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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Prevalence of *Salmonella* infection in village chickens and determination of the tetracycline resistance genes in the *Salmonella* isolates in the Sistan region, Iran

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Abstract

Recently, an increasing number of multi drug resistant *Salmonella* species have been emerged due to overuse of antibiotics in veterinary and human medicine which has adverse consequences on public health. The present study was conducted with the aim of investigating the prevalence of *Salmonella* infection in village chickens in Sistan region and determining the prevalence of the antibiotic resistance genes in *Salmonella* isolated from these birds. In this study, 100 chickens were randomly selected from five counties of Sistan region. A cloacal swab sample was taken from each bird and also information about age, gender, breed, proximity with other birds, proximity with waterfowl, proximity with livestock, and receiving different antibiotics especially tetracycline were obtained using a questionnaire. Conventional culture methods used for *Salmonella* detection and isolation. Then, amplification of *invA* gene by PCR was used to confirm *Salmonella* colonies. Finally, 27 samples were confirmed to be infected with *Salmonella* by both culture and PCR methods. Disk diffusion method was used to determine the sensitivity to 4 antibiotics including; tetracycline, gentamicin, cefepime, and difloxacin. The results of the present study showed that proximity to waterfowl (OR = 0.273) significantly mitigates the risk of *Salmonella* infection. For the isolates, the highest resistance was recorded against cefepime and the highest susceptibility was to difloxacin. The presence proportion of *tet*A and *tet*B in tetracycline resistant isolates was higher than that in susceptible ones but this difference was not statistically significant.

Keywords Salmonellosis · Tetracycline resistance genes · Zoonotic transmission · East Middle

Introduction

Salmonella has worldwide distribution but some serotypes has more prevalence in some countries and is considered an important zoonotic pathogen. *Salmonella* is a Gram-negative bacillus, belonging to the family Enterobacteriaceae [1]. Avian salmonellosis can cause clinical disease in the poultry and also is a source of food borne transmission disease to humans [2]. Foodborne Salmonellosis cause gastroenteritis that can affect all ages, but the incidence, severity, and potential complications of the disease are higher in young children, the elderly, and immunocompromised people [3].

Antimicrobial resistant serotypes of *Salmonella* are increasingly common worldwide, both of human origin and also animal sources. These *Salmonella*s are now major risks to global health and food security [4]. Antimicrobial resistance can occur naturally, but the overuse of antibiotics in poultry, livestock, and humans is accelerating the process [5].

Tetracycline is widely used in poultry due to its cheapness, oral administration, and few side effects [6]. Tetracycline resistance genes are generally coded in plasmids and transposons and are transmitted through conjugation. However, in some isolates of bacteria the relevant genes are also found in the chromosome [7, 8]. The *tet* genes found at the highest

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frequency in gram-negative bacteria are related to efflux pumps, which are coded by the *tet*A, *tet*B, *tet*C, *tet*D, and *tet*G genes [9, 10]. Active efflux and ribosome protection are most widely distributed mechanisms of tetracycline resistance [7].

The Sistan region is located at the north of Sistan and Baluchestan province bordering Afghanistan country. Sistan and Baluchestan are the second largest province of Iran, situated in the southeast of the country. In rural areas of Sistan, poultry are mostly reared in Backyard or small household farms. Low biosecurity measures in backyard production systems cause the high risk of infectious diseases [11]. Also, arbitrary use of antibiotics by livestock farmers may lead to the antimicrobial resistance, the present study was conducted with the aim of determining the prevalence of *Salmonella* infection in village chickens of Sistan region and the effect of some factors of this infection. Also, determining the antibiotic resistance of *Salmonella* isolates and determining the prevalence of *tet* genes in these isolates were other aims behind this research.

