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ORIGINAL ARTICLE

High prevalence of antibiotic resistance and biofilm formation in *Salmonella* Gallinarum

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ABSTRACT

Background and Objectives: Antibiotic resistance is an indicator of the passively acquired and circulating resistance genes. *Salmonella* Gallinarum significantly affects the poultry food industry. The present study is the first study of the *S.* Gallinarum biofilm in Iran, which is focused on the characterization of the *S.* Gallinarum serovars and their acquired antibiotic resistance genes circulating in poultry fields in central and northwestern Iran.
Materials and Methods: Sixty isolates of *S*. Gallinarum serovar were collected from feces of live poultry. The bacteria were isolated using biochemical tests and confirmed by Multiplex PCR. Biofilm formation ability and the antibacterial resistance were evaluated using both phenotypic and genotypic methods. The data were analyzed using SPSS software.
Results: According to Multiplex PCR for *ratA, SteB*, and *rhs* genes, all 60 *S.* Gallinarum serovars were Gallinarum biovars. In our study, the antibiotic resistance rate among isolated strains was as follows: Penicillin (100%), nitrofurantoin (80%), nalidixic acid (45%), cefoxitin (35%), neomycin sulfate (30%), chloramphenicol (20%), and ciprofloxacin (5%). All isolates were susceptible to imipenem, ertapenem, ceftriaxone, ceftazidime, and ceftazidime+clavulanic acid. All sixty isolates did not express the resistance genes *IMP, VIM, NDM, DHA*, bla_{OXA48}, and *qnrA*. On the other hand, they expressed *GES* (85%), *qnrB* (75%), *Fox M* (70%), *SHV* (60%), *CITM* (20%), *KPC* (15%), *FOX* (10%), *MOXM* (5%), and *qnrS* (5%). All *S.* Gallinarum isolates formed biofilm and expressed *sdiA* gene.
Conclusion: Considering that the presence of this bacteria is equal to the death penalty to the herd, the distribution of resistance genes could be a critical alarm for pathogen monitoring programs in the region. This study showed a positive correlation between biofilm formation and 50% of tested resistance genes. Also, it was found that the most common circulating *S. gallinarum* biovars are multidrug-resistant.

Keywords: *Salmonella*; Poultry disease; Antibiotic resistance

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INTRODUCTION

Salmonella contamination is a major expense in the poultry industry. Contamination with *S. gallinarum* serotypes causes host death or reduced chicken production. Close monitoring to eliminate *Salmonella* serovars (such as Typhi, Typhimurium and Enteritidis) from food are needed (1). Also, some *Salmonella* serovars such as *S*. Enteritidis, *S*. Heidelberg, *S*. Kentucky and *S*. Gallinarum, which could spread to the reproductive organs and contaminate the next generation, must be omitted (2).

Presently, Gallinarum and Pullorum are categorized as biotypes of *Salmonella* Gallinarum serovar (3). *S. enterica* serotype Gallinarum is responsible for fowl typhoid (1), affecting the mature chicken and spreading horizontally (4). Fundamentally, in such infections, the herd should be destroyed, all the rodents and insects should be eradicated, and the cages should be kept empty for some time. Therefore, Gallinarum biovars lead to considerable economic losses in the poultry industry worldwide. Moreover, since they are found in other farm animals, the chickens are considered as a source of transmitting the microbiota to a variety of hosts.

Both *Salmonella* biovar Gallinarum and biovar Pullorum are non-motile bacteria with shared biochemical traits and somatic antigens (1). The results of various studies on the worldwide prevalence of *S*. Gallinarum between the years 1981 and 2020 showed that the prevalence of *S*. Gallinarum decreased until 2006, but from that year, there was an increasing prevalence rate of *S*. Gallinarum worldwide (5). Thus, the identification of biovar is vital due to the mentioned consequences for the poultry industry.

Biofilm is a biologically active matrix composed of persistent cells and extracellular substances formed on surfaces inside and outside the host body (6). The ability of bacteria to form biofilms and also the frequency of transferred genetic material encoding multidrug resistance (MDR) traits among biofilm-forming bacteria are important (7-10). The correlation between antibiotic resistance and severity of biofilm formation is an interesting field of study.

