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Original article

Molecular detection, typing, and virulence potential of *Salmonella* Serotypes isolated from poultry feeds

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Abstract

Salmonella contamination in poultry feed is one of the main issues in poultry industry and public health. The aim of the present study was molecular detection and typing of *Salmonella* serotypes isolated from poultry feeds. Moreover, we determined the antibiotic resistance pattern and the ability of biofilm formation in the serotypes. To this end, eighty feed samples were collected from aviculture depots. *Salmonella* serotypes were identified by culture and PCR methods. For serological identification, a slide agglutination test was used. BOXAIR and rep-PCR methods were applied to evaluate the diversity of serotypes. The disc diffusion method was performed to evaluate the antibiotic susceptibility of serotypes to sixteen antibiotics. Biofilm formation was also assessed by the microtiter-plate test. From a total of 80 feed samples, 30 samples were contaminated with *Salmonella* spp., which were divided into 5 different serotypes belonging to B, C, and D serogroups. BOXAIR-PCR (D value [DI] 0.985) and rep-PCR (DI 0.991) fingerprinting of isolates revealed 23 and 19 reproducible fingerprint patterns, respectively. A higher antibiotic resistance was observed to ampicillin and doxycycline (100% each), followed by chloramphenicol (83.33%) and tetracycline (73.33%). Multidrug resistance (MDR) was detected in all *Salmonella* serotypes. Half of the serotypes possessed the ability of biofilm formation with varied adhesion strengths. These results revealed the high and unexpected prevalence of *Salmonella* serotypes in poultry feed with MDR and biofilm formation ability. BOXAIR and rep-PCR revealed a high diversity of *Salmonella* serotypes in feeds and subsequently indicated variation in the source of *Salmonella* spp. The unknown sources harboring high diversity of *Salmonella* serotypes indicated poor control, which could cause problems for feed manufacturing.

Keywords: *Salmonella*, feed, BOXAIR-PCR, Rep- PCR, drug resistance, biofilm

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Introduction

Particular serotypes of *Salmonella* have widely distributed foodborne pathogens. This bacterium, as one of the primary enteric pathogens, infects humans and animals. In addition to affecting the poultry, *salmonella* can cause disease in humans through the consumption of contaminated products (Antunes et al. 2016).

Excessive use of antibiotics in poultry diet, which has increased the resistance of bacteria to existing antibiotics, has issued a threat to the global population in recent years (Mehdi et al. 2018, Andrew Selaledi et al. 2020). Multidrug resistance in *Salmonella* serotypes has been frequently reported in many studies (Khademi et al. 2020, Vaez et al. 2020). Moreover, besides the increasing antimicrobial resistance, biofilm formation by bacteria has caused a reduced response to antibiotic treatment in both humans and animals. It is noteworthy that in the food industry, biofilm formation by bacteria has been one of the most important issues (Palma et al. 2020, Carrascosa et al. 2021). Biofilm formation is a dominant lifestyle of microorganisms. Biofilms, as an aggregation of bacteria, are enclosed in self-produced extracellular polymeric substances and attached to all surfaces (Yin et al. 2019). The ability of biofilm formation among *Salmonella* spp. has been widely reported (Obe et al. 2021). *Salmonella* can form biofilm on different processing surfaces such as food contact surfaces in the poultry industry (Wang et al. 2013). Furthermore, there is an increasing concern about the possibility of transmitting biofilm-producing and antibiotic-resistant bacterial strains through food production chain to the humans (Guéneau et al. 2022). Considering the issues raised and regarding the increase of antibiotic resistance, priority should be given to the monitoring of antibiotic resistance among pathogenic bacteria that pose a threat to the human and animal health.

Feed is one of the dangerous sources of *Salmonella* for livestock and poultry that can cause diseases in humans through a direct or indirect contact of contaminated feed and animals shedding *Salmonella* into food and water sources (Sargeant et al. 2021). Although the results of effective control have shown decreased *Salmonella* contamination in feed samples in the developed countries, this problem has remained unresolved in the developing countries (Hazards et al. 2019).