Materials and work methods

Sampling procedures

The study population consisted of village chickens in 5 counties of Sistan region. The chicks originated from different breeder flocks. The sampling in this study was done in winter 2022, 100 chickens that are kept traditionally were randomly selected from different villages in the Sistan region, Southeast of Iran (20 birds from each county). Cloacal swab samples were collected by inserting sterile swab in the cloaca and turning it slowly several times to get the swab stained with feces, and then, the swab was placed in 5 ml of tryptic soy broth medium (Merck, Germany). Information about age, gender, breed, proximity with other birds, proximity with waterfowl, proximity with livestock, and receiving different antibiotics especially tetracycline in the last months were obtained using a questionnaire. The samples were placed in a container near the ice and transferred to the microbiology laboratory of the Veterinary Faculty of Zabol University within 6 h at most.

Microbiological methods

Bacterial culture

The TSB media for 24 h incubated at 37 °C and then cultured in SS agar, McConkey agar, and eosin methylene blue (EMB) media (Merck, Germany). In other to confirm the isolates as *Salmonella*, the bacteria were subcultured in urea, TSI, Simon citrate, lysine decarboxylase, and SIM media (Merck, Germany), and biochemical reactions and color changes in the media were checked.

DNA extraction

The samples that were culture positive for *Salmonella* were selected for molecular testing. The boiling method was used to extract DNA. First, a small amount of bacteria suspected to be *Salmonella* was scraped into 5 ml of LB medium in a sterile tube. The LB media for 24 h were incubated at 37°C and then were centrifuged at 4800 rpm for 5 min at room temperature, the supernatant was eliminated, and remained sediment was mixed with PBS to a 1.5 ml microtube. Then, the microtube was centrifuged at 4800 rpm for 5 min at room temperature and the supernatant was eliminated. Then this step was repeated for one more time. In the next step, 200 µL of TE buffer was added to the sediment, and the microtube was placed in a thermomixer (Eppendorf, Germany) for 10 min at 95°C. Then, the microtubes were centrifuged at 15000 rpm for 10 min. Finally, the contents were transferred to a new microtube and stored in a freezer at −20°C until the next use.

PCR procedure

Amplification of *invA* gene by PCR was used to confirm *Salmonella* suspected samples. The program used in the thermocycler PCR device (Eppendorf, Germany) for *invA* gene was one cycle of predenaturation at 95°C for 5 min following that, 35 thermal cycles, including denaturation at 94°C for 1 min, primer annealing at 56°C for 30 s, and extension at 72°C for 30 s; after completing these cycles, a final extension was performed at 72°C for 10 min.

Also, multiplex-polymerase chain reaction was used in order to identify the *tet*A, *tet*B, *tet*C, and *tet*D genes. The master mix solution was purchased from Pishgam Industrial Company, Iran. The program used in the thermocycler PCR device for *tet*A, *tet*B, *tetC*, and *tet*D genes was one cycle of predenaturation at 94°C for 5 min and then 35 thermal cycles including denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension cycle at 72 °C for 5 min [12]. The primers used in this study are shown in Table 1 (Pishgam, Iran). The known clinical isolates of *tet*A, *tet*B, *tet*C, and *tet*D genes producing *Salmonella* were used as the quality control, and double-distilled water as the negative control instead of DNA in PCR.

The PCR products were electrophoresed with 1% agarose gel which had been made by TAE buffer; then, it was stained with ethidium bromide (CinnaGen, Iran) and subsequently visualized by a Gel Doc Machine (Cambridge, Germany).