Administration of antibiotics and food containing antibiotics are the main causes of antibiotic resistance in poultry. These antibiotics promote biofilm formation and prevent bacterial eradication by conventional antibiotics (11). Diagnosis of *Salmonella* serovars in the field and information on their antibiotic resistance could define the protocol for administering the antibiotics in the poultry industry (12).

Salmonella serovars are differentiated by cultural, biochemical, and molecular techniques. These techniques have been used to distinguish *S. entrrica* serovar Gallinarum from other *Salmonella* species (13, 14). Although *Salmonella* biovar Gallinarum and Pullorum are distinguished primarily on the basis of biochemical tests, including tests for ornithine and dulcite decarboxylase, it is widely believed that some atypical biovars are difficult to distinguish. Recent molecular techniques have suggested some genes for the differentiation of these two biovars. *ratA* and *SteB* represent hypothetical proteins and fimbrial usher genes respectively. *ratA* is a pseudogene without a premature stop codon in open reading frames in each of the biovars. The RHS family (*rhs*) pseudogenes encode type II toxin-antitoxins. Hq703462 is a partial coding sequence for the putative RHS protein. The amplification result differs between the biovars Gallinarum and Pullorum. Although the *rhs* gene is shared by these two biovars, SteB is unique to biovar Gallinarum (15).

Due to the importance of *S*. Gallinarum contamination in the poultry industry and increasing antibiotic resistance, we decided to assess the prevalence of *S*. Gallinarum and also the pattern of antibiotic resistance of *S*. Gallinarum in samples collected from different farms. Furthermore, since bacteria in the biofilm are more resistant to antibiotics, we estimated the relationship between the ability to form biofilms and the pattern of antibiotic resistance. We hope that the results of our study can help expose the misuse of antibiotics in the poultry industry. According to these results, the urgent need for appropriate antimicrobial regimen surveillance programs can be highlighted in order to prevent the increasing rate of antimicrobial resistance.

MATERIALS AND METHODS

Isolation, diagnosis of *Salmonella* from feces samples. Feces samples were collected from 18 farms of different provinces of Iran, including Tehran, Qom, Qazvin, Fars, West Azerbaijan, and East Azerbaijan from 2012 to 2017 based on the A Laboratory Manual for the Isolation, Identification, and Characterization of Avian Pathogens, fifth Edition, Salmonellosis. Then the group D non-motile *Salmonella* (60 samples

(was isolated. The bacteria were confirmed by microbiological analysis based on (ISO6579), including culture on xylose lysine desoxycholate agar (XLD) (Merck, germany) and RVS broth (Rappaport-Vassiliadis Soy Peptone) (Merck, germany). Then, biochemical analysis, including Lysine decarboxylase, Voges-Proskauer, indole reaction, beta-galactosidase reaction, urease, and H2S production, was performed. Subsequently, the isolates were serotyped with specific O and H *Salmonella* antisera (Mast, UK) and classified based on the Kauffman White scheme.

Extraction of DNA. The genomic DNAs of the 60 *S*. Gallinarum serovar isolates were extracted using the kit (Roch life science Cat. No. 11796828001).

Differentiation of *Salmonella gallinarum* biovars. To differentiate between *Salmonella enetrica* biovar Gallinarum from Pullorum, *ratA, steB*, and *rhs* genes were amplified by Multiplex PCR (16). The amplified *Hq703462* gene was used as an internal control to confirm the isolated *S*. Gallinarum serotype by PCR. The standard strains of both biovars were obtained from the OIE (World Organization for Animal Health, Padua, Italy). PCR was performed in a 25 µl of the reaction mixture using primer pairs shown in Table 1; the following PCR program was used: 1 cycle for initial denaturation at 95ºC for 5 minutes, 40 cycles for denaturation at 94ºC for 40 seconds, annealing stage at 56ºC (or 60 for *Hq703462*) for 30 seconds, elongation step at 72ºC for 40 seconds, and final elongation cycle at 72ºC for 7 minutes. A PCR reaction without the template was used as a negative control.

