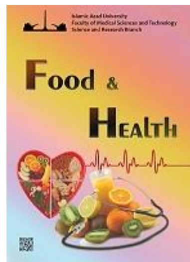
Food & Health 2022, 5(3): 1-7



Food & Health

Journal homepage: fh.srbiau.ac.ir

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Prevalence, virulence factor, and antibiotics susceptibility patterns of *Salmonella* spp. from poultry products in Ardabil province

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ARTICLE INFO

Original Article

Article history:
Received 22 June 2022
Revised 06 August 2022
Accepted 30 August 2022
Available online 20 September 2022

Keywords:
Salmonella spp.
BOXAIR-PCR
REP- PCR
Drug resistance
Biofilm

ABSTRACT

Contamination of poultry products by *Salmonella* spp. is a critical issue in the poultry industry and public health. The present study aimed at molecular detection and typing of *Salmonella* isolated from poultry products. Moreover, antibiotic resistance patterns and the biofilm formation ability of isolates were determined. Eighty poultry product samples were collected from chicken supply and distribution centers. *Salmonella* spp. were identified by culture as well as the genus-specific PCR. A slide agglutination test using O grouping polyvalent sera were used for serological identification. BOXAIR and REP-PCR methods were evaluated for the discrimination of *Salmonella* isolates at the serotype level. Antibiotic susceptibility testing of serotypes against sixteen antibiotics was performed using the standard Kirby-Bauer disc diffusion method. The Microtiter-plate biofilm formation assay measured the extent of biofilm formation. From 80 samples, 11 *Salmonella* spp. were identified, divided into two serotypes belonging to B and A serogroups. BOX repeat-based PCR (BOXAIR-PCR) and Repetitive element-based PCR (REP-PCR) banding results of isolates revealed 7 and 6 reproducible fingerprint patterns, respectively. The highest resistance was observed in response to ampicillin and doxycycline, followed by chloramphenicol, sulfamethoxazole, trimethoprim, neomycin, and nalidixic acid. Multidrug resistance was detected in all *Salmonella* serotypes. Seven isolates possessed the ability to produce biofilm with varied adhesion strength. These results revealed the high and unexpected prevalence of *Salmonella* spp. in poultry products with multiple antibiotic resistance and biofilm production ability. Also, BOXAIR and REP-PCR results revealed high diversity in Salmonella serotypes and subsequently indicated variety in the origin of *Salmonella* spp.

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1. Introduction

Salmonella spp. is the primary bacterial pathogen that causes foodborne infectious diseases worldwide and is a significant public health problem in most countries (1). Poultry production is one of the most widespread food industries worldwide (2). Consumption of contaminated poultry and poultry products, including eggs, are the primary foodborne sources of *Salmonella* (3). Salmonellosis is caused by various serotypes of *Salmonella,* which are highly diverse (4). Antimicrobial-resistant *Salmonella* in meats, particularly in poultry, is a significant risk in treating foodborne diseases caused by this bacterial pathogen (5, 6). In recent years, the wide use of antibiotics in the diet of domestic animals has become a threat to the world's population as it increases bacterial resistance against available antibiotics (7). Many antimicrobial agents are used to raise poultry in most countries (8). A large number of such antimicrobials are considered to be essential in human medicine (9). The indiscriminate use of such essential antimicrobials in animal production will likely accelerate the development of antibiotic resistance in pathogens and commensal organisms. This would result in

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treatment failures and economic losses and could act as the source of a gene pool for human transmission. In addition, there are human health concerns about antimicrobial residues in meat, eggs, and other animal products (7). The problem of antibiotic resistance of *Salmonella* becomes more critical when the biofilm formation ability of these bacteria is considered (10). It is worth noting that biofilms are becoming one of the critical issues related to the food industry. Concerning the genus *Salmonella*, biofilm plays a vital role in antimicrobial resistance (11). *Salmonella* can form biofilms on produced food; on poultry farm processing surfaces.