Determination of antibiotic resistance of isolates

Four antibiotics including tetracycline (30 µg), gentamicin (10 µg), cefepime (30 µg), and difloxacin (25 µg) were used for determination of antimicrobial resistance of isolates

Table 1 Nucleotide sequence of primers used in the present study

Gene	Primer type	Primer sequences (5′ to 3′)	Product length	Source
InvA	Forward	GTGAAATTATCGCCACGTTCGGGCAA	285 bp	[13]
	Reverse	TCATCGCACCGTCAAAGGAACC		
*tet*A	Forward	CGCCTTTCCTTTGGGTTCTCTATATC	182 bp	[12]
	Reverse	CAGCCCACCGAGCACAGG		
*tet*B	Forward	GCCAGTCTTGCCAACGTTAT	975 bp	[12]
	Reverse	ATAACACCGGTTGCATTGGT		
*tet*C	Forward	TTCAACCCAGTCAGCTCCTT	560 bp	[12]
	Reverse	GGGAGGCAGACAAGGTATAGG		
*tet*D	Forward	GAGCGTACCGCCTGGTTC	780 bp	[12]
	Reverse	TCTGATCAGCAGACAGATTGC		

(Hamoon Teb, Iran). Agar disk diffusion test was used to identify the antibiotic resistance profile of isolates [14]. According to the instructions of the Clinical and Laboratory Standards Institute [15], disk diffusion method on Mueller Hinton agar was used. Briefly, the antibacterial paper discs were placed on agar, and after 24 h of incubation at 37°C, the diameter of the growth inhibition zones was measured to determine the resistance of *Salmonella* isolates.

Statistical analysis

Since, the response variable (*Salmonella* infection) was a dichotomous (Yes/No) nominal one. Therefore, the logistic regression model was used for statistical analysis. In the first stage, the relationship between each explanatory variable with the *Salmonella* infection was determined using chi square or Fisher's exact tests. The related variables to *Salmonella* infection (*P*-value at univariate analysis<0.25) were used in the multivariate logistic regression model. For similar variables, collinearity was assessed using the chi-square test. Variables with a significant *P*-value on this test ($P<0.05$) were considered collinear, and only the variable that was more closely related to the *Salmonella* infection were entered in the model. All the explanatory variables were considered categorical and entered in a single step in the regression model.

The relationship between the presence of *tet* genes and resistance to different antibiotics was investigated using the chi-square likelihood ratio test. The SPSS version 26 software was used for data analysis. A significant level of $P<0.05$ was considered statistically.

Results

In the present research, out of 100 samples examined, 31 samples were positive for *Salmonella* by using the conventional culture methods, of which 27 samples (27%, 95% CI: 18.6–36.8%) were confirmed positive by the PCR method. The prevalence of *Salmonella*-infected chickens according to risk factors is specified in Table 2.

A logistic regression model was fitted with four variables for factors associated with *Salmonella* infection. Due to the collinearity of proximity to waterfowl and proximity to other birds, only proximity to waterfowl was used in the model. The model showed that among studied chickens, being 1 year or less (OR=1.683) increase and being male (OR=0.332) and being around waterfowl (OR=0.233) decrease the risk of getting *Salmonella* infection. Also, the risk of *Salmonella* infection in Nimrooz, Zabol, Zahak, and Hamoon was lower than in Hirmand (all odds ratios<1), but only the proximity to waterfowl was significantly related to *Salmonella* infection (Table 3).

The explanatory variables included in the model express 0.226 of the variation of the dependent variable (Nagelkerke R Square = 0.226)

Figures 1, 2, 3, and 4 show agarose gel electrophoresis of PCR products amplified by studies primers. PCR products related to *InvA*, *tet*A, *tet*B, and *tet*C genes were single DNA fragments of 285, 182, 975, 560, and 780 bp, respectively.*tet*A, *tet*B, *tet*C, and *tet*D genes were detected in 24, 6, 6, and 0 (zero) isolates out of a total 27 *Salmonella* isolates. The sensitivity of these isolates to tetracycline according to the presence of *tet* genes is shown in Table 4. As can be seen in this table, the relationship between presence of *tet* genes and susceptibility of isolates to tetracycline was not statistically significant.

The susceptibility of *Salmonella* bacteria isolates to different antibiotics is shown in Diagram 1. For the isolates, the highest resistance was recorded against cefepime and the highest susceptibility was to difloxacin.