Antibacterial susceptibility testing. Antibiotic susceptibility test was performed using the disk diffusion method on Muller-Hinton Agar media with various antibiotics of different classes based on CLSI 2022 guidelines suggestions (19). The antibiotics were purchased from Mast Company (UK), including penicillin (10 µg), nitrofurantoin (50 µg), nalidixic acid, (30 µg), amoxicillin (25 µg) amoxicillin (20 µg) + clavulanic acid (10 µg), cefoxitin (30 µg), colistin sulfate (10 µg) chloramphenicol (30 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), ceftazidime (30 µg) + clavulanic acid (10 µg), ceftriaxone (30 µg), cefepime (30 µg) ertapenem (10 µg), kanamycin (30 µg), trimethoprim (1.25 µg) + sulfamethoxazole (23.7 µg), and imipenem (10 µg).

Determination of antimicrobial resistance genes. In this study, the frequency of 14 antimicrobial resistance genes was evaluated. *Salmonella* isolates underwent a PCR test to detect the presence of resistance genes mentioned in Table 2. PCR was accomplished in a 25 µl final volume with a reaction mixture containing 1 µl of each primer using primer sequences presented in Table 2. The following PCR program was used: one cycle for initial denaturation at 95ºC for 5 minutes, 30 cycles with denaturation at 94ºC for 40 seconds, annealing step at 56ºC for 30 seconds, extension stage at 72ºC for 50 seconds, and final extension stage in 72ºC for 10 minutes.

Biofilm formation. Biofilm formation was inspected phenotypically by microplate assay. Briefly, 230 µl of fresh Tryptic soy broth (TSB) (Merck, Germany) was poured into each well of a polystyrene plate

Table 1. The sequences of paired primers used in this study

Target gene	Primer name	Oligonucleotide sequences (5' to 3')	Biovar	Annealing temperature (ºC)	PCR product size (bp)	Reference
steB	steB-F	TGTCGACTGGGACCCGCCCGCCCGC	Gallinarum (D1)	56	636	(17)
	steB-R	CCATCTTGTAGCGCACCAT	the gene is absent in pullorum			
rhs locus	rhs-F	TCGTTTACGGCATTACACAAGTA	Gallinarum +Pullorum	56	402	(15)
	rhs-R	CAAACCCAGAGCCAATCTTATCT				
ratA	ratA-f	GACGTCGCTGCCGTCGTACC	Gallinarum +Pullorum	56	SG:1047	(18)
	ratA-r	TACAGCGAACATGCGGGCGG			SP:243	
Hq703462	Hq-f	CGATATAGCTTACTGTGTCCCG	Gallinarum	60	145	(13)
	Hq-r	TCATGCACTACCACCATAACG				

SG is *Salmonella* Gallinarum and SP: *Salmonella* Pullorum

Table 2. Oligonucleotide primers used for detection of antimicrobial resistance and biofilm genes

Primers	Sequences	Amber classification	Genes	Size of PCR-amplified product (bp)	Annealing temperature (ºC)	References
IMP-F	GGAATAGAGTGGCTTAATTCTC	B	*IMP*	232	56	(20)
IMP-R	GGTTTAACAAAACAACCACC					
VIM-F	GTTTGGTCGCATATCGCAAC	B	*VIM*	389	56	(21)
VIM-R	AATGCGCAGCACCAGGATAG					
GES-F	ATGCGCTTCATTCACGCAC	-	*GES*	591	56	(22)
GES-R	CTATTTGTCCGTGCTCAGG					
NDM-F	GGTTTGGCGATCTGGTTTTC	B	*NDM*	621	56	(20)
NDM-R	CGGAATGGCTCATCACGATC					
bla_{OXA48} -F	GCGTGGTTAAGGATGAACAC	D	bla_{OXA48}	438	56	(20)
bla_{OXA48}-R	CATCAAGTTCAACCCAACCG					
SHV-F	ATGCGTTATATTCGCCTGTG	A	*SHV*	896	56	(22)
SHV-R	AGATAAATCACCACAATGCGC					
KPC-F	CGTCTAGTTCTGCTGTCTTG	A	*KPC*	798	50	(Saffar
KPC-R	CTTGTCATCCTTGTTAGGCG					et al., 2016)
qnrB-F	GATCGTGAAAGCCAGAAAGG	-	*qnrB*	469	50	(23)
qnrB-R	ACGATGCCTGGTAGTTGTCC					
FOX-F	CACCACGAGAATAACC	-	bla_{FOX}	1184	50	(24)
FOX-R	GCCTTGAACTCGACCG					
QnrA-F	ATTTCTCACGCCAGGATTTG	-	*qnrA*	516	52	(23)
QnrA-R	GATCGGCAAAGGTTAGGTCA					
QnrS-F	ACGACATTCGTCAACTGCAA	-	*qnrS*	417	52	(23)
QnrS-R	TAAATTGGCACCCTGTAGGC					
CITMF	TGG CCA GAA CTG ACA GGC AAA	Amp C	*LAT-1 TOLAT-4,CYM-2*	462	55	(25)
CITMR	TTT CTC CTG AAC GTG GCT GGC		*TO CYM-7, BIL-1*			
MOXMF	GCT GCT CAA GGA GCA CAG GAT	Amp C	*MOX1,2*	520	55	(25)
MOXMR	CAC ATT GAC ATA GGT GTG GTG C		*CYM1, 8 to 11*			
DHAMF	AAC TTT CAC AGG TGT GCT GGG T	Amp C	*DHA1,2*	405	55	(25)
DHAMR	CCG TAC GCA TAC TGG CTT TGC					
FOXMF	AACATGGGGTATCAGGGAGATG	Amp C	*FOX-1 TO FOX-5b*	190	55	(25)
FOXMR	CAAAGCGCGTAACCGGATTGG					
sdiA-for	AATATCGCTTCGTACCAC	-	*sdiA*	274	53	(26)