In addition, there is growing concern about the possibility of transmission of resistant bacterial strains, including those able to form biofilms, through the food production chain to consumers (10). Pulsed-field gel electrophoresis (PFGE) is considered the gold standard in typing *Salmonella* and is known for its ability to discriminate isolates and for tracking the source of outbreaks. However, it does not produce equal discrimination power with different serovars; it is time-consuming and labour-intensive (12). Many other DNA-based typing methods, such as BOX repeat-based (BOXAIR) and repetitive extragenic palindromic (REP) have been employed to delineate epidemiological relationships between various *Salmonella* isolates (13). These PCR-based fingerprinting techniques are relatively straightforward, rapid, and sensitive for discriminating between closely related strains (14). Several studies have demonstrated the high discriminatory potential of BOXAIR and REP-PCR in the epidemiological studies of closely related bacterial strains, including *Salmonella* (13, 15-17). The objectives of this study were to evaluate *Salmonella* infection and pathogenesis in poultry products to identify the serotypes diversity and subsequently to determine the antibiotic resistance pattern and ability of biofilm formation of isolates.

2. Materials and methods

2.1. Sample collection and sampling size

Sampling was carried out from February 2021 through July 2021. Eighty poultry product samples (meat= 40, egg= 40) were randomly selected from chicken supply and distribution centres with health licenses in Ardabil province, northwest of Iran, to participate in this study. Samples were collected in sterilized 30-gram plastic containers and transferred to the laboratory.

2.2. Salmonella isolation and identification

Samples prepared in selenite F-rich medium (Himedia, India), were cultured at 37°C for 24 hours and transferred and cultured in the selected solid mediums of *Salmonella* such as MacConkey Agar (Merk, Germany), *Salmonella-Shigella* agar (Himedia, India) and brilliant green agar (Himedia, India). The colonies were investigated after incubation for 24 hours at 37°C. Selective colonies were confirmed by IMViC (Indol, MR, VP, Citrate) test, TSI (Triple Sugar Iron) reaction, urease test, nitrate reduction test, and other biochemical tests (18). The biochemical results were further confirmed by PCR amplification using the *16S rRNA* gene previously described by Lee et al. (19, 20). PCR amplification was conducted in a 25 µL reaction mixture in Touchgene Gradient (Model FTGRAD2D, UK). The cycling program was performed according to described reference. Finally, PCR products were resolved by electrophoresis in stained 1% (w/v) agarose gel. Visualizations were undertaken using a UV transilluminator (BTS-20, Japan), and the 100 bp plus DNA ladders were used as molecular size markers.

2.3. Serotyping

A slide agglutination test using O grouping polyvalent sera were used for serological identification. The agglutination test used serogroups A to D antisera (Baharafshan Co., Iran). Briefly, All the reagents were left to reach room temperature. On a clean microscope slide, a drop of the antiserum was placed at one end, and sterile normal saline (0.85% NaCl) was placed at the opposite end of the same slide. Three to four colonies from the non-selective media were suspended in 0.3 ml sterile saline, and a dense cell suspension was made. One loopful of the cell suspension was put onto each serum and standard saline drop and mixed well. The cell suspension and normal saline served as controls. The slide was gently shaken for 1 min. Agglutination within 1 min was regarded as positive for polyvalent O-group. Whenever any isolate was agglutinated with one of the polyvalent O-sera, it was again tested against the corresponding monovalent antisera, using the same procedure as above.

2.4. Fingerprinting of Salmonella isolates based on BOX and RER-PCR

The primers REP 1 (5'-IIIGCGCCGICATCAGGC-3') and REP 2 (5'- ACGTCTTATCAGGCCTAC-3') were used for REP-PCR in the present study (21). 50-CTACGGCAAGGCGACGCTGACG-30 was employed for BOXAIR-PCR (21). The PCR reactions were performed in 25 µl volume for BOX and 30 µl volume for REP and according to the described by Hashemi and Baghbani-arani (15).

2.5. Antimicrobial susceptibility test

All *Salmonella* isolates were subjected to *in vitro* antibiotic susceptibility testing against 16 antibiotics. The disk diffusion method was used following the guidelines of the Clinical and Laboratory Standards Institute (16). Antibiotics used in the study were ampicillin (10 µg), piperacillin (100 µg), ceftazidime (30 µg), doxycycline (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), enrofloxacin (5 µg), flumequine (30 µg), neomycin (30 µg), gentamicin (10 µg), kanamycin (30 µg), sulfamethoxazole and trimethoprim (1.25/23.75 µg), nalidixic Acid (30 µg), ciprofloxacin (5 µg), colistin (10 µg), lincospectin (15/200 µg). All antibiotic disks were procured

from PadtanTeb Company (Iran). *Salmonella* isolates that demonstrated resistance to 2 or more antibiotics were considered multidrug-resistant strains (22).

2.6. Biofilm formation ability assay

The procedure of biofilm formation of isolates in poly-styrene microtiter plates was based on the previously described method with some modifications (23). Briefly, 200 µl of bacterial suspension with OD600 = 0.1 (10^7 log CFU/ml) was inoculated directly to each well, using three wells per isolate. Plates were wrapped with parafilm and incubated at 37°C for 24 hours. Then, the plates were washed thrice by phosphate buffered saline (PBS) (Sigma-Aldrich) and allowed to air-dry for 20 minutes. In the next step, biofilms were stained with 150 µl of 1% w/v crystal violet (CV) for 30 minutes, washed twice with taped water to remove excess stain, and then air-dried for 30 minutes. Biofilm was quantified by eluting CV with 150 µl 95% v/v ethanol and determining the optical absorbance of the eluted dye at 570 nm. Ethanol (95%) was used as blank control. The optical density cut-off (OD_c) was defined as the mean OD of the negative control (culture medium), and the isolates were classified as follows: non-adherent ($OD \leq OD_c$); weak adherent ($OD_c < OD \leq 2xOD_c$), moderate adherent ($2x\ OD_c < OD \leq 4\ x\ OD_c$); and adherent solid ($OD > 4xOD_c$) (23).

3. Results

3.1. Salmonella confirmation

Based on biochemical tests from 80 poultry product samples, 11 (13.75 %) samples (egg= 4 and meat= 7) they have belonged to *Salmonella* spp. Amplification with the genus-specific PCR based on 16s rRNA gene confirmed the isolates as Salmonella spp. by amplifying DNA fragments in size 498bp (Fig. 1).

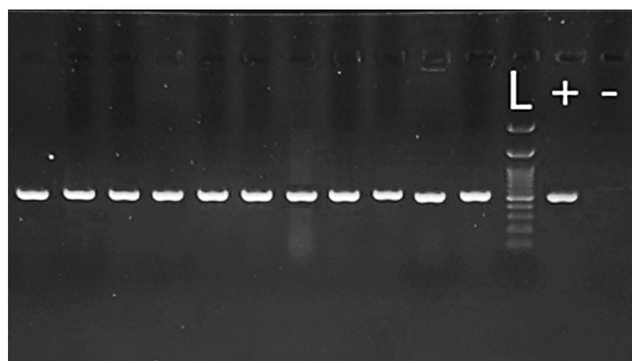

Fig. 1. Agarose gel electrophoresis of the PCR product of 16S rRNA gene. Lane L, molecular weight marker (100bp plus DNA ladder).

3.2. Serotyping

A total of 2 different serotypes were divided among 11 *Salmonella* spp. The most commonly isolated serogroup was group B (n= 6; 54.54%), followed by group A (n= 5; 45.45%). None of the *Salmonella* isolates belonged to serogroups C and D.

3.3. Antimicrobial susceptibility test

Antibiotic resistance patterns of *Salmonella* spp. strains isolated from poultry product samples are shown in Table 1. The highest resistance was observed in response to ampicillin and doxycycline (100% each), followed by chloramphenicol, sulfamethoxazole, and trimethoprim (72.72% each), neomycin (45.45), nalidixic acid (36.36). All strains in this study were susceptible to ceftazidime, enrofloxacin, gentamicin, ciprofloxacin, flumequine, lincospectin and piperacillin. As shown in Table 1, the resistance rate of each antibiotic differed among *Salmonella* spp. Resistance to one antibiotic was not observed in samples, while multidrug resistance, defined as resistance to two or more antibiotics, was detected in all *Salmonella* spp.

3.4. Biofilm Formation

Among the 11 *Salmonella* isolates, seven isolates (63.63%) adhered to polystyrene microtiter plates and were considered presumptive biofilm producers. According to the results, four isolates (36.36%) could produce strong biofilm. Also, two (18.18%) and one (0.09%) isolates could produce moderate and weak biofilm, respectively.

3.5. Fingerprinting of Salmonella isolates based on BOX and REP-PCR

Visual comparison of the BOXAIR-PCR banding results of 11 *Salmonella* isolates revealed seven reproducible fingerprint patterns. The multiple DNA fragments generated ranged in size between 200 and 2500 bp (Fig. 2a). In all tested *Salmonella* isolates, four standard bands and seven distinct polymorphic bands were observed (Fig. 2b). REP-PCR of genomic DNA from 11 *Salmonella* strains generated six reproducible fingerprint patterns. Multiple DNA fragments were obtained in sizes ranging from 200 to 2000 bp. In all *Salmonella*-tested isolates, five standard bands and six distinct polymorphic bands were observed (Fig. 2b).

4. Discussion

Non-typhoidal *Salmonella* plays a significant role in foodborne human salmonellosis worldwide. It can be transmitted to humans, mainly through consuming foods of animal origin, including poultry, eggs, and meat (24). Therefore, continuous monitoring of contamination in these animal-origin foods with *Salmonella* and determining the pattern of drug resistance to prevent the spread of resistance in public health is necessary. In this study, 11 (13.75%) *Salmonella* spp. were identified in 80 poultry product samples by cultural as well as the genus-specific PCR. Recent studies from Iran and other world regions could support this finding.

Table 1. Antibiogram results of the studied *Salmonella* isolates.

Antibiotics	Symbol	Concentration (µg/disc)	Sensitive [n (%)]	Intermediate [n (%)]	Resistant [n (%)]
Doxycycline	D	30	0 (91.30)	0 (8.69)	11 (100.00)
Ceftazidime	CAZ	30	11 (100.00)	0 (0.00)	0 (0.00)
Enrofloxacin	ENR	5	11(100.00)	0 (0.00)	0 (0.00)
Neomycin	N	30	3 (27.27)	3 (27.27)	5 (45.45)
Nalidixic Acid	NA	30	7 (63.63)	0 (0.00)	4 (36.36)
Gentamicin	GM	10	11 (63.63)	0(0.00)	0 (0.00)
Sulfamethoxazole/Trimethoprim	SXT	1.25/23.75	3 (27.27)	0 (0.00)	8 (72.72)
Ciprofloxacin	CIP	5	11 (100.00)	0 (0.00)	0 (0.00)
Flumequine	FM	30	11 (100.00)	0 (0.00)	0 (0.00)
Kanamycin	K	30	10 (90.90)	1 (9.09)	0 (0.00)
Colistin	CL	10	0 (100.00)	1 (9.09)	10 (90.90)
Chloramphenicol	C	30	3 (27.27)	0 (0.00)	8 (72.72)
Lincospectin	LS	15/200	11 (100.00)	0 (0.00)	0 (0.00)
Tetracycline	TE	30	2 (18.18)	0 (0.00)	9 (81.81)
Piperacillin	PIP	100	11 (100.00)	0 (0.00)	0 (0.00)
Ampicillin	AM	10	0 (0.00)	0 (0.00)	11 (100.00)

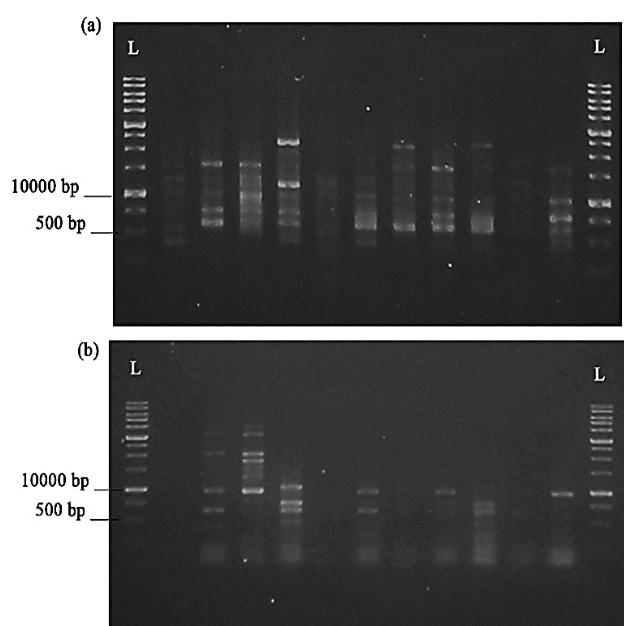

Fig. 2. Representative gel showing amplification for Salmonella isolates sequences by (a) BOXAIR-PCR, (b) REP-PCR. Lane L, molecular weight marker (1 kb DNA ladder).

In the study of Asadpour et al. (25), *Salmonella* was isolated at a rate of 15% from 20 slaughtered broiler chicken flocks. In another study, Amirmozaffari et al. (26) determined the contamination level of poultry eggs and meat with *Salmonella* and reported 19%, 4%, 21%, and 5% *Salmonella* isolated from industrial eggs, domestic eggs, industrial chicken meat, and domestic chicken meat, respectively. In the Chaharmahal and Bakhtiari province of Iran, 620 samples of meat were collected from supermarkets, and 4.51% of samples were contaminated with *Salmonella* (27). Another study in Ardabil province showed that 260 samples of chicken and viscera on the market were contaminated with *Salmonella* at a rate of 10% (28). Sodageri et al. (29) reported that out of 560 chicken samples (liver, heart, and gizzard), *Salmonella* was isolated in 19.8% of samples. Ghaderi et al. (30) also showed that of the 585 samples from poultry, 25.5% were infected with *Salmonella*. The contamination rate of poultry samples with *Salmonella* was 3–66% in various epidemiological studies from different countries (31). In this study, the rate of contamination of poultry egg samples with *Salmonella* was higher than the rates reported in some other studies, such as 0% in Cairo, Egypt (32), 0.3% in Dhaka, Bangladesh, 2.9% in Eastern Ethiopia, 3% in Belgium (33), 3.3% in North India (34),5.40% in Guangdong, China (35), South India (7.7%), Nigeria (24.17%) (33), and Spain (34%) (36). These differences in the rate of *Salmonella* contamination in poultry samples and egg samples can be related to differences in the hygienic control and management programs of different regions and countries. There are different reports on the prevalence of various *Salmonella* serogroups in Iran and other regions worldwide. In our study, serogroup B and A were the dominant *Salmonella* serogroup. In a study by Ezzatpanah et al., the serogroup D1 of *Salmonella* was reported as the dominant serogroup isolated from poultry (37). Goncagul et al. (38) in Turkey, isolated the groups A, B, C, and D on the skin of the wing part of slaughtered poultry and represented that serogroup D was the majority. According to Mahmud et al. (39) results in Bangladesh, 43% of isolates were assigned to serogroup B and 57% to serogroup C. Different results for serogroups can be due to differences in geographic regions, time and type of sampling. Due to the existing rotational situation between serotypes, it seems that serotypes can replace each other in a particular region and period. Numerous reports have been published about *Salmonella* antibiotic resistance in different areas. Our results indicate that all serotypes of *Salmonella* showed resistance to three or more antibiotics. Also, results of the present study revealed that high levels of drug resistance to ampicillin, doxycycline, chloramphenicol, sulfamethoxazole, and trimethoprim were found in *Salmonella* serotypes. Recent studies from Iran and other world regions could support this finding. Molla et al. (40) indicated that among 23 tested antibiotics, the highest levels of resistance were related to sulfamethoxazole (51.2%), amoxicillin and ampicillin

(46.2%), tetracycline (2/41%), chloramphenicol (30%), florfenicol (27.5%), streptomycin (22.5%) and cotrimoxazole (21%), and there was no resistance against nitrofurans, quinolones, cephalosporins, kanamycin, and neomycin. Capita et al. (41) observed that the highest resistance levels were against sulfonamides, fluoroquinolones, and tetracyclines. According to reports by Yang et al. (42), the highest percentages of resistance were related to sulfamethoxazole +trimethoprim (67%), tetracycline (56%), nalidixic acid (35%), ciprofloxacin (21%) and ceftriaxone (16%). Ruichao et al. reported the highest percentages of resistance to tetracycline (77%), sulfamethoxazole + trimotropil (43%), nalidixic acid (41%), streptomycin (41%) and ampicillin (25%), and the lowest percentages to gentamycin (15%), amoxicillin (14%), ciprofloxacin (12%), and florfenicol (10%) (43). Abdelghany et al. (44) observed the highest percentages of resistance to nalidixic acid (98.8%), sulfamethoxazole +trimethoprim (96.4%), oxytetracycline (95.2%), and ampicillin (91.06%). In Iran, studies conducted in different regions indicate the emergence and increase of drug resistance in the *Salmonella* isolates. Peighambari et al. reported the highest resistances to tetracycline (66.6%), furazolidone (52.8%), nalidixic acid (43.8%), linoecspectin (42.3%), flumequine (40.6%) and streptomycin (39.1%); while ciprofloxacin and imipenem had 100% sensitivity (45). Asadpour et al. (25) showed that all isolates resisted tetracycline, streptomycin, nalidixic acid, cefazolin, and sulfamethoxazole + trimethoprim. Raeisi and Ghiyami observed the highest resistance rates to streptomycin and nalidixic acid (100%), tetracycline (92.3%), neomycin and furazolidone (84.6%), and chloramphenicol (73.3%). Also, the lowest resistance rates were against amoxicillin and ampicillin (11.5%), ciprofloxacin (7.7%), and gentamicin (3.7%) (28). Sodagari et al. (29) introduced tetracycline as the most resistant antibiotic against *Salmonella* isolated from poultry. Also, in the present study, all isolates were susceptible to ceftazidime, enrofloxacin, gentamicin, ciprofloxacin, flumequine, lincospectin, and piperacillin. Therefore, these drugs may be effective in the treatment of salmonellosis. In total, the reasons behind the resistance of isolates are the uncontrolled use of antibiotics in veterinary medicine that cause the destruction of sensitive bacteria and the selection of resistant species to multiple antibiotics. Biofilm formations by bacteria are directly associated with many infections. Different studies were conducted to compare the ability of different *Salmonella* serotypes for biofilm production (46, 47). Seven isolates (63.63%) in our study possessed the ability to produce biofilm on polystyrene microtiter plates with varied adhesion strength. This result is in accordance with recent studies indicating that *Salmonella* serovars could produce biofilm (46, 48). The differences in biofilm formation could be attributed to strain variation (49). As all serotypes had multiple antibiotic resistance, it can be suggested that biofilm formation is a mechanism for bacteria to survive better, especially in isolates with insufficient resistance levels. Several studies have demonstrated the high discriminatory power of BOXAIR and REP-PCR in the differentiation of *Salmonella* strains (15-17). This study used BOXAIR and REP-PCR methods to investigate different *Salmonella* isolates. Also, our study's BOXAIR-PCR and REP-PCR banding results of 11 *Salmonella* isolates revealed seven and six reproducible fingerprint patterns, respectively. Analysis by BOXAIR and REP-PCR revealed high diversity in serotypes of *Salmonella* in poultry products. This indicates diversity in the origin of *Salmonella*. The unidentified source of the isolated *Salmonella* serotypes implies inadequate control possibilities and may therefore pose a problem to poultry production regarding food safety.

5. Conclusion

The data analysis obtained during this study shows Salmonella's high and unexpected prevalence in the region's poultry products. These bacteria also showed dangerous activities regarding pathogenicity, prevalence, and high diversity of contaminant sources. The contamination of poultry meat and eggs samples with *Salmonella*, besides the presence of antimicrobial-resistant and MDR *Salmonella,* isolates in these samples, could highly impress on food safety and human health. Therefore, continuous monitoring of animal source foods, especially poultry meat and eggs, for the occurrence of contamination, antibiotic resistance patterns, and virulence characteristics of *Salmonella* is essential to improve food safety and to reduce the zoonotic risk of this foodborne pathogen for consumers, and also to choose effective antibiotics for the treatment of salmonellosis. Based on the results, we recommend enhancing the poultry farm control measures and limiting the use of antibiotics, particularly those critical in human medicine for prophylaxis purposes in food-producing animals.

Acknowledgments

This paper is derived from the first author's thesis, and the Department of Animal Science of Islamic Azad University, Shabestar Branch, is highly appreciated.

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