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ORIGINAL ARTICLE

Determination of Tetracycline and Enrofloxacine Resistance in *Salmonella* Isolated From Poultry

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ABSTRACT

Background: In recent years, an increase in antibiotic resistance has been observed in *Salmonella* in different countries. The aim of this study was to determine the tetracycline and enrofloxacine resistance in *salmonella* isolated from poultry.

Methods: The pattern of antibiotic resistance to tetracycline and enrofloxacin in isolated *Salmonella* of fecal broiler chickens from Shiraz, southern Iran, was assessed using minimum inhibitory concentration (MIC) and PCR methods.

Results: Of 100 fecal samples of broiler chickens, 5 samples (5%) were infected to *Salmonella*. The antimicrobial susceptibility showed that MIC_{90} of isolated *Salmonella* strains for enrofloxacin and tetracycline was less than 0.2 µg/mL and 180 µg/mL, respectively, indicating a high sensitivity to these antibiotics. In two samples the presence of tetracycline resistance plasmid was also found, while all the strains were susceptible to enrofloxacin.

Conclusion: According to the results, the isolated *Salmonella spp.* showed higher resistance to tetracycline than enrofloxacin, which seems due to the excessive usage of this antibiotic in poultry industry.

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Introduction

Non-typhoidal *Salmonella* is known as one of the most important food-producing pathogens with high prevalence in worldwide. Raw foods, including poultry meat, eggs, and red meat are considered as the main sources of *Salmonella* infection. Nowadays, global concerns about *Salmonella* multi-drug resistance, as a human health threatening, are increasing. Several outbreaks of food-borne diseases have been reported for *Salmonella enterica* (1-3). A common way caused multi-drug resistance is transferring of mobile genetic materials, including transposons and plasmids (2-4).

Since one of the main problems in food industry is infection of chicken meat with this pathogen, antibiotics are commonly used in the treatment and prevention of the disease. Regarding that the antibiotic resistance can easily be transfer by plasmid genes, this study was carried out using phenotypic and molecular assays in order to identify accurate genetic patterns of antibiotic resistance and their incidence in broiler chickens in Shiraz, southern Iran.

Materials and Methods

In this study, 100 broiler chickens from Shiraz

farms were collected in 2011. In order to obtain fecal specimens from broiler chickens, direct swabs from stool were provided. All samples were transferred to glass tubes containing 10 ml of selenite medium and incubated at 37°C for 24 hours. Following the enrichment procedure, the specimens were sub-cultured onto the selective culture media, brilliant green agar and xylose lysine deoxycholate (XLD) agar and incubated at 37°C for 24 hours. Biochemical tests for urea, lysine, sulfuric acid, methyl ester and indole were used to confirm the suspected colonies.

DNA Extraction was undertaken using DNA extraction kit (Cinna Gene, Iran). Then, the bacterial specimens were transferred to 1.5 µL tubes and centrifuged for 10 minutes at 12000 rpm. The supernatant was discarded and 200 µL of lysis buffer and 40 µL of proteinase K were added and incubated for 15 minutes at 65°C. Then, 30 µl of distilled water was added to the purified DNA and kept at -20°C until use. The DNA concentration was estimated by optical absorption at 260 nm and its purity was determined using nanodrop in 260/280 nm absorbance.

PCR assay was performed to identify tetracycline (*tetA*) and enrofloxacin (*gyrA*) resistance using specific primers. For *tetA* as F 5' GTGAAACCCAACCATACCCC 3' and R 5' GAAGGCAAGGAGGATGTAG 3'. For *gyrA* as F 5'ATGAGCGAATTAGCCAAAGA 3' and R 5'GCAACCGTCCAACACTTCAT 3'. The components of the PCR reaction in a final volume of 25 µL contain 1 µL template DNA, 2.5 µL PCR buffer, 0.5 µL of Taq DNA polymerase enzyme (5 µg/µL), 0.5 µL of each primer (10 µmol) 1 µL of the DNTP mixture (2.5 mmol), and 1.5 µL of MgCl2 (50 mmol) which were mixed together (CinnaGen, Iran).

The PCR reaction was conducted as 30 cycles, using thermocycler (Ependorf, Germany). The initial denaturing was performed at 94°C for 3 minutes. Then, 25 cycles of denaturation for 1 min at 94°C was performed. For *gyrA*, 1 min at 55°C and for *tetA*, 1 min at 53°C were undertaken, and repeated at 72°C for another 1 minute. The final proliferation was performed at 72°C for 10 minutes (2). To evaluate the PCR, 5 µL of the product was transferred to 1% agarose gel and stained with ethydium bromide and observed under ultraviolet light at 582 and 890 bp for enrofloxacin and tetracycline, respectively.

Inducing the antibiotic resistance was also performed in Salmonella. The sensitive bacterial specimens to enrofloxacin and tetracycline were further assessed by minimum inhibitory concentration (MIC) test. For this purpose, *Salmonella* was initially cultured in a tryptic soy broth (TSB) medium, and incubated at 37°C for 24 hours. The bacterial suspensions and each antibiotic (tetracycline at the concentration of 300 µg/mL or enrofloxacin at the concentration of 1 µg/mL) were added to the TSB medium and incubated at the same condition. The preparations were then sub-cultured onto the McConkey agar medium and incubated as before. The growing colonies showed their resistant to each antibiotic which were further confirmed by a PCR assay.

The MIC experiment was performed using a 96-well microplate. At first, 100 µL TSB medium was added to each well, then two folded dilutions of oxytracycline at the concentration of 300 µg/mL and enrofloxacin at the concentration of 1 µg/mL were added to the culture medium. Ten microliter of *Salmonella* suspension (both resistant and non-resistant ones), with 10^7 CFU/mL was added to each well. Each experiment was performed in four replications. Also, the TSB medium containing *Salmonella typhimurium* as positive control and antibiotic and the TSB medium as a negative control were also transferred into wells. The minimum concentration of the antibiotic inhibiting bacterial growth was considered as MIC (5, 6).

Results

Out of 100 fecal samples collected from broiler chickens, 5% of the samples were infected with *Salmonella*. Of which, 2% were found resistance to tetracycline (40%, Figure 1); while all 5 samples were sensitive to the enrofloxacin antibiotic. Samples that were resistant to tetracycline and enrofloxacin antibiotics were also tested by PCR, to visualize the antibiotic resistance patterns (Figure 1 and 2).

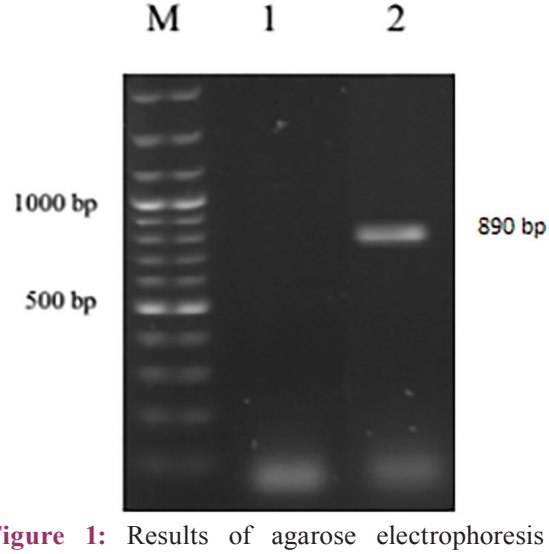

Figure 1: Results of agarose electrophoresis of tetracycline resistance gene in the 1% gel. M: Marker 100 bp, line 1: Negative control, line 2: Positive control (Plasmid (pMOBGIII) 890 bp, line 3: Positive sample.

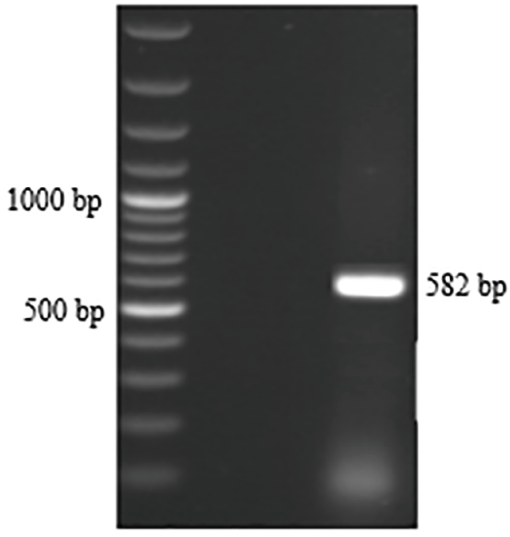

Figure 2: Results of agarose electrophoresis of enrofloxacin resistance gene in a 1% gel: 100 bp marker, line 1: Negative control, line 2: Positive sample 582 bp.

Details of the resistance bacteria isolated from the fecal samples to both antibiotics are given in Tables 1-4 and Figures 3 and 4. The MIC values of non-resistant and resistant strains were respectively 99.1±1.2% and 41.8±3.8%, at the concentration of 300 µg/mL tetracycline. Furthermore, MIC_{90} was 180 µg/mL for non-resistant tetracycline strains, while the MIC_{50} was 300 µg/mL for resistant strains. However, the MIC values for non-resistant and resistant strains were respectively 99.7±0.4% and 47.5±3.2%, at the concentration of 1 µg/mL enrofloxacin. MIC_{90} for non-resistant strains to enrofloxacin was less than 0.2 µg/mL and MIC_{50} for resistant strains was about 0.9 µg/mL.

Discussion

Antibiotic resistance is one of the problems and obstacles of treating bacterial diseases in animals,

Table 1: Percent of growth inhibition of isolated non-resistant *Salmonella* to tetracycline in MIC test.

Positive samples	Concentration of tetracycline (µg/L)							
	300	150	75	37.5	18.75	9.38	4.68	2.34
1	99.9	89	80.9	66.1	60.3	49.3	30.8	25.8
2	97	90	84.1	74.4	70.2	60.9	54.7	40.5
3	100	89.7	83.5	79.9	76.4	65.1	62	58.9
4	99	96	89.8	83.5	80.6	72.5	69.3	65.2
5	100	96.5	94.3	91	89.9	80.8	79.9	77.1

Table 2: Percent of growth inhibition of isolated resistant *Salmonella* to tetracycline in MIC test.

Positive samples	Concentration of tetracycline (µg/L)							
	300	150	75	37.5	18.75	9.38	4.68	2.34
1	42.4	38.6	32.9	27.7	23.2	21.9	17.3	12.4
2	40	39.6	34.4	34.1	27.2	24.7	19.2	9.9
3	46	44.2	40.6	37.4	26.1	21.7	18.2	10.5
4	36.3	35.8	31.2	29.3	25.5	20	16	9.4
5	44.7	40	38	32.8	30	26.6	19.8	7.6

Table 3: Percent of growth inhibition of isolated non-resistant *Salmonella* to enrofloxacine in MIC test.

Positive samples	Concentration of enrofloxacine (µg/L)							
	1	0.5	0.25	0.125	0.06	0.03	0.015	0.007
1	100	99.9	97.6	97.4	96.6	95.7	91.7	97.1
2	100	99.2	98.7	97.1	96.6	94.9	92.9	95.2
3	99.9	98.3	97.2	96.6	95.7	90.9	90.6	90.3
4	99.8	98.8	97.9	96.9	95.4	92.6	91.9	88.3
5	98.9	98.4	97.7	97.1	96.6	98	96.9	90.5

Table 4: Percent of growth inhibition of isolated resistant *Salmonella* to enrofloxacine in MIC test.

Positive samples	Concentration of enrofloxacine (µg/L)							
	1	0.5	0.25	0.125	0.06	0.03	0.015	0.007
1	49.7	47.4	38.9	32	27.2	24.2	16.2	107
2	50	46.6	40	39.4	35.9	26.5	15.6	10.6
3	47.5	43.5	39.5	37.5	35	20.1	16.6	4.4
4	48.6	43.8	35.4	32.7	25.9	19	13.1	10
5	42	40	39.7	37.8	33.9	25.5	19.6	12.5

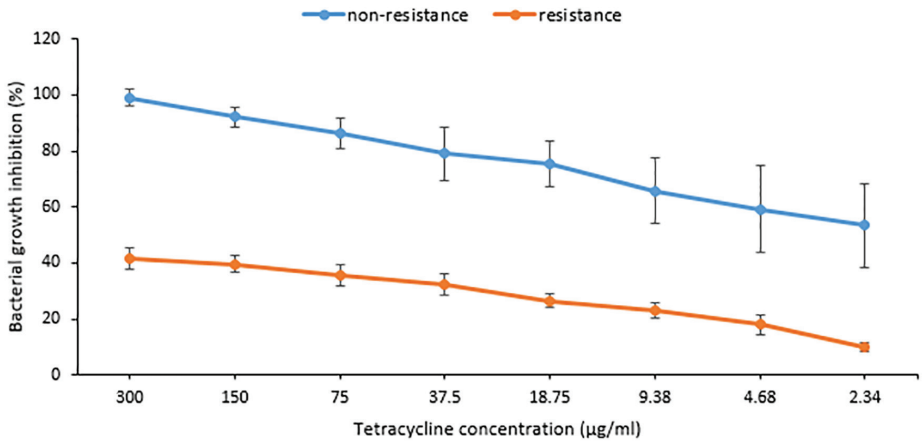

Figure 3: Mean percent of growth inhibition of resistant and non-resistant *Salmonella* to tetracycline.

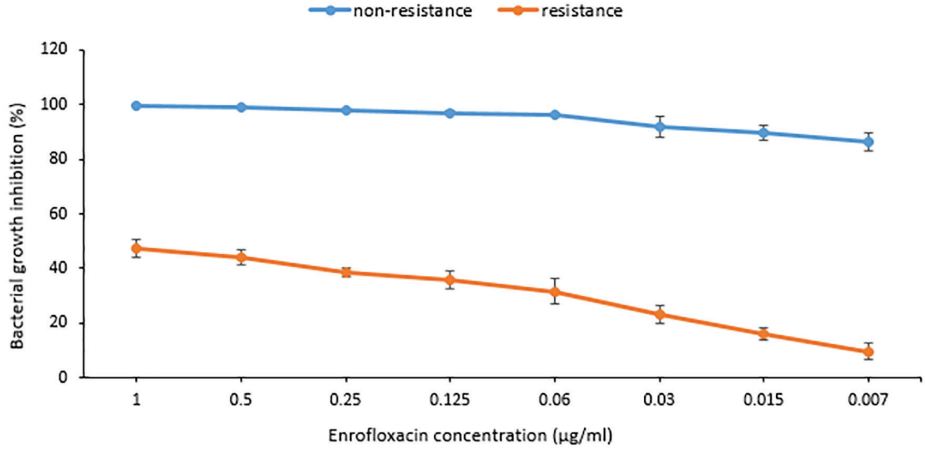

Figure 4: Mean percent of growth inhibition of resistant and non-resistant *Salmonella* to enrofloxacin.

poultry and humans. As such, in a short period of time, resistance to a new and effective antibiotic is problematic and practically its use becomes limited. This resistance has been mainly observed in human pathogens, the causes of common diseases between humans and animals and indigenous bacteria in food. According to a previous study, it was shown that antibiotic resistance was rapidly transferred to *Enterobacteriaceae*. In the event of contamination of foodstuff to the bacteria with multi-drug resistance, the resistance would be transferred to intestinal microflora, and this flora remained an antibiotic resistance reservoir (7).

In this study, the transmission pattern of antibiotic resistance was investigated in *Salmonella* isolated from slaughtered poultry carcasses in Shiraz slaughterhouse using a PCR assay. The results showed that the resistance of this pathogen to tetracycline (40%) was higher than that of enrofloxacin (None). In a previous study, antibiotic resistance in *Salmonella* isolates between 1980 and 1998 was increased from 20% to 40% (8). The increased antibiotic resistance in *S. typhimurium* isolated from chickens to ampicillin, tetracycline and chloramphenicol led to the emergence of multidrug resistance strains (9, 10).

In another study, the resistance of isolated *Salmonella* of dietary intake to tetracycline and enrofloxacin were 90% and 15%, respectively (2). Previous studies revealed that *Salmonella* was a major reservoir of antibiotic resistance in both human and animals. It was also demonstrated that 75% of *Salmonella* isolated from pigs were resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and kanamycin antibiotics. In human a record of resistance to 10 types of antibiotics was also remarkable finding (11). It was shown that a high proportion of *Escherichia coli* isolated from faeces and poultry carcasses remained resistant to tetracycline, streptomycin, ampicillin and sulfonamides. The occurrence of the resistances was directly associated with the rate of antibiotic use in the poultry industries both as preventive and growth-promoting factor. It was shown that 15.4% of isolated strains were able to transfer their resistance mobile genes to the laboratory strain of *Escherichia*

coli K12 (12).

In another study, *Salmonella enterica* isolated from chickens revealed the highest resistance to chloramphenicol, ampicilin and tetracycline, respectively, from which, 43.7% had multi-drug resistance and 10.7% had single-drug resistance (13). The extensive use of antibiotics for the purpose of treatment or prevention, led to a significant increase in the transferable plasmid antibiotic resistance. Use of enrofloxacin promoted the spread of strains with a low sensitivity to quinolones (14). It was demonstrated that the emergence of resistant strains of *S. enterica* to fluoroquinolones is increasing (15).

In this study, antimicrobial susceptibility to enrofloxacin and tetracycline was also assessed by applying MIC method. All strains isolated from *Salmonella* were susceptible to enrofloxacin, of which, MIC_{90} sensitivity to enrofloxacin for the isolated strains was less than 0.2 mg/mL. This was similar to the results of other studies on *Salmonella* isolated from poultry meat, as well as some *Salmonella* strains isolated from animal sources (5, 16). In addition, MIC_{90} sensitivity to tetracycline for the isolated strains was about 180 µg/mL. The unlimited use of antibiotics to prevent diseases and as growth promotion, can be considered as the main reasons for the emergence of antibiotic resistance. Due to the consumption of contaminated livestock products, resistant organisms in the human digestive system transmit the resistance factor to the natural flora of the digestive tract. When a bacterial disease containing resisting factor, the treatment process becomes difficult, the possibility of recovery decreases, and the dose of the drug intake raises. The development of antibiotic resistance in recent decades requires the cautious use of antimicrobial agents in veterinary and medical care, and there should be strategies to stop the increasing trend of antibiotic resistance.

Conclusion

Based on the results of this study, the prevalence of tetracycline resistance *Salmonella* was higher than enrofloxacin, which seems to be due to the extensive use of this antibiotic in the poultry industry. However, studies on the multi-drug antibiotic resistance mechanisms are advised.

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Conflict of Interest

None declared.

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