were as follows: human isolates (88.2%), poultry (94.1%), and chicken/eggs (78.6%). The resistance to nalidixic acid in the current study was higher than those reported by other studies in Brazil (21.5%) and Europe (26%) (31). To the best of the author's knowledge, fluoroquinolones are widely used in veterinary for therapeutic purposes; they are also selectively used for mutant *Salmonella* isolates resistant to nalidixic acid or for cases with decreased susceptibility to fluoroquinolones (32, 33). As mentioned in the results section, all the isolates showed a high susceptibility to ceftazidime-clavulanic acid (100%), ceftriaxone (92.1%), ceftazidime (94.7%), and cefepime (7.1%), which may indicate the limited and controlled administration of such antibiotics in poultry and animal food products in Iran. These results were inconsistent with those reported from Turkey (34); but in agreement with the current study findings, a study conducted in Brazil showed the resistance about 88.8% to nalidixic acid (35). Also, Ghasemmahdi et al. showed the resistance (97%) to nalidixic acid (36).

The current study aimed at investigating the correlation between biofilm formation potential and antibiotic resistance of isolates species, if any. Based on the results of biofilm formation assessments, 34.5% of the isolates were strong biofilm producers, while 59.6% and 5.7% were moderate and weak biofilm producers, respectively. In line with our findings, a study conducted by Sereno et al., reported that 72.7% of all the isolates could form biofilms, although 13.6%, 59.1%, and 34% were moderate, weak, and strong biofilm producers, respectively. Nonetheless, 27.3% did not form biofilms (15) and inconsistent with the current study, they found no strong biofilm producers. Furthermore, there was a statistically significant correlation between strong biofilm formation and resistance to colistin, ceftazidime, penicillin, gentamicin, chloramphenicol, trimethoprim, and trimethoprim-sulfamethoxazole. However, contrary to the current study findings, a research conducted by Reza et al., reported that biofilm formation potential could not change the antibiotic resistance profile in the microorganisms (37).

In general, owing to the administration of antibiotics to improve growth and prevent diseases in food animals, the augmentation of human salmonellosis started with the emergence of foodborne MDR *Salmonella* (23, 38). The information acquired from various studies could be utilized to develop better strategies to eliminate biofilms in the food chain, and accordingly, reduce *Salmonella* contamination in animal/food chain. Furthermore, to ensure the effectiveness of control programs, it is necessary to constantly monitor antibiogram results reported worldwide.

5.1. Conclusions

The presented results showed that more than half of all the assessed strains were MDR. Moreover, there was a significant correlation between biofilm formation potential and antibiotic resistance. Due to the isolation of MDR strains both from clinical and chicken/egg samples, and the likely transmission of resistant species through food chain to human, it seems that there was a significant correlation between biofilm formation and antibiotic resistance; thus, control programs are needed to prevent uncontrollable outbreaks.

Acknowledgments

Authors would like to thank the laboratories staff for their help with the study.

Footnotes

Authors' Contribution: All authors contributed to the study design. Azad Khaledi: study design; Reza Khaltabadi Farahani: performing the experiments and preparing the manuscript; Parastoo Ehsani and Mina Ebrahimi Rad: revising of the manuscript and analysis of the data.

Conflict of Interests: This study did not receive any financial support.

Funding/Support: All authors contributed to the study design. Azad Khaledi: study design; Reza Khaltabadi Farahani: performing the experiments and preparing the manuscript; Parastoo Ehsani and Mina Ebrahimi Rad: revising of the manuscript and analysis of the data.

References

1. Taylor AJ, Lappi V, Wolfgang WJ, Lapierre P, Palumbo MJ, Medus C, et al. Characterization of foodborne outbreaks of Salmonella enterica serovar Enteritidis with whole-genome sequencing single nucleotide polymorphism-based analysis for surveillance and outbreak detection. *J Clin Microbiol*. 2015;**53**(10):3334–40. doi: 10.1128/JCM.01280-15. [PubMed: 26269623]. [PubMed Central: PMC4572550].
2. Galanis E, Lo Fo Wong DM, Patrick ME, Binsztein N, Cieslik A, Chalermchikit T, et al. Web-based surveillance and global Salmonella distribution, 2000-2002. *Emerg Infect Dis*. 2006;**12**(3):381–8. doi: 10.3201/eid1205.050854. [PubMed: 16704773]. [PubMed Central: PMC3291443].
3. Mezal EH, Sabol A, Khan MA, Ali N, Stefanova R, Khan AA. Isolation and molecular characterization of Salmonella enterica serovar Enteritidis from poultry house and clinical samples during 2010. *Food Microbiol*. 2014;**38**:67–74. doi: 10.1016/j.fm.2013.08.003. [PubMed: 24290628].
4. Shomer I, Avisar A, Desai P, Azriel S, Smollan G, Belausov N, et al. Genetic and phenotypic characterization of a Salmonella enterica serovar enteritidis emerging strain with superior intramacrophage replication phenotype. *Front Microbiol*. 2016;**7**:1468. doi: 10.3389/fmicb.2016.01468. [PubMed: 27695450]. [PubMed Central: PMC5025531].
5. Liu F, Barrangou R, Gerner-Smidt P, Ribot EM, Knabel SJ, Dudley EG. Novel virulence gene and clustered regularly interspaced short palindromic repeat (CRISPR) multilocus sequence typing scheme for subtyping of the major serovars of Salmonella enterica subsp. enterica. *Appl Environ Microbiol*. 2011;**77**(6):1946–56. doi: 10.1128/AEM.02625-10. [PubMed: 21278266]. [PubMed Central: PMC3067318].
6. Gould LH, Walsh KA, Vieira AR, Herman K, Williams IT, Hall AJ, et al. Surveillance for foodborne disease outbreaks - United States, 1998-2008. *MMWR Surveill Summ*. 2013;**62**(2):1–34. [PubMed: 23804024].
7. Gal-Mor O, Boyle EC, Grassl GA. Same species, different diseases: How and why typhoidal and non-typhoidal Salmonella enterica serovars differ. *Front Microbiol*. 2014;**5**:391. doi: 10.3389/fmicb.2014.00391. [PubMed: 25136336]. [PubMed Central: PMC4120697].
8. Fluit AC. Towards more virulent and antibiotic-resistant Salmonella? *FEMS Immunol Med Microbiol*. 2005;**43**(1):1–11. doi: 10.1016/j.femsim.2004.10.007. [PubMed: 15607630].
9. Murugkar HV, Rahman H, Dutta PK. Distribution of virulence genes in Salmonella serovars isolated from man & animals. *Indian J Med Res*. 2003;**117**:66–70. [PubMed: 12931840].
10. Rahman H, Prager R, Tschape H. Occurrence of sef & pef genes among different serovars of Salmonella. *Indian J Med Res*. 2000;**111**:40–2. [PubMed: 10824465].
11. Schwarz S, Kehrenberg C, Walsh TR. Use of antimicrobial agents in veterinary medicine and food animal production. *Int J Antimicrob Agents*. 2001;**17**(6):431–7. [PubMed: 11397611].
12. Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, et al. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother*. 2004;**53**(1):28–52. doi: 10.1093/jac/dkg483. [PubMed: 14657094].
13. Vaz CS, Streck AF, Michael GB, Marks FS, Rodrigues DP, Dos Reis EM, et al. Antimicrobial resistance and subtyping of Salmonella enterica subspecies enterica serovar Enteritidis isolated from human outbreaks and poultry in southern Brazil. *Poult Sci*. 2010;**89**(7):1530–6. doi: 10.3382/ps.2009-00453. [PubMed: 20548083].
14. Ghaderi R, Moradi Bidhendi S, Khaki P. Occurrence of multidrug-resistant Salmonella enterica serovar Enteritidis isolates from poultry in Iran. *Arch Razi Inst*. 2016;**71**(1):43–9.
15. Sereno MJ, Ziech RE, Druziani JT, Pereira JG, Bersot LS. Antimicrobial susceptibility and biofilm production by Salmonella sp. strains isolated from frozen poultry carcasses. *Braz J Poultry Sci*. 2017;**19**(1):103–8. doi: 10.1590/1806-9061-2016-0268.
16. Shi X, Zhu X. Biofilm formation and food safety in food industries. *Trends Food Sci Tech*. 2009;**20**(9):407–13. doi: 10.1016/j.tifs.2009.01.054.
17. Wang H, Ye K, Wei X, Cao J, Xu X, Zhou G. Occurrence, antimicrobial resistance and biofilm formation of Salmonella isolates from a chicken slaughter plant in China. *Food Contr*. 2013;**33**(2):378–84. doi: 10.1016/j.foodcont.2013.03.030.
18. Khaltabadi Farahani R, Karami P, Ehsani P, Khaledi A, Khaltabadi Farahani AH. Molecular detection of virulence genes of Salmonella gallinarum from Iranian poultry. *J Res Med Dent Sci*. 2018;**6**(2):404–8. doi: 10.5455/jrmds.20186262.
19. Lu Y, Zhao H, Sun J, Liu Y, Zhou X, Beier RC, et al. Characterization of multidrug-resistant Salmonella enterica serovars Indiana and Enteritidis from chickens in Eastern China. *PLoS One*. 2014;**9**(5). e96050. doi: 10.1371/journal.pone.0096050. [PubMed: 24788434]. [PubMed Central: PMC4008530].
20. Kasturi KN, Drgon T, Dozois CM. Real-time PCR method for detection of Salmonella spp. in environmental samples. *Appl Environ Microbiol*. 2017;**83**(14). doi: 10.1128/aem.00644-17.
21. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing*. Wayne, PA: Clinical and Laboratory Standards Institute; 2007.
22. Lim BK, Thong KL. Application of PCR-based serogrouping of selected Salmonella serotypes in Malaysia. *J Infect Dev Ctries*. 2009;**3**(6):420–8. [PubMed: 19762954].
23. Turki Y, Mehr I, Ouzari H, Khessairi A, Hassen A. Molecular typing, antibiotic resistance, virulence gene and biofilm formation of different Salmonella enterica serotypes. *J Gen Appl Microbiol*. 2014;**60**(4):123–30. [PubMed: 25273985].
24. Batista DF, de Freitas Neto OC, Lopes PD, de Almeida AM, Barrow PA, Berchieri A Jr. Polymerase chain reaction assay based on ratA gene allows differentiation between Salmonella enterica subsp. enterica serovar Gallinarum biovars Gallinarum and Pullorum. *J Vet Diagn Invest*. 2013;**25**(2):259–62. doi: 10.1177/1040638713479361. [PubMed: 23512922].
25. Zhu C, Yue M, Rankin S, Weill FX, Frey J, Schifferli DM. One-step identification of five prominent chicken Salmonella serovars and biotypes. *J Clin Microbiol*. 2015;**53**(12):3881–3. doi: 10.1128/JCM.01976-15. [PubMed: 26378281]. [PubMed Central: PMC4652083].
26. Paiao FG, Arisitides LG, Murate LS, Vilas-Boas GT, Vilas-Boas LA, Shimokomaki M. Detection of Salmonella spp, Salmonella Enteritidis and Typhimurium in naturally infected broiler chickens by a multiplex PCR-based assay. *Braz J Microbiol*. 2013;**44**(1):37–41. doi: 10.1590/S1517-83822013005000002. [PubMed: 24159281]. [PubMed Central: PMC3804175].
27. Stepanovic S, Cirkovic I, Ranin L, Svabic-Vlahovic M. Biofilm formation by Salmonella spp. and Listeria monocytogenes on plastic surface. *Lett Appl Microbiol*. 2004;**38**(5):428–32. doi: 10.1111/j.1472-765X.2004.01513.x. [PubMed: 15059216].
28. Akiyama T, Khan AA, Cheng CM, Stefanova R. Molecular characterization of Salmonella enterica serovar Saintpaul isolated from imported seafood, pepper, environmental and clinical samples. *Food Microbiol*. 2011;**28**(6):1124–8. doi: 10.1016/j.fm.2011.03.003. [PubMed: 21645810].

29. Stevens A, Kerouanton A, Marault M, Millemann Y, Brisabois A, Cavin JF, et al. Epidemiological analysis of Salmonella enterica from beef sampled in the slaughterhouse and retailers in Dakar (Senegal) using pulsed-field gel electrophoresis and antibiotic susceptibility testing. *Int J Food Microbiol*. 2008;**123**(3):191–7. doi: 10.1016/j.ijfoodmicro.2008.01.007. [PubMed: 18325615].
30. Van TT, Moutafis G, Tran LT, Coloe PJ. Antibiotic resistance in food-borne bacterial contaminants in Vietnam. *Appl Environ Microbiol*. 2007;**73**(24):7906–11. doi: 10.1128/AEM.00973-07. [PubMed: 17951438]. [PubMed Central: PMC2168151].
31. Meakins S, Fisher IS, Berghold C, Gerner-Smidt P, Tschape H, Cormican M, et al. Antimicrobial drug resistance in human nontyphoidal Salmonella isolates in Europe 2000-2004: A report from the enter-net international surveillance network. *Microb Drug Resist*. 2008;**14**(1):31–5. doi: 10.1089/mdr.2008.0777. [PubMed: 18366323].
32. Ferrari R, Galiana A, Cremades R, Rodriguez JC, Magnani M, Tognim MC, et al. Plasmid-mediated quinolone resistance (PMQR) and mutations in the topoisomerase genes of Salmonella enterica strains from Brazil. *Braz J Microbiol*. 2013;**44**(2):651–6. doi: 10.1590/S1517-83822013000200046. [PubMed: 24294265]. [PubMed Central: PMC3833171].
33. Ferrari R, Galiana A, Cremades R, Rodriguez JC, Magnani M, Tognim MC, et al. Plasmid-mediated quinolone resistance by genes qnrA1 and qnrB19 in Salmonella strains isolated in Brazil. *J Infect Dev Ctries*. 2011;**5**(6):496–8. [PubMed: 21727652].
34. Arslan S, Eyi A. Occurrence and antimicrobial resistance profiles of Salmonella species in retail meat products. *J Food Prot*. 2010;**73**(9):1613–7. [PubMed: 20828466].
35. de Oliveira MMM, Brugnetra DF, Piccoli RH. Microbial biofilms in the food industry: A review. *J Adolfo Lutz Institute*. 2010;**69**(3):277–84.
36. Ghasemmahdi H, Tajik H, Moradi M, Mardani K, Modaresi R, Badali A, et al. Antibiotic resistance pattern and biofilm formation ability of clinically isolates of Salmonella enterica serotype typhimurium. *Int J Enteric Pathog*. 2015;**3**(2). doi: 10.17795/ijep27372.
37. Raza A, Sarwar Y, Ali A, Jamil A, Haque A, Haque A. Effect of biofilm formation on the excretion of Salmonella enterica serovar Typhi in feces. *Int J Infect Dis*. 2011;**15**(11):e747–52. doi: 10.1016/j.ijid.2011.06.003. [PubMed: 21816646].
38. Yang B, Qu D, Zhang X, Shen J, Cui S, Shi Y, et al. Prevalence and characterization of Salmonella serovars in retail meats of marketplace in Shaanxi, China. *Int J Food Microbiol*. 2010;**141**(1-2):63–72. doi: 10.1016/j.ijfoodmicro.2010.04.015. [PubMed: 20493570].