Identification and control of *Salmonella* in poultry feeds require research approaches including diagnostic tests' development and validation. Multiple methods have been investigated to identify *Salmonella* serotypes. Hence, in this study we carried out biochemical and molecular tests to identify the *Salmonella* serotypes isolated from the poultry feed. The tests included: (1) biochemical tests, (2) serotyping tests, (3) molecular methods to predict *Salmonella* serotypes and, (4) fingerprinting of *Salmonella* serotypes based on BOX repeat-based PCR (BOXAIR-PCR) and repetitive extragenic palindromic (rep) PCR. Moreover, the other objectives of the present study were: determination of antibiotic resistance pattern and evaluation of biofilm formation in the serotypes.

Materials and Methods

Sample collection and sampling size

Sampling was carried out from February to July, 2021. Eighty feed samples were collected from aviculture depots (holding the health license) in Ardabil province, northwest of Iran. Four Samples of approximately 100 g each, by donning a pair of sterile surgical gloves, were collected from different locations in the stored commodity and combined in a sterile sample bag. Finally, 25 g of each was used as the test sample in this study. The samples were then transported to the laboratory in cooled containers and ground into powder in a coffee grinder. The grinder was sanitized with 70% ethanol between each sample grinding and allowed to dry. The samples were then collected into sterile 50-mL screw-top tubes and refrigerated until analysis.

Isolation and identification of *Salmonella*

Forty mL of tetrathionate broth (Difco, Sparks, MD) was added to each 25 g of feed sample, and the tubes were incubated at 42°C for 18-24 h. The tubes were then vortexed and sterile swabs were used to streak these samples to Xylose Lysine Deoxycholate (XLD) agar plates (Hardy Diagnostics, Santa Maria, CA), and subsequently incubated at 37°C for 48 h. The selected colonies were confirmed by IMViC test, TSI reaction, urease test, Nitrate reduction test, and other biochemical tests (Sedeik et al. 2019). The biochemical results were further confirmed by PCR amplification using the *16S rRNA* gene that was previously described (Lee et al. 2009, Hemmati et al. 2020).

Serotyping

Slide agglutination test using O grouping polyvalent sera was done for serological identification. The agglutination test was carried out using the serogroups A to D antisera (Baharafshan Co., Iran). Briefly, a drop of antiserum and a drop of sterile normal saline were placed on the opposite side of a clean microscope slide. One loopful of bacterial suspension was placed onto each of the drops of serum and normal saline

Table 1. Primers used in PCR.

Serotype	Primer Sequence	Product Size (bp)
S. Typhimurium*	F: GAGTTGAAACGGTTCTGTAC R: ACTACCTGCGACAGCAACT	495
S. Infantis	F: TCGAGATGGGTATGTAGC R: CGCAACCATGAACTTTTAC	97
S. Gallinarum	F: GATCTGCTGCCAGCTCAA R: CGCCCTTTTCAAAACATAC	173
S. Pullorum	F: CGTACAATAAGGGATTATG R: GTAAAGACCAGTTAACAC	237
S. Dublin	F: ATGACTTCAAATGTACCA R: CAATTCTGGAACTGATTCG	836

* This primer also binds to *Salmonella* Kentucky; This serotype has not been reported in Iran yet.

and shaken. Agglutination within 1 min was regarded as positive for polyvalent O-group.

Molecular tests

DNA extraction

DNA extraction was performed using the boiling method recommended by Güler et al. (2008), Briefly, bacterial colonies were suspended in 100 µL sterile water, then boiled for 10 min and subsequently centrifuged at 13000 rpm for 2 min to remove cell debris. Finally, the supernatant was used as a source of DNA template.

PCR-based detection of serotypes of isolates

Detection of *Salmonella* serotypes was carried out by PCR for specific genes. The PCR analyses were conducted using the candidate primers designed for this study (Table 1). The primers were designed for five classical *Salmonella* serotypes, including *S.* Pullorum, *S.* Typhimurium, *S.* Infantis, *S.* Gallinarum, and *S.* Dublin. PCR cycling programs are listed in Tables 1 and 2. Briefly, PCR amplification was conducted in a 25-µL reaction mixture containing 2 µL of the template DNA, 12.5 µL of 2X PCR master mix (AMPLIQON, Denmark), and 1 µL (0.4 Mm) of each primer (Sinaclon, Iran). PCR was conducted in a Touchgene Gradient (Model FT GRAD 2D, UK). Electrophoresis of the PCR products was conducted in 1% (w/v) agarose gel and visualized by a UV transilluminator (BTS-20, Japan). For electrophoresis, 100 bp DNA ladders were used as molecular size markers. The PCR results were confirmed by sequencing and subsequent blasting in NCBI GenBank DNA database (data not shown).