Discussion

Nowadays, we are witnessing an increase in high-resistance and multi-resistance *Salmonella* strains, which is the result of the indiscriminate use of antimicrobials in both

Table 2 Univariate analysis of association between explanatory variables with *Salmonella* infection

Variable[1]	Levels	Number of tested animals	Number of positive animals	Prevalence	Statistical test	*P-value*
Location (counties)	Nimrooz	20	4	20%	Pearson chi square	0.208
	Zabol	20	2	10%		
	Zahak	20	8	40%		
	Hamoon	20	6	30%		
	Hirmand	20	7	35%		
Age	One year or less	76	24	31.6%	Pearson chi square	0.066
	More than 1 year	24	3	12.5%		
Sex	Male	18	2	11.1%	Fisher's exact test	0.142
	Female	82	25	30.5%		
Proximity with other birds	Yes	60	13	21.7%	Pearson chi square	0.141
	No	40	14	35.0%		
Proximity with waterfowl	Yes	48	7	14.6%	Pearson chi square	0.007
	No	52	20	38.5%		
Proximity with Livestock	Yes	85	24	28.2%	Fisher's exact test	0.753
	No	15	3	20.0%		
Receiving antibiotics in the last month	Yes	10	3	30.0%	Fisher's exact test	1.000
	No	90	24	26.7%		
Receiving tetracycline in the last month	Yes	10	3	30.0%	Fisher's exact test	1.000
	No	90	24	26.7%		

[1]Breed of all the chickens was heritage; therefore, this variable was removed from statistical analysis

Table 3 Factors associated with *Salmonella* infection in multivariate logistic regression model; *RC*, reference for comparison

Variable and level		*B*	Wald	*P*-value	Adjusted odds ratio
Location (counties)			5.146	0.273	
	Nimrooz	−1.512	3.219	0.073	0.220
	Zabol	−1.777	3.331	0.068	0.169
	Zahak	−0.674	0.710	0.400	0.510
	Hamoon	−0.991	1.625	0.202	0.371
	Hirmand (RC)	0			1
Age	One year or less	0.521	0.453	0.501	1.683
	More than 1 year (RC)	0			1
Sex	Male	−1.103	1.595	0.207	0.332
	Female (RC)	0			1
Proximity with waterfowl	Yes	−1.455	5.779	0.016	0.233
	No (RC)	0			1

humans and animals [16]. It is estimated that over 607 tons of antibiotic active ingredient were consumed in poultry farms in Iran in 2010. Tetracyclines have the highest rate of consumption in both livestock and poultry farms of the country. Tetracyclines are used for therapeutic, prophylactic and growth promotion purpose [17].

The *tet* genes contribute to tetracycline resistance in *Salmonella* bacteria. The *tet*A, tetB, *tet*C, and *tet*D genes were detected in 89%, 22%, 22%, and 0% of *Salmonella* isolates in the present research. The presence proportion of *tet*A in tetracycline resistant isolates was higher than that in susceptible ones (100% of the resistant isolates contained the *tet*A gene, while 86% of the susceptible isolates contained the *tet*A gene). But this difference was not statistically significant. The same relationship was also observed in the case of *tet*B gene.

In the present study, 3%, 15%, 33%, and 100% of *Salmonella* isolates were resistance to difloxacin, tetracycline,

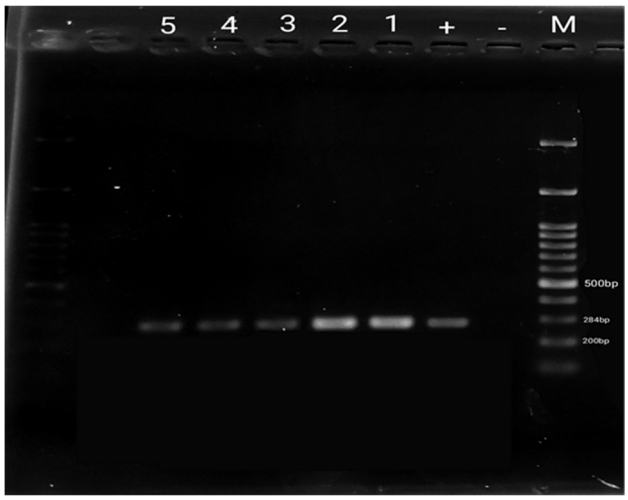

Fig. 1 PCR reactions for detection of *InvA* gene. *InvA* characterized by identifying a fragment of 284 bp

Fig. 2 PCR reactions for detection of *tet*A gene. *tet*A characterized by identifying a fragment of 182 bp

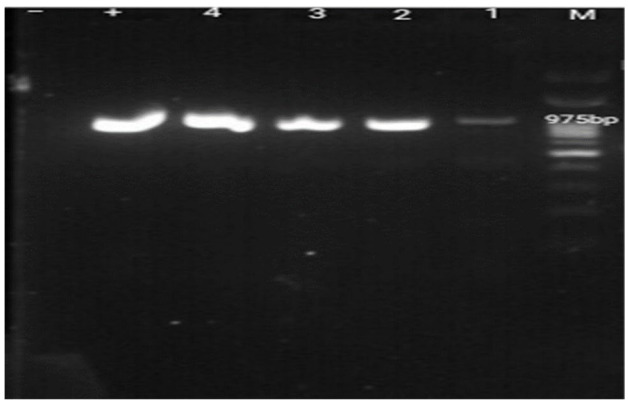

Fig. 3 PCR reactions for detection of *tet*B gene. *tet*B characterized by identifying a fragment of 975 bp

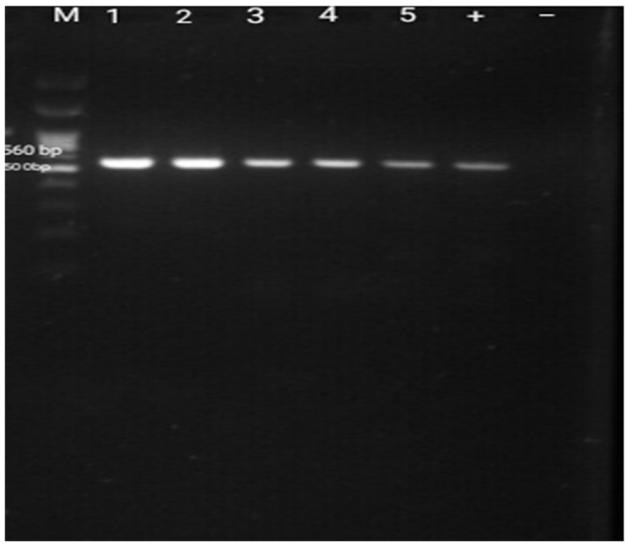

Fig. 4 PCR reactions for detection of *tet*C gene. *tet*C characterized by identifying a fragment of 560 bp

gentamicin, and cefepime, respectively. A meta-analysis on the prevalence of fluoroquinolone-resistant *Salmonella* serotypes in Iran shows that the resistance rate to ciprofloxacin in *Salmonella* strains was low (2.9%) [18]. The fluoroquinolones are a class of potent orally absorbed antimicrobial agents that have proved effective in many infections especially gram-negative infections [19]. The results obtained in the present study revealed that resistance of *Salmonella* isolates to difloxacin (a fluoroquinolone antibiotic) is less than other studied antibiotics.

In the present study, the prevalence of *Salmonella* infection was 27% by serial testing with culture and PCR methods. In the villages of Sistan region, chickens are reared together with other poultry and livestock. A few chickens (usually between 5 and 50 ones) are kept in each yard and these chickens are generally of indigenous breeds and different ages. Housing in this system is a cage or a brick room where animals are kept at night. Chicken feed is mostly poured on the soil or on a tray. Poor hygiene and lack of quarantine can cause of the high prevalence of *Salmonella* infection in the present research.

In a study on broiler chickens in Kerman (another eastern province of Iran), poultry manures were collected from commercial broiler houses that *Salmonella* was isolated from 13 out of 110 poultry houses (12%) using conventional culture methods [20]. While in the present study, the prevalence of *Salmonella* infection by culture method was 31%. In another study that was conducted in 21 cities of Iran, fecal samples were collected from poultry farms and microbiological cultivation was used for *Salmonella* isolation that was revealed 7.5% of fecal samples were infected with *Salmonella* [21]. The less prevalence of *Salmonella* infection in these studies

Table 4 Antimicrobial susceptibility to tetracycline (TE30) depending on the presence or absence of the *tet*A, *tet*B, *tet*C, and *tet*D genes, among 27 isolates of *Salmonella*. The table percentages were calculated in the columns

Antimicrobial agent	Susceptibility	All *Salmonella* isolates	*Salmonella* isolates containing *tet* genes			
			*tet*A	*tet*B	*tet*C	*tet*D
TE30	Susceptible (*n*=21)	21 (77.8%)	18 (75%)	4 (66.7%)	6 (100%)	0 (-)
	Intermediate (*n*=2)	2 (7.4%)	2 (8.3%)	1 (16.7%)	0 (0%)	0 (-)
	Resistant (*n*=4)	4 (14.8%)	4 (16.7%)	1 (16.7%)	0 (0%)	0 (-)
Statistical association between TE30 and *tet*s			0.447[1] (ns[2])	0.643 (ns)	0.176 (ns)	-[3]
Number of isolates with *tet* genes		27	24	6	6	0

[1] *P*-value in chi-square likelihood ratio test

[2] *NS*, non-significant

[3] Due to the lack of data in this column, it is impossible to perform a statistical test

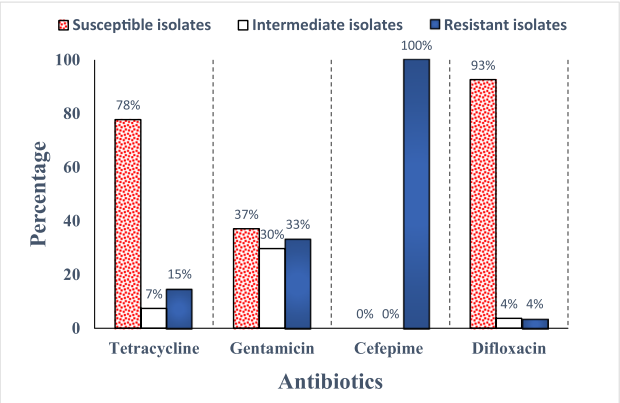

Diagram 1 The susceptibility of *Salmonella* bacteria isolates to different antibiotics

might be due to better biosecurity implementation in industrial farming compared to backyard production system.

In the present study, it was found that proximity with waterfowl mitigate the risk of *Salmonella* infection. Although in many studies, the prevalence of *Salmonella* infection in waterfowl was higher than that of chickens [22–24]. However, some studies have shown that the prevalence of *Salmonella* in waterfowl is lower than that in chickens. Including, in a study that was conducted in Egypt, the samples were collected from internal organs of chickens, ducks, turkeys, quail, pigeons, and geese that 12%, 10%, 10%, 10%, 7%, and 0% of chickens, ducks, turkeys, quails, pigeons, and geese respectively were positive using by bacteriological and serological tests [25]. In another study, cloacal swabs were collected from different live poultry species including broilers, breeders, layers, turkeys, and ducks, beside litter samples from various poultry farms. That 17%, 10%, 2%, 6%, and 2 % in broilers, breeders, layers, ducks, and turkeys respectively were positive for *Salmonella* by bