Short Communication

Prevalence of *aadA1*, *aadA2*, *aadB*, *strA* and *strB* genes and their associations with multidrug resistance phenotype in *Salmonella* Typhimurium isolated from poultry carcasses

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Abstract

This study aimed to assess the prevalence of aminoglycoside resistance genes in *S*. Typhimurium isolated from poultry carcasses in Iran, and to reveal the most prevalent patterns of antimicrobial resistance. A total number of 300 samples of poultry carcasses were analyzed. *Salmonella* was isolated from 245 samples (81.66%). Multiplex PCR showed that 56.3% of the samples belonged to serovar *S*. Typhimurium and the remainder (43.6%) contained the rest of serovars. The highest rate of drug resistance was observed for tetracycline (97.0%), nalidixic acid (87.0%) and amoxicillinclavulanic acid (67.4%). These serovars, however, were sensitive to cefotaxime (84.8%), sulfamethoxazole trimethoprim (77.6%) and gentamicin (71.0%). *aadA1* gene was detected in 63 isolates (45.6%), *aadA2* in 48 isolates (34.7%), *aadB* in 43 isolates (31.1%), *strA* in 52 isolates (37.6%) and *strB* in 31 isolates (22.4%). High prevalence of aminoglycoside resistance genes in *S*. Typhimurium was shown. Furthermore, there was a significant association ($P < 0.02$) between the presence of *aadA1*, *aadA2*, *strA* and *strB* genes and resistance to streptomycin. Also, there was a significant association ($P < 0.001$) between the presence of *aadB* gene and resistance to kanamycin and gentamicin.

Keywords: *Salmonella* Typhimurium, aminoglycoside resistance genes, poultry

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Introduction

Salmonellosis is an infection caused by *Salmonella*, the major cause of foodborne infections and the second most common foodborne illness after *Campylobacter* infection (Soltan-Dallal et al., 2014). The genus *Salmonella* presently includes two species, *Salmonella enterica* (*S. enterica*) and *Salmonella bongori* (*S. bongori*). *S. enterica* is divided into subspecies I (enterica), II (salamae), IIIa (arizonae), IIIb (diarizonae), IV (houtenae), and VI (indica) (Switt et al., 2009). *S.* enterica serovar Typhimurium causes yearly 1.3 billion cases of disease worldwide (Meyer et al., 2012; Coburn et al., 2007). Salmonellosis in human are often the result of ingestion of contaminated foods such as beef, undercooked pork, milk, poultry, eggs, seafood, fresh produce, and non-alcoholic beer. Additionally, direct contact with animals is the cause of transmission of *Salmonella* to humans (Meyer et al., 2012; Solltan Dallal et al., 2009; Doosti et al., 2016). The emergence of antimicrobial resistant bacteria pathogens has become a great public health concern (Mokhtari-Farsani et al., 2015). The use of antimicrobials in disease treatment and growth promotion in domestic livestock can potentially lead to expansion of antimicrobial resistant bacteria. Multidrug resistant strains of *S.* enterica serovar Typhimurium were first isolated in year 1980 (Gallardo et al., 1999) and are typically identified by penta drug resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (type ACSSuT) (Faldynova et al., 2005). Currently, at least 2,610 serovars of *S.* enterica have been recognized worldwide, and almost all are capable of causing disease in humans and animals (Sabol and Ali, 2010). Therefore, *S.*enterica remains a microbial threat in terms of standards and food safety, and molecular detection and analysis of *Salmonella*, specifically *S.*enterica, is of high importance (Hadjinicolaou et al., 2009; Regan et al., 2008).

aadA family of genes encodes aminoglycoside-3″-adenylyltransferases (AAD), which confer resistance to streptomycin and spectinomycin by adenylylation (White and Rawlinson, 2001). The nucleotide sequence of *aadB* gene, which confers resistance to kanamycin, gentamicin and tobramycin, has been specified (Cameron et al., 1986). *strA* and *strB* genes encode streptomycin-inactivating enzymes and confer streptomycin resistance in at least seventeen genera of gram-negative bacteria such as *Salmonella* (Sundin and Bender, 1996). Since few studies have been done of the prevalence of these five important genes, especially in the Middle East region, therefore, the present research aimed to study the frequency of aminoglycoside resistance genes in *S.* Typhimurium isolated from poultry carcasses in Iran, and also the relationship between these genes and resistance to antimicrobial agents.

Materials and Methods

Sampling: In this cross-sectional study, a total number of 300 poultry carcass samples were collected during April of 2015 from an industrial slaughterhouse in Chaharmahal va Bakhtiari province (south-west of Iran). Immediately after cutting the feathers and skin, about 5 g of each poultry carcass sample (meat) was transferred to a sterile tube (by sterile scalpels), placed on ice and transported to Biotechnology Research Center of Shahrekord Azad University in an insulating foam box with ice.

Isolation and identification of Salmonella*: Salmonella* serovars were isolated using standard (culture) methods. One gram of each sample in buffered peptone water (BPW, Oxoid, USA) was inoculated. The samples were incubated for 18 h at 37°C. In the next step, 0.1 ml of the pre-enriched cultures were transferred to Rappaport Vassiliadis (RV, Oxoid) and incubated at 42°C. After 24 h of incubation, 10 µl of the culture from each enriched broth was streaked on *Salmonella* Shigella (SS) agar (Difco) and incubated at 37°C for 24 h. Colonies were cultured in nutrient broth for 24 h at 37°C.

Colonies grown on SS medium (one colony in 100 µL deionized water) were subjected to genomic DNA isolation using DNA Extraction Kit (CinnaGen Co, Iran) according to the manufacturer's protocol as described previously (Jami et al., 2008). For molecular diagnosis of *Salmonella* from other bacteria, PCR assays with specific *16s rDNA* primers (Table 1) were carried out (for isolation of other bacteria) and the PCR products showed a 178 bp fragment on 1.5% agarose gel for *Salmonella* (Fey et al., 2004).

A 3-plex-PCR was performed for detection and isolation of *S.* Typhimurium from the rest of *Salmonella* spp. (Lim et al., 2003). Primer sequences used for the 3-plex-PCR in this study are shown in Table 1. PCR reactions were done and the amplification products were analyzed by 1.5% agarose gel electrophoresis. After electrophoresis, generated bands were screened and digitally photographed under UV light.

Antimicrobial susceptibility testing: Following standard culture methods, bacteria were taken from the nutrient broth medium with a sterile swab and transferred onto Mueller Hinton culture media for antimicrobial drug susceptibility test. Evaluation of drug resistance was performed using standard Bauer-Kirby disk diffusion (KB testing) method for *S.* Typhimurium samples. A total of 11 antibiotic discs (Padtan Teb, Iran) with tetracycline (30 µg), ciprofloxacin (5 µg), streptomycin (10 µg), ampicillin (10 µg), chloramphenicol (30 µg), kanamycin (30 µg), gentamicin (10 µg), amoxicillin clavulanic acid (30 µg), cefotaxime (30 µg), nalidixic acid (30 µg) and sulfamethoxazole-trimethoprim (25 µg) were chosen and used based on their importance in human or animal treatment depending on *Salmonella* infections. Diameters of the inhibition zones were interpreted based on the CLSI sub committee's recommendations (2014).

Detection of aminoglycoside resistance genes: PCR was performed for identification of *aadA1*, *aadA2*, *aadB*, *strA* and *strB* genes (Table 1) and PCR reactions were prepared separately for each primer set. PCR test was performed in a final volume of 25 µl containing 2 µL of template DNA, 2.5 µL of 10X PCR buffer, 1.5 mM of MgCl2, 200 µM of dNTPS, 2 mM of each primers and

one unit of Taq DNA polymerase enzyme (CinnaGen Co, Iran). Thermal PCR conditions consisted of initial 5 min at 95°C and then 32 cycles of denaturation at 94°C for 1 min, annealing temperature for the 5 genes at 65°C, 64°C, 62°C, 61°C and 65°C (respectively) for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min, with a final hold at 10°C in a thermal cycler (Mastercycler gradient, Eppendrof, Germany). The amplification products were analyzed by 1% agarose gel electrophoresis. After electrophoresis, generated bands were screened and digitally photographed under UV light.

Table 1 Primers for identification of *S.* Typhimurium

Gene target	Primer name	Sequence (5'–3')	Accession number	size (bp)
	16s-F	CGGGGAGGAAGGTGTTGTG		
Salm16S	*16s-R*	GAGCCCGGGGATTTCACATC	Z49264	178 bp
	aadA1-F *aadA1*-	CTCCGCAGTGGATGGCGG		
aadA1	R	GATCTGCGCGCGAGGCCA	KC292125	311 bp
	aadA2-F *aadA2*-	CATTGAGCGCCATCTGGAAT		
aadA2	R	ACATTTCGCTCATCGCCGGC	KC292190	432 bp
	aadB-F	CTAGCTGCGGCAGATGAGC		
aadB	*aadB*-R	CTCAGCCGCCTCTGGGCA	HQ639678	219 bp
	strA-F	TGGCAGGAGGAACAGGAGG		
strA	*strA*-R	AGGTCGATCAGACCCGTGC	HQ639678	608 bp
	strB-F	GCGGACACCTTTTCCAGCCT		
strB	*strB*-R	TCCGCCATCTGTGCAATGCG	HQ639678	256 bp
	fliC-F	ATAGCCATCTTTACCAGTTCCCCC		
FliC	*fliC-R*	GCTGCAACTGTTACAGGATATGCC	D13689	183 bp
	rfbJ-F	CCAGCACCAGTTCCAACTTGATAC		
RfbJ	*rfbj-R*	GGCTTCCGGCTTTATTGGTAAGCA	AE008792	663 bp
	fljB-F	ACGAATGGTACGGCTTCTGTAACC		
FljB	*fljB-R*	TACCGTCGATAGTAACGACTTCGG	AF045151	526 bp

Statistical analysis: All data were analyzed by Excel 2007 and SPSS software (Version 17.SPSS Inc, USA). P-values less than 0.05 were considered statistically significant.

Results and Discussion

Estimated prevalence of S. Typhimurium: In the present study, a total of 300 samples of poultry carcasses were tested for the presence of *Salmonella*. Two hundred and eighty (n=280; 93.3%) samples were grown in culture. A total of 245 (n=300; 81.66%) *Salmonella* were isolated from the samples by PCR using *16s rDNA* gene primers. To identify *S*. enterica serovar Typhimurium using 3-PCR, three primer sets were used in the reaction mixture. Several distinct results were obtained from the 3-PCR according to *Salmonella* serovar (Fig 1). Three amplified products derived from *fliC*, *rfbj* and *fljb* genes including 183, 663, and 526 bp were found in serovar Typhimurium. The presence or absence of *fliC*, *rfbj* and *fljb* genes was used as criteria to differentiate serovar Typhimurium from other *Salmonella* serovars (Table 2) (Lim et al., 2003). Out of the 245 samples, 138 (56.3%) samples were found to be serovar Typhimurium and 43.6% to be the rest of serovars (Table 2).

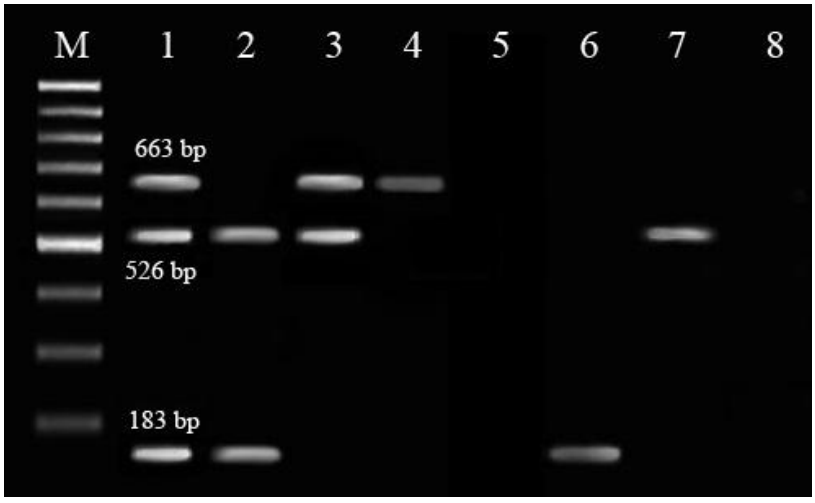

Figure 1 Gel electrophoresis for detection of *S.* Typhimurium using the three primers sets, *fliC*, *rfbj* and *fljb*. Lane M is 100 bp DNA ladder (Fermentas, Germany), lane 1 serovar Typhimurium, lane 2 serovar Landau, lane 3 serovar Saintpaul, lane 4 serovar Agona, lane 5 serovar Enteritidis, lane 6 serovar Kentucky, lane 7 serovar Newport and lane 8 negative sample.

Susceptibility of Salmonella: The resistance pattern of *S.* Typhimurium isolates to 11 antimicrobial agents listed in this study is shown in Table 3. All *S.* Typhimurium isolates (n=138) were resistant to more

than one antimicrobial agent. The highest rate of drug resistance was against tetracycline (97.0%), nalidixic acid (87.0%) and amoxicillinclavulanic acid (67.4%). The highest rate of drug sensitivity was against cefotaxime (84.8%), sulfamethoxazole trimethoprim (77.6%) and gentamicin (71.0%). Thirty-three isolates (24.0%) were sensitive to 7, and 89 isolates (64.4%) had antimicrobial resistance to 3 or more than 3 antimicrobial agents.

Prevalence of aminoglycoside resistance genes: In order to identify the resistance genes, PCR test was carried out for each gene separately. PCR products showed 311, 342, 219, 608 and 256 bp fragments for *aadA1*, *aadA2*, *aadB*, *strA* and *strB* genes, respectively. The distribution of aminoglycoside resistance genes varied among the *S.* Typhimurium isolates. Sixty-three isolates (45.6%) possessed the *aadA1* gene, 48 (34.8%) possessed the *aadA2* gene, 43 (31.2%) possessed the *aadB* gene, 52 (37.7%) possessed the *strA* gene and 31 (22.5%) possessed the *strB* gene. The highest prevalence rate was observed for the *aadA1* gene. Also, in 38 *S.* Typhimurium there was more than one antimicrobial resistance gene. A significant association was determined between the resistance to streptomycin, gentamicin and kanamycin and the aminoglycoside resistance genes. For instance, the results showed a significant association ($P < 0.02$) between the presence of *aadA1*, *aadA2*, *strA* and *strB* genes and the resistance to streptomycin. Also, there was a significant association ($P < 0.001$) between the presence of *aadB* gene and the resistance to kanamycin and gentamicin.

Table 2 Diagnosis patterns of different serovars of *S. enterica* subsp. enterica and prevalence of *Salmonella* serovars in the present study

Salmonella enterica serovars	PCR results			Number
	Flic	*Rfbj*	*Fljb*	
Typhimurium	+	+	+	138
another serovars				21
Landau	+	-	+	2
Saintpaul	-	+	+	5
Agona	-	+	-	8
Enteritidis	-	-	-	62
Kentucky	+	-	-	5
Newport	-	-	+	4

Table 3 Susceptibility of 138 *S.* Typhimurium isolated from poultry carcasses

Antibiotic discs	Resistance	Sensitivity
Ampicillin (10 µg)	46 (33.3%)	92 (66.7%)
Chloramphenicol (30 µg)	46 (33.3%)	92 (66.7%)
Streptomycin (10 µg)	88 (63.7%)	50 (36.3%)
Tetracycline (30 µg)	133 (97.0%)	5 (3.0%)
Ciprofloxacin (5 µg)	65 (47.0%)	73 (53.0%)
Kanamycin (30 µg)	44 (31.8%)	94 (68.2%)
Gentamicin (10 µg)	40 (29.0%)	98 (71.0%)
Sulfamethoxazole trimethoprim (25 µg)	31 (22.4%)	107 (77.6%)
Cefotaxime (30 µg)	21 (15.2%)	117 (84.8%)
Nalidixic acid (30 µg)	120 (87.0%)	18 (13.0%)
Amoxicillinclavulanic acid (30 µg)	93 (67.4%)	45 (32.6%)

A wide range of antibiotics are extensively used for the control of infectious diseases in poultry and other animal species, which in turn can result in a significant increase in antimicrobial resistance in bacteria (Leite et al., 2005). Development of antimicrobial resistance in *Salmonella* serotypes has been considered as a public health problem worldwide. Therefore, it is important to determine the prevalence of disease caused by *Salmonella* together with the identification of antimicrobial resistance pattern. Some studies have been performed to evaluate the prevalence of *S.* Typhimurium, frequency of aminoglycoside resistance genes and also resistance of this bacterium to antimicrobial agents. Results of a study from Solltan Dallal et al. (2009) in Tehran showed that 51 (38.3%) out of 133 chicken and beef samples were identified as *Salmonella* strains. They also showed the highest resistance to nalidixic acid (90.6%), tetracycline (71.9%), trimethoprim (56.6%) and streptomycin (25%) in chicken and to nalidixic acid (36.8%), tetracycline (21%), trimethoprim (26.3%) and streptomycin (5.3%) in meat. The results were almost similar to ours particularly in the case of resistance to tetracycline and nalidixic acid. Dilmaghani et al. (2011) showed that 181 (9.6%) out of 1,870 intestine samples were infected with *Salmonella* in the north, north-west and capital city (Tehran) of Iran. Among the *Salmonella*-positive samples, 52 were identified as serovar Typhimurium. While they showed low (28.7%) prevalence of *S.* Typhimurium in the north of the country, our present study indicates relatively higher (56.3%) prevalence of *S.* Typhimurium in the south-west of Iran.

In a study by Ma et al. (2007), 30 *Salmonella* isolates were analyzed and resistance genes were characterized by PCR. Results showed that the genes *aadA1*, *aadA2*, and *aadB* could confer resistance to aminoglycoside antimicrobials. In a similar work, the presence of relevant resistance genes such as *aadA* and *strA* were investigated using PCR method by Thong et

al. (2010). Among 47 samples of *S*. Typhimurium, a total of 57.4% of the *S*. Typhimurium strains were multidrug resistant and showed high resistance rates to tetracycline (70.2%), streptomycin (53.1%), ampicillin (29.7%), nalidixic acid (27.6%), kanamycin (23.4%), chloramphenicol (21.2%), and trimethoprim (19.1%). Resistance genes including *aadA* and *strA* were detected among the drug-resistant strains. Unlike Douai's study, *Salmonella* was found to be resistant to nalidixic acid (87.0%) in the present study.

Molecular methods have been widely used for the identification of microbial strains and serovars. Lim et al. (2003) proposed that the results of PCR for samples containing *fliC*, *rfbj* and *fljb* genes (specific primers Typhimurium) were indicative of *S*. Typhimurium. El-Aziz (2013) collected one hundred samples of retail raw chicken meat and giblets in Egypt to detect *S*. Typhimurium by PCR against *rfbJ* and *fliC* genes. *S*. Typhimurium was detected (40%-48%), but this serovar was not detected in the gizzard. Similar to our study, El-Aziz's results showed high prevalence of *S*. Typhimurium in the examined samples.

The above information suggests that both the prevalence and the antimicrobial resistance pattern for *Salmonella* spp. vary among countries and different geographical zones, which can clearly reflect the difference in healthcare programs in developing and developed countries. Our study showed the high prevalence of *S*. Typhimurium in the south-west of Iran. It is shown that the frequency of aminoglycoside resistance genes is relatively high in poultry carcasses, which is likely due to the immethodical use of antimicrobial agents. Furthermore, two significant associations were found between the resistance to streptomycin, gentamicin and kanamycin and the aminoglycoside resistance genes. Considering the fact that most of the *Salmonella* strains are common among humans and domestic animals (Zoonotic), it is suggested that more epidemiological studies should be conducted to detect any change in resistance pattern. Epidemiological studies together with the results of our study as well as others (for instance, similar works in the north of Iran) may provide more insights to design new therapeutic plans including limited use of antimicrobial drugs in humans and animals, perform antimicrobial susceptibility tests to select suitable antimicrobial agent, and apply suitable dosage of antimicrobial agents. The new programmed and controlled treatment plans can widely impact domestic healthcare in terms of both economy and public health aspects.

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บทคัดย่อ

อุบัติการณ์ ของยีน *aadA1*, *aadA2*, *aadB*, *strA* และ *strB* และความความสัมพันธ์ กับการดื้อยาหลายชนิด ของเชื้อ *Salmonella* Typhimurium ที่แยกได้จากสัตว์ปีก

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การศึกษาครั้งนี้ มีวัตถุประสงค์เพื่อ ศึกษาอุบัติการณ์ ของยีนดื้อยา aminoglycoside ของเชื้อ *S.* Typhimurium ที่แยกได้จากสัตว์ปีกในประเทศอิหร่าน และเพื่อหารูปแบบการดื้อยาที่พบบ่อยของเชื้อ จากการตรวจตัวอย่างจำนวน 300 ตัวอย่างจากสัตว์ปีก พบเชื้อ *Salmonella* จำนวน 245 ตัวอย่าง คิดเป็น 81.66%. และเมื่อตรวจพิสูจน์ด้วยวิธี multiplex PCR พบเชื้อ *S.* Typhimurium 56.3% และ *Salmonella* serovar. อื่นๆ 43.6% และพบเชื้อที่ดื้อต่อยา tetracycline (97.0%), nalidixic acid (87.0%) และ amoxicillinclavulanic acid (67.4%) ในขณะที่พบเชื้อไวรับต่อยา cefotaxime (84.8%), sulfamethoxazole trimethoprim (77.6%) และ gentamicin (71.0%) และเมื่อตรวจหายีนดื้อยาพบ ยีน *aadA1* ในเชื้อ 63 ตัวอย่าง (45.6%) ยีน *aadA2* ในเชื้อ 48 ตัวอย่าง (34.7%) ยีน *aadB* ในเชื้อ 43 ตัวอย่าง (31.1%), ยีน *strA* ในเชื้อ 52 isolates (37.6%) และยีน *strB* ในเชื้อ 31 isolates (22.4%). ซึ่งแสดงให้เห็นถึงอุบัติการณ์สูงในยีนดื้อยา aminoglycoside นอกจากนี้ยังพบความสัมพันธ์อย่างมีนัยสำคัญระหว่างยีน *aadA1, aadA2, strA* และ *strB* genes กับการดื้อต่อยา streptomycin. (P<0.02) และความสัมพันธ์ ระหว่างยีน *aadB* gene กับการดื้อต่อยา kanamycin และ gentamicin.(P<0.001)

คำสำคัญ: *Salmonella* Typhimurium aminoglycoside ยีนดื้อยา สัตว์ปีก

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