in triplicate. Non-cultured media was used for negative control. 20 µl of the freshly cultured bacteria was added to each of the wells and incubated overnight at 37ºC. The wells were washed three times using 300 µl of PBS. Then, 250 µl of methanol was added to each well and kept for 15 minutes at ambient temperature for air-drying. Next, wells were incubated with 250 µl of crystal violet 2% for 5 minutes (24).

The content of plates was removed and rinsed three times with distilled water and further air-dried. Following the addition of 250 µl of acetic acid 33% to each well, the absorbance of supernatants was measured at 570 nm (23, 24).

The ODt, which represents the mean OD of the three wells for each isolates, and the ODc, which represents the mean OD of the three wells for the control, were recorded. Biofilm formation levels were classified based on the OD as follows (27).

ODt < ODc Non-biofilm

ODc < ODt < 2× ODc Weak biofilm

2× ODc < ODt < 4×ODc Moderate biofilm

ODt ≥ 4×ODc Strong biofilm

The genotype of the bacteria for biofilm production was assessed by the PCR using two *sdi* primers to explore the presence of the *SDI* gene (Table 2).

Statistical analysis. The data were analyzed using SPSS software (version 22.0; Chicago, Illinois, USA). Consensus tables and chi-square tests have been used to investigate the correlation. The P-values <0.05 were considered statistically significant.

Ethical consideration. Chickens were not manipulated for sampling. Samples were collected from yards as a random surveillance program done by the National Veterinary Reference Laboratory.

RESULTS

Differentiation of *Salmonella entrica* biovar Gallinarum from Pullorum. As shown in Fig. 1, *ratA* and *stepB* were used to differentiate between *S.* Gallinarum biovars of Gallinarum and Pullorum according to PCR. *S.* Gallinarum produces 1047 bp band; however, *S.* Pullorum produces 243 bp band for *ratA* gene (Fig.1A). Moreover, *S.* Gallinarum produces 402 bp for *steB* gene and 636 bp for the *rhs* gene; however, in *S.* Pullorum, only 402 bp fragments for the step B gene were amplified using the respective primers shown in Fig. 1B. The present study showed that all 60 *S.* Gallinarum isolates were *S.* Gallinarum biovar.

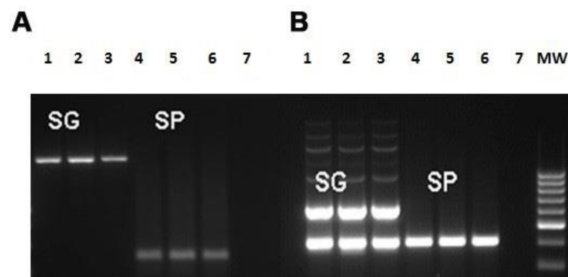
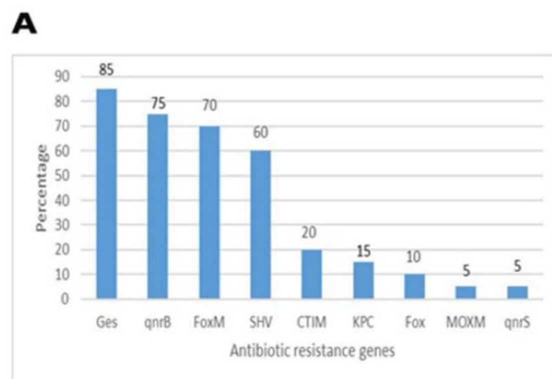


Fig. 1. Differentiation of *Salmonella* Gallinarum biovars using Multiplex PCR:
A: Amplification of *ratA* gene for *S.* Gallinarum biovars as 1047 bp band for *S.* Gallinarum and 243 for *S.* Pullorum: lane 1, standard strain 2, 3, are isolated *S.* Gallinrum, lanes 4, 5, 6; three standard *S.* Pullorum and number 7 is negative control with no DNA template
B: Amplification of *steB* and *rhs* genes as 636 bp and 402 bp bands, respectively for detection and confirmation of **S.** Gallinarum. Lane number 1 is standard *S.* Gallinarum, 2, 3 are the isolated ones. The lanes 4, 5, 6 are three standard *S.* Pullorum, that only *rhs* gene is amplified and the lane number 7 is a negative control.

Antimicrobial resistance. Antimicrobial resistance genes Amplification in *S.* Gallinarum and the presence of resistance genes are shown in Fig. 2. The Distribution of resistance genes in 60 isolated *S.* Gallinarum from different provinces of Iran is shown in Fig. 3.

Fig. 2. Result of detection of resistance genes using Multiplex PCR. From the left: M: marker 100 bp, lane 1; *GES* gene (591 bp) and *IMP* (232 bp), lane 2; *Ges* gene (591 bp) and *VIM* (389 bp), lanes 4, 9, 13 (T0) are negative control, lanes 6 and 7 are related with *SHV* (896 bp) and bla_{OXA48} (438 bp), lane 8; bla_{OXA48} (438 bp), lanes 10 and 11; show the *qnrA* (516 bp), lane 12; products with size 568 bp, 264 bp and 516 bp are related with *marR, parC* and *gyrA* genes, respectively. lanes 14, 18 and 19; show the band 798 bp of *KPC*, lane 15; corresponds with *FOX* (1184 bp), *qnrB* (469 bp) and lanes 16 and 17; show the *qnrB* (469 bp).



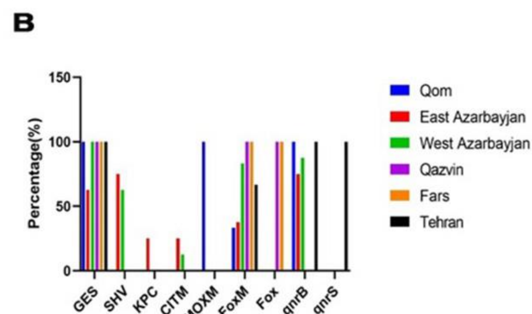


Fig. 3. Distribution of resistance genes in 60 isolated *S.* Gallinarum from different provinces of Iran. (A) distribution of resistance genes in collected samples. (B) distribution of resistance genes based on the samples collected from each province.

Antibacterial susceptibility assay. In our study, the pattern of susceptibility to selected antibiotics for the collected *S.* Gallinarum strains is as follows: penicillin (100%), nitrofurantoin (80%), and amoxicillin (75%), amoxicillin+clavulanic acid (50%), nalidixic acid (45%), neomycin sulfate (30%), chloramphenicol (20%), and ciprofloxacin (5%). On the contrary, all bacteria were susceptible to imipenem, ertapenem, ceftriaxone, ceftazidime, and ceftazidime+ clavulanic acid (Table 3).

As shown in Table 4, the resistant phenotype (+ve) was observed in 12 of 17 antibiotics tested. For cefepime, kanamycin, trimerhoprime, sulfamethoxazole, and colistin sulfate antibiotics, only intermediate resistance was observed. 85% of *S*. Gallinarum isolates showed intermediate resistance for ciprofloxacin, and only 5% were highly resistant.

In this study, all isolates were susceptible to imipenem, ertapenem, and extended-spectrum of cephalosporins, 3rd, and 4th generation cephalosporins, including ceftriaxone, ceftazidime, and ceftazidime+clavulanic acid, except cefepime which showed intermediate resistance in 15% of the isolates.

The rate of multidrug resistance is presented in Table 4. Twenty-four *S*. Gallinarum serovars were resistant to less than two classes of antibiotics (pattern 1, 2, 5); however, most of the biovars 36/60 (60%) were multidrug-resistant (MDR), in 7 categories, with different patterns as presented in Table 4.

Statistical analysis. Results showed that all 60 *S.* Gallinarum isolates produced biofilm according to amplification of the *sdiA* gene. As shown in Table 5, biofilm formation were reported as strong (n=21 isolates or 35%), intermediate (n=24 or 40%), weak (n=15 or 25%). The results showed that the isolates with higher biofilm production are more antibiotic-resistant than weak biofilm producers in a planktonic form (shaded area). Moreover, a positive correlation is shown between some resistance genes such as *FOX M, GES, Fox, KPC,* and *qnrB* and the severity of biofilm formation. However, no correlations were found for the *SHV* gene, bla_{OXA48}, *MOXM* (Ampc), and *CITM* (AmpC).

DISCUSSION

Monitoring for *Salmonella* infections is vital to the poultry industry. They are responsible for economic losses by harming the industry worldwide. In addition, they are a source of diseases transmitted to humans through diet and the environment. For economic and pathogenetic reasons, detection of *S*. Gallinarum in older birds and *S*. Pollurum in chickens is crucial (13). In this study, 60 isolates of

Table 3. Antimicrobial resistance frequency of *S.* Gallinarum against different classes of antibiotics (different shadings).

	Antibiotic name	Susceptible N (%)	Intermediate N (%)	Resistant N (%)
B-lactamse	Penicilin 10 µg (P10C)	0	0	60 (100)
	Amoxicilin 25 µg (A25c)	12 (20)	3 (5)	45 (75)
	Amoxicilin 20 µg+ Clavulanic acid 10 µg (Aug)	30 (50)	0	30 (50)
	Ceftazidime 30 µg (CAZ30c)	60 (100)	0	0
	Ceftazidime 30 µg+ Clavulanic acid (CAZ+Clave)	60 (100)	0	0
	Ceftriaxone 30 µg (CRO 30c)	60 (100)	0	0
	Cefepime 30 µg (CPM30c)	51 (85)	9 (15)	0
Carbapenemase	Imipenem 10 µg (IMI10c)	60 (100)	0	0
	Ertapenem 10 µg (ETP10c)	60 (100)	0	0
	Nitrofurantoein 50 µg (FM50)	6 (10)	6 (10)	48 (80)
Quinolones	Nalidxic acid 30 µg (NA30c)	27 (45)	6 (10)	27 (45)
	Ciprofloxacin 5 µg (CIP 5c)	6 (10)	51 (85)	3 (5)
	Chloramphenicol 30 µg (C30c)	27 (45)	21 (35)	12 (20)
Aminoglycoside	Neomycin sulphate 10 µg (KF30c)	33 (55)	9 (15)	18 (30)
	Kanamycin 30 µg (K30c)	51 (85)	9 (15)	0
	Trimethoprim 1.25 µg+ Sulfametoxazole 23.7 µg (TS 25c)	54 (90)	6 (10)	0
	Colistin sulphate 10 µg (CO 10c)	48 (80)	12 (20)	0

Table 4. Multi drug resistance patterns of the 60 isolated *S.* Gallinarum between 2012-2017.

Antibiotic resistance Patterns	Antibiotics categories									MDR
	ß-lactamase inhibitors			Aminoglycosides	Fluoroquinolones	Polymyxins	Antimetabolite (nitrofuran)	Quinolone	Chloramphenicol	
	Penicillin	Amoxicillin	Cefoxitin	Neomycin	Ciprofloxacin	Colistin	Nitrofurantoin	Nalidixic acid	Chloramphenicol	
1	R									-
2	R						R			-
3	R					R	R			+
4	R						R			+
5	R	R					R	R		-
6	R	R					R			+
7	R					R	R	R	R	+
8	R	R			R	R	R	R		+
9	R	R	R				R			+
10	R	R	R					R		+
11	R	R	R	R			R	R		+
12	R	R	R	R		R		R		+
13	R	R	R	R			R	R	R	+
14	R	R	R	R	R		R	R	R	+

*Resistance

S. Gallinarum were confirmed by biochemical test. PCR-based detection of *Salmonella* biovars is sensitive, easy, and rapid (28, 29). Up to now, Xiong et al. have detected several genes; they have amplified *ratA* (ROD) gene that shows a deletion in biovar Pullorum compared with Gallinarum. They showed that the combined amplification of *stn*, I137_08605, and *ratA* ROD could be 100% specific for each biovar (30, 31). In our study, the *ratA* that is common between two biovars showed different sizes; however, the *rhs* and *SteB* genes were used for biovar classification. All 60 *S*. Gallinarum were identified as Gallinarum; therefore, the following results of our study are beneficial to the industry of mature or growing chickens, ducks, and turkeys of farms in Iran.

To confirm the identification of *S*. Gallinarum biovars, Paiva et al. employed the RFLP-based amplification of the *Flic* gene of flagellar antigen and digestion with a restriction enzyme (Hinp1l) followed by running on the agarose gel. The technique is a two-step process that is expensive and time-consuming compared to standard PCR (32).

All *Salmonella* strains were *S*. Gallinarum. *S*. Pullorum was not detected; the reason could be related to the community of collected samples, i.e., adult farm chickens, and not the young ones, which are more susceptible to *S*. Pullorum.

A variety of bacteria is present in the gastrointestinal tract of poultry, such as *Enterobacteriaceae*, that exchange the genetic materials, including resistance genes (21, 33).

Inappropriate antibiotic use in poultry has led to the emergence of resistant bacteria and horizontal resistant gene transfer to environmental and transient *Salmonella* (34).

Studies have shown that the *GES* and *KPC* genes are detected in *Klebsiella*, with the respective prevalence of 11% and 23% (35). However, our results showed the respective prevalence of 15% and 85% for *KPC* and *GES* genes. Moreover, the *S*. Gallinarum with KPC resistance gene does not contain *GES* and vice versa, which has not been reported up to now.

The existence of a variety of β-lactam genes such as *KPC, SHV, GES, Fox, qnrB,* and *qnrS* in *S*. Gallinarum, could be a significant warning due to their transmissibility to other bacteria of the ecosystem, arising a dilemma in the treatment of pathogenic bacteria in the poultry industry which would finally contaminate human (36-38).

Our result on the origin of resistance contrasts with the study conducted in Brazil from 2006 to 2013. They found no *PMQR* gene in 17 isolates of *S*. Gallinarum or *S*. Pullorum isolates. However, they re-

Table 5. Correlation between the strength of biofilm formation and the presence of antibiotic resistance genes in 60 *Salmonella* SPP.

Antibiotic resistance gene	Number of Isolates (%)	Strong Biofilm Formation (%)	Weak and moderate Biofilm Formation (%)	P-value
GES gene (*bla*)	51 (85)	15 (29.4)	36 (70.6)	0.054
Positive	9 (15)	6 (66.7)	3 (33.3)	
Negative				
Fox gene (*bla*)	6 (10)	6 (100)	0 (0)	0.001
Positive	54 (90)	15 (27.8)	39 (72.2)	
Negative				
Kpc gene (*bla*)	9 (15)	69 (66.7)	3 (33.3)	0.054
Positive	51 (85)	15 (29.4)	36 (70.6)	
Negative				
FoxM (Ampc)	42 (70)	9 (21.4)	33 (78.6)	0.001
Positive	18 (30)	12 (66.7)	6 (33.3)	
Negative				
qnrB	45 (75)	12 (26.7)	33 (73.3)	0.022
Positive	15 (25)	9 (60)	6 (40)	
Negative				
qnrS	36 (60)	12 (33.3)	24 (66.7)	0.039
Positive	24 (40)	9 (37.5)	15 (62.5)	
Negative				
SHV gene	36 (60)	12 (33.3)	24 (66.7)	0.476
Positive	24 (40)	9 (37.5)	15 (62.5)	
Negative				
bla_{OXA48} Positive	36 (60)	12 (33.3)	24 (66.7)	0.476
Negative	24 (40)	9 (37.5)	15 (62.5)	
MOXM (Ampc)	57 (95)	21 (36.8)	36 (63.2)	
Positive	3 (5)	0 (0)	3 (100)	0.545
Negative				
CITM (AmpC)	12 (20)	6 (50)	6 (50)	0.312
Positive	48 (80)	15 (31.2)	33 (68.8)	
Negative				

ported resistance to quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin) (39).

The high prevalence of multidrug-resistant *Salmonella* in poultry may increase the rate of MDR *Salmonella* in humans (40). In our study, all the isolates were susceptible to imipenem, ertapenem, ceftriaxone, ceftazidime, and ceftazidime + clavulanic acid supported by the previous studies conducted in Vietnam (41). To the best of our knowledge, the fluoroquinolones and third-generation of cephalosporins are relatively effective for the treatment of salmonellosis (22), though in recent years, the resistance to routine antibiotics has increased (41). The absence of resistance against cephalosporins antibiotics in the present study possibly shows restricted use in poultry (41, 42). As mentioned above, 45 (75%) of our isolates were resistant to amoxicillin, and 30 (50%) of the isolates were resistant to amoxicillin-clavulanic acid, which is an indicator of the presence of ESBLs genes in the isolates. Moreover, the results are warning for the possible increase in the prevalence of ESBLs genes in human populations. The previous studies have also shown an increase in resistance for *S*. Pullorum/Gallinarum over time (12). In contrast to our study, Ramya et al. showed that the susceptibility of *Salmonella* spp. for ciprofloxacin and amoxicillin were 100% and 82%, respectively (43). Furthermore, studies in geographical areas such as Bangladesh have also shown approximately 50% resistance to five antibiotics among 16 *Salmonella* spps. isolates in

2016 (44). Results from another study in Bangladesh from 2021 showed an increase in the frequency of antibiotic resistance: This study reports high levels of resistance to penicillin and nalidixic acid, sulfometaxazole trimethoprim , ampicillin and amoxicillin (45).

A total of 130 *S*. Gallinarum isolates from chickens were collected in a study conducted in Korea from 2014 to 2018. In general, these isolates showed higher resistance to nalidixic acid, gentamicin, ciprofloxacin and ampicillin (46). The antimicrobial susceptibility profiles of *Salmonella* isolated from poultry in Pakistan were as follows: highest resistance to nalidixic acid, ampicillin, amoxicillin, moderate resistanceto gentamicin, chloramphenicol, tetracycline, ciprofloxacin, ceftazidime and low resistance to cefotaxime, ceftriaxone, sulfamethoxazole and cefixime (45). Studies of resistance gene in *S*. Gallinarum in India in 2016 have shown that the 25.6% are resistant to ciprofloxacin 81.81% to amoxicillin, doxycycline, kanamycin, gentamycin, and tetracycline (44).

In many countries, control and prevention programs to eradicate salmonellosis are ineffective due to the use of antibiotics as growth factors in poultry (47). In the present study, the high antibiotic resistance could result from the same process and lead to a disastrous outcome.

The production of bacterial biofilms enhances the ability of bacteria to endure harsh environmental conditions and sanitation procedures (48). Therefore, the prevalence of biofilm formation and the level of biofilm production are essential parameters for biofilm eradication (13, 16). The biofilm formation was studied using both molecular and phenotypic techniques in the present study. This study confirmed the variable biofilm formation; however, a relation was found between biofilm formation and antibiotic resistance. Our results also showed that biofilm formation is significantly related to the prevalence of antibiotic resistance genes for *Fox*, *GES, KPC, qnrB,* and *FOXM* (P<0.05) and could be considered a factor that increases the virulence of *S*. Gallinarum.

The present study is the first study on *S*. Gallinarum biofilm in Iran focusing on the characterization of *S*. Gallinarum biovar and their acquired antibiotic resistance genes circulating in poultry farms in central and northwestern of Iran. Furthermore, our results demonstrated the association between biofilm production ability and resistance to commonly administered antibiotics.

CONCLUSION

Considering that the presence of this bacteria is equal to the death penalty to the herd, the distribution of resistance genes could be a critical alarm for pathogen monitoring programs in the region. This study showed a positive correlation between biofilm formation and 50% of tested resistance genes. Also, it was found that the most common circulating *S*. Gallinarum biovars are multidrug-resistant.

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