Fingerprinting of *Salmonella* isolates based on BOX and Rep-PCR

The primers REP 1 (5'-IIIGCGCCGICATCAGGC-3') and REP 2 (5'- ACGTCTTATCAGGCCTAC-3') were used for rep-PCR in the current study, and 50-CTACG GCAAGGCGACGCTGACG-30 was employed for BOXAIR-PCR (Dombek et al. 2000). PCR amplification tests were conducted in a volume of 25 µL for BOX and a volume of 30 µL for rep, according to a previously-described protocol (Hashemi and Baghbani-arani, 2015).

Antimicrobial susceptibility test

To determine the antibiotic susceptibility, all serotypes of *Salmonella* were subjected to 16 antibiotics. The Kirby-Bauer disc diffusion method was performed following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The antibiotic disks used in this 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), and lincospectin (15/200 µg). All antibiotic discs had been provided from PadtanTeb Co., Iran. The *Salmonella* strains that showed resistance to at least three antibiotics were considered as multidrug resistant (Pokharel et al. 2006).

Biofilm formation

Biofilm formation was tested by the quantification of biofilms formed in 96-well microtiter plates, with minor modifications (Majtán et al. 2008, Hosseinzadeh et al. 2018). Biofilm was quantified by eluting the crystal violet (CV) with 30% (v/v) glacial acetic acid and

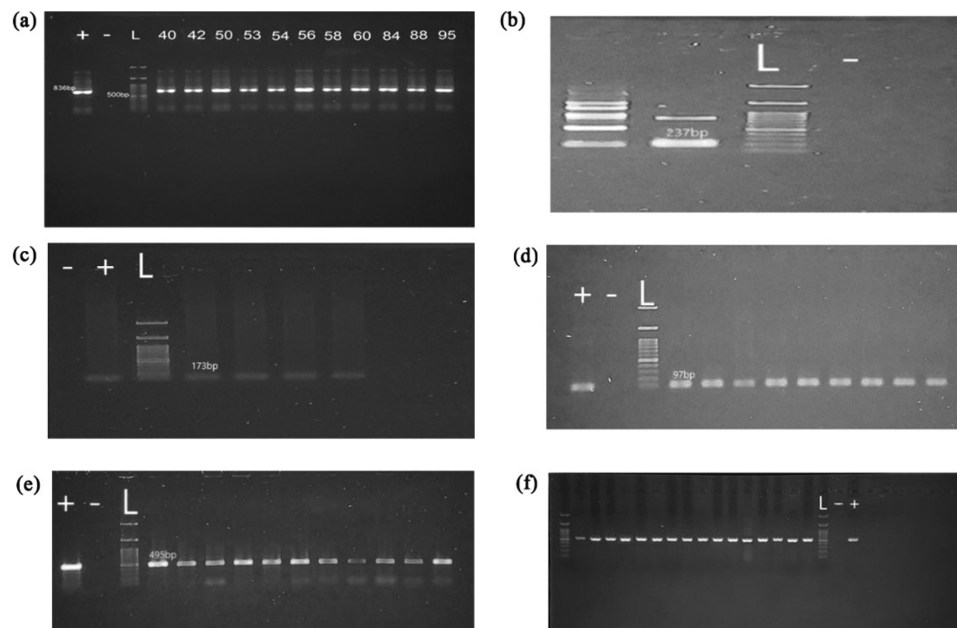

Fig. 1. Agarose gel electrophoresis of the PCR product of specific *Salmonella* serotypes and *16sr RNA* genes. (a) *S.* Dublin; (b) *S.* Pullorum; (c) *S.* Gallinarum; (d) *S.* Infantis; (e) *S.* Typhimurium; (f) *16sr RNA* gene. Lane L, molecular weight marker (1 kb DNA ladder).

Table 2. PCR Schedule.

Pathogenic agent	Initial denaturation		cycles	Denaturation		Annealing		Extension		Final extension	
	Temp. (°C)	Time (min)		Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	Temp (°C)	Time (min)	Temp (°C)	Time (min)
S. Typhimurium	95	10	35	95	30	53	20	72	20	72	5
S. Infantis	95	5	35	95	15	55	40	72	40	72	10
S. Gallinarum	95	5	35	95	15	52	60	72	90	72	7
S. Pullorum	95	5	35	95	15	51	30	72	60	72	15
S. Dublin	95	5	35	95	15	54	30	72	60	72	15

determining the optical absorbance of the eluted dye at 570 nm. The optical density cut-off (OD_c) was defined as the mean OD of the negative control (culture medium). Therefore, the isolates were categorized into four groups: non-biofilm former ($OD \leq OD_c$); weak biofilm former ($OD_c < OD \leq 2x\ OD_c$); moderate biofilm former ($2x\ OD_c < OD \leq 4x\ OD_c$); and strong biofilm former ($OD > 4x\ OD_c$).

Results

Salmonella confirmation

Based on the biochemical test, from a total of 80 feed samples, 30 samples (37.5%) were contaminated with *Salmonella* spp. The *16s rRNA* gene-based PCR confirmed the isolates as *Salmonella* spp., producing an amplicon of 498 bp (Fig. 1).

Serotyping

All isolated *Salmonella* spp. were divided into five different serotypes (Table 3). The most commonly isolated serogroup was group D (n=18; 60%), followed by group C (n=10; 33.33%) and group B (n=2; 6.66%). None of the *Salmonella* isolates belonged to serogroup A.

PCR-based detection of *Salmonella* serotypes

Among the 30 *Salmonella* isolates, 5 serotypes were identified according to the PCR test. PCR amplification with the specific primer sets produced single amplicons of 495 bp, 97 bp, 173 bp, 237 bp, and 836 bp for *S.* Typhimurium, *S.* Infantis, *S.* Gallinarum, *S.* Pullorum, and *S.* Dublin isolates, respectively (Fig. 1). Overall, *S.* Typhimurium (n=2; 6.66%), *S.* Infantis (n=10; 33.33%), *S.* Gallinarum (n=5; 16.66%), *S.* Pullorum (n=2; 6.66%), and *S.* Dublin (n=11; 36.66% each) were identified.

Table 3. Serotyping, biofilms and gene fingerprinting results.

Serotype	No. of strains (%)	Somatic Antigen Serogroup	Biofilm ability				Box PCR Profiling	Rep PCR profiling
			Strong (%)	Moderate (%)	Weak (%)	Negative (%)	Profile (NO.)	Profile (NO.)
S. Typhimurium	2 (6.6)	B (2)-6.66%	50%	-	-	50%	C (1), D (1)	A (1), E (1)
S. Infantis	10 (33.3)	C (10)-33.33%	10%	10%	30%	50%	A (3), B (1), E (1), F (1), G (1), H (1), I (1), J (1)	A (1), B (2-), C (1), D (1), F (1), G (1), H (1), I (1), V (1)
S. Gallinarum	5 (16.6)	D (5)-16.66%	40%	20%	--	40%	M (1), N (1), O (1), P (1), Q (1)	A (1)-L (1), J(1), M(1),N(1)
S. Pullorum	2 (6.6)	D (2)-6.66%	50%	--	--	50%	K (1), L (1)	A (1), K (1)
S. Dublin	11 (36.6)	D (11)-36.66%	27.27%	--	18.18%	54.54%	A (2-), B (1), R (1), S (1), T (1), V (1), W (1), X (1), Y (1)	A (1), B (2-), C (1), O (1), P (1), Q (1), R (1), T (1), U (1), S (1)

Table 4. Antibiotic Resistance Frequency of *Salmonella* spp. isolated from feed of poultry. Percent (number).

Serotype	D	CAZ	ENR	N	NA	GM	SXT	CIP	FM	K	CL	C	LS	TE	PIP	AM
S. Typhimurium	100% (2)	0	0	50% (1)	50% (1)	0	50% (1)	0	50% (1)	50% (1)	50% (1)	100% (2)	0	50% (1)	0	100% (2)
S. Infantis	100% (10)	0	0	0	0	0	0	0	10% (1)	0	10% (1)	80% (8)	0	70% (7)	0	100% (10)
S. Gallinarum	100% (5)	0	0	0	20% (1)	0	0	40% (2)	0	40% (2)	0	80% (4)	0	80% (4)	0	100% (5)
S. Pullorum	100% (2)	0	0	0	0	0	50% (1)	0	0	0	50% (1)	0	0	100% (2)	0	100% (2)
S. Dublin	100% (11)	0	0	27.27% (3)	18.18% (2)	0	27.27% (3)	9.09% (1)	0	18.18% (2)	27.27% (3)	100% (11)	0	72.72% (8)	0	100% (11)

D, Doxycycline; CAZ, Ceftazidime; ENR, Enorfloxacin; N, Neomycin; NA, Nalidixic Acid; GM, Gentamicin; SXT, Sulfamethoxazole and Trimethoprim; CIP, Ciprofloxacin; FM, Flumequine; K, Kanamycin; CL, Colistin; C, Chloramphenicol; LS, Lincospectin; TE, Tetracycline; PIP, Piperacillin; AM, Ampicillin.

Antimicrobial susceptibility test

The antibiotic resistance patterns of *Salmonella* strains isolated from the poultry feed samples are shown in Table 4. Higher antibiotic resistance was observed to ampicillin and doxycycline (100% each), followed by chloramphenicol (83.33%), tetracycline (73.33%), colistin (20%), sulfamethoxazole and trimethoprim and kanamycin (16.66%, each), nalidixic acid and neomycin (13.33%, each), ciprofloxacin (10%), and flumequine (6.66%). Moreover, all *Salmonella* spp. isolated from the poultry feed samples were susceptible to ceftazidime, enorfloxacin, gentamicin, lincospectin, and piperacillin. As shown in Table 4, *Salmonella* serotypes had different rates of resistance to each antibiotic. *S.* Typhimurium and *S.* Dublin showed higher resistance than other serotypes. Overall, resistance to one antibiotic was not observed in the isolates, while resistance to more than two antibiotics was detected in all *Salmonella* serotypes.

Biofilm formation

Among the 30 *Salmonella* isolates, 50% (15/30) were able to produce biofilm. Another half of the isolates were not able to form biofilm (Table 3). According to the results shown in Table 3, among the serotypes, *S.* Dublin (n=3), *S.* Galinarum (n=2), *S.* Typhimurium, *S.* Infantis, and *S.* Pullorum (n=1, each) produced strong biofilms, respectively. Additionally, two and five isolates were able to produce moderate and weak biofilms, respectively.

Fingerprinting of *Salmonella* isolates based on BOX and rep-PCR

The results of BOXAIR-PCR banding pattern revealed 23 reproducible fingerprints among 30 *Salmonella* isolates. The multiple DNA fragments generated ranged in sizes between 200 and 2500 bp (Fig. 2a). Furthermore, the rep-PCR banding pattern

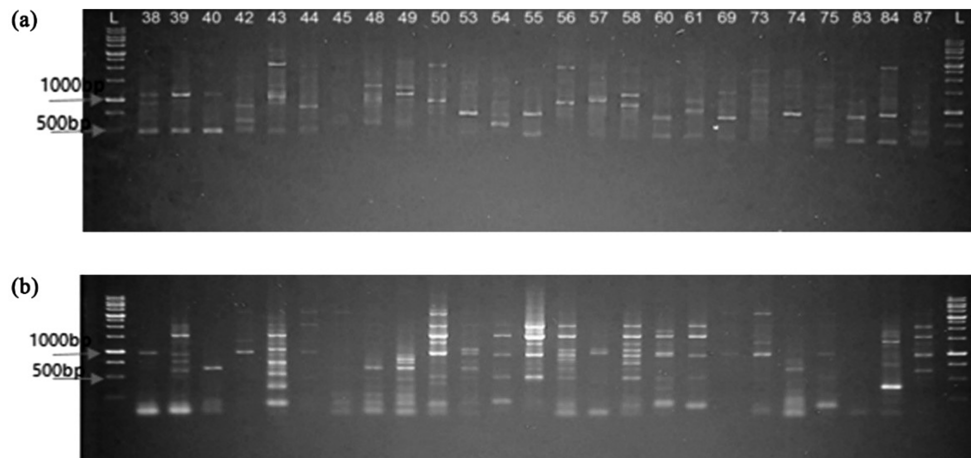

Fig. 2.Representative gel showing amplification for *Salmonella* isolates sequences by (a) BOXAIR-PCR, (b) REP-PCR. Lane L, molecular weight marker (1 kb DNA ladder).

revealed 19 reproducible fingerprints. The multiple DNA fragments were obtained in sizes ranging from 200 to 2000 bp. In all *Salmonella* isolates tested, one common band and 17 distinct polymorphic bands were observed (Fig. 2b). The D values of BOXAIR-PCR and rep-PCR in this analysis were 0.985 and 0.991, respectively (Table 3).

Discussion

Feed is a potential major carrier for the transmission of microorganisms such as *Salmonella* to the poultry (Olson et al. 2022). Therefore, the poultry feed is considered a major factor in the control of *Salmonella* and subsequently its transmission to the humans. Hence, detecting *Salmonella*, and determining the pattern of drug resistance to prevent the spread of resistant strains is of great importance.

In the current study, 30 (37.5%) *Salmonella* spp. were identified in 80 feed samples by culture and PCR methods. Moreover, in our study, serogroup D was the dominant *Salmonella* serogroup, followed by group C, and group B. A few studies have assessed the prevalence of *Salmonella* spp. in the poultry feed in Iran; and our results are in agreement with the findings of some previous studies. In the study of Azizpour and Ghazaei, the rate of *Salmonella* infection in 50 animal feed samples was 8%, of which 50% belonged to serogroup D and 25% to serogroup B (Azizpour and Ghazaei 2019). In another study conducted by Mayahi et al. (2017) 62 broilers and their feeds were sampled in 21 provinces of Iran. These authors showed that all feed samples were negative for *Salmonella* bacteria. One of the reasons of the contamination of animal feed with *Salmonella* is the presence of meat and bone meal (Parker et al. 2022). However, in our study, the poultry feed did not contain these substances and therefore the presence of *Salmonella* in the feed samples could be attributed to environmental factors. It has been reported that the contamination of animal feed with *Salmonella* and/or other microorganisms can occur at various stages including feed preparation, transportation, processing, and storage (Gosling et al. 2021). Furthermore, *Salmonella* infections have been shown to persist in dry soil for years (Jechalke et al. 2019). This persistence greatly increases the possibility of their growth and reproduction in feed mills and grain warehouses. Moreover, after proving the presence of this pathogen in such environments, it will be difficult to eradicate it (Kisluk and Yaron 2012). In this study, *S.* Typhimurium (n=2; 6.66%), *S.* Infantis (n=10; 33.33%), *S.* Gallinarum (n=5; 16.66%), *S.* Pullorum (n=2; 6.66%), and *S.* Dublin (n=11; 36.66%) were identified. The relationship between animal feed, human, and animal salmonellosis has been proven years ago. The serotypes that were detected in the animal feeds were among the serotypes that usually cause diseases in the humans and animals. This highlights the complexity and hygienic importance of Salmonella in the animal feeds. Although certain serotypes always have a high incidence, our study indicated *S.* Dublin and *S.* Infantis were the significant serotypes identified.

Numerous reports have been published about the antibiotic resistance of *Salmonella* in different areas (Thung et al. 2016, V T Nair et al. 2018, Wang et al. 2019). According to the previous studies, multidrug resistant *Salmonella* spp. are resistant to more than two antibiotics (Pokharel et al. 2006). Our results indicated that all serotypes of *Salmonella* showed resistance to 3 or more antibiotics. As well, in this study high levels of drug resistance were observed to ampicillin, doxycycline, and chloramphenicol. This finding of the current study is consistent by recent studies. In the

study of Li et al. (2013) resistance to tetracycline, ampicillin, trimethoprim-sulfamethoxazole, and nalidixic acid in *Salmonella* spp. was reported. Resistance to chloramphenicol was also found in different serotypes of *Salmonella* (Hsu et al. 2013, Asif et al. 2017). Resistance to conventional and newer antibiotics among different serotypes of *Salmonella* has also been reported in some studies (Su et al. 2004, Sodagari et al. 2015). In our study, resistance to conventional antibiotics such as trimethoprim-sulfamethoxazole and flumequine was observed with a low percentage (Table 4). One of the probable reasons for resistance of serotypes to different antibiotics can be the excessive use of antibiotics in the livestock industry, which causes the destruction of sensitive bacteria and the selection of antibiotic-resistant ones. In this study, *Salmonella* serotypes were susceptible to ceftazidime, enrofloxacin, gentamicin, lincospectin, and piperacillin. These results are similar to those reported by other studies (Mayrhofer et al. 2004, Dallal et al. 2010, Fallah et al. 2013, Kuang et al. 2015, Wu et al. 2021). The lack of resistance to these antibiotics could be ascribed to their uncommon use in the livestock farms.

Biofilm forming bacteria contribute to many microbial infections. The ability of different *Salmonella* serotypes to produce biofilm has been reported in several studies (Silva et al. 2019, Akinola et al. 2020). Half of the serotypes in our study were able to form biofilm with varied adhesion strengths (Table 3). This result corroborates the results of recent studies that show *Salmonella* serotypes are capable of producing biofilms with various intensities (Díez-García et al. 2012, Akinola et al. 2020). The variety of strains can be seen as the reason for the difference in the ability of biofilm formation (Kalai Chelvam et al. 2014). Regarding the fact that all serotypes had multiple antibiotic resistance, it can be concluded that biofilm formation is an important mechanism for bacterial resistance, especially antibiotic resistance (González et al. 2018).

Molecular typing of *Salmonella* serotypes is a common method that is used to investigate the genetic relatedness, and is capable of distinguishing closely related *Salmonella* isolates and identifying the source responsible for foodborne disease outbreaks. Molecular methods have been developed as alternatives due to the low ability of commercial sera kits in evaluating the diversity of serotypes in a sensitive manner (Yoshida et al. 2016, Tang et al. 2019). The high discriminatory power of BOXAIR and rep-PCR in analyzing the diversity of *Salmonella* serotypes in comparison with other fingerprinting methods has been reported in some studies (Weigel et al. 2004, Hashemi and Baghbani-Arani 2015, Poonchareon et al. 2019). In this study, we used BOXAIR and rep-PCR methods to differentiate the *Salmonella* isolates. Moreover, in our study BOXAIR-PCR and rep-PCR banding results revealed 23 and 19 reproducible fingerprint patterns, respectively. In Iran, determining the *Salmonella* serotypes isolated from the poultry feed using the BOX and rep-PCR methods has not been reported so far. Our results are in line with prior studies in which the same methods were used for differentiating the *Salmonella* isolates at the serogroup level (Rasschaert et al. 2005, Hashemi and Baghbani-arani 2015). Hashemi and Baghbani-arani reported that BOX-PCR and rep-PCR are appropriate methods for the discriminatory typing of *Salmonella* spp. (Hashemi and Baghbani-arani 2015). As recommended by Hunter and Gaston, the D value >0.9 is desirable for good differentiation (Hunter and Gaston 1988). In our study, the D value for the BOXAIR and rep-PCR methods were 0.985 and 0.991, respectively. Based on this criterion, the rep-PCR method has the highest discriminatory power for the *Salmonella* serotyping. The high discriminatory power ($D > 0.9$) of the method used in this study shows the accuracy of this method in molecular typing, analysis of genetic relatedness, and fulfilling epidemiological purposes in *Salmonella* isolates.

Analysis by BOXAIR and rep-PCR revealed high diversity of *Salmonella* serotypes in the poultry feeds. This indicates variation in the source of *Salmonella*. The unknown sources of *Salmonella* isolated from the feed samples indicate poor hygiene controls, which can cause problems for feed manufacturing. Understanding the prevalence and diversity of *Salmonella* serotypes in a region will help producers to develop food safety practices.

In total, due to the high number of contamination sources of *Salmonella* in flock, different strategies should be employed to reduce the risks through the identification of different serotypes of *Salmonella*. Control measures are most effective at the feed level. The control measures to reduce the prevalence of *Salmonella* in the poultry farms will lead to a reduction in the incidence of salmonellosis in the humans. Among these measures, nutritional approaches can greatly help in avoiding *Salmonella* during feed processing and subsequently safer feed manufacturing.

To sum up, the analysis of findings obtained in the current study shows the high and unexpected prevalence of *Salmonella* in the poultry feed used in the region. These bacteria also showed dangerous activities in terms of pathogenicity, prevalence, and high diversity of contamination sources (given the wide variety of gene profiles). This issue can lead to the spread and even outbreak of *Salmonella*-related diseases in the humans and animals in the study area, which requires more attention, prevention, and monitoring the health

care. In addition, the antibiotic resistance, especially the multi-drug resistance (MDR) observed in this study, can be a good guide for veterinary clinicians in the prevention and treatment of *Salmonella* diseases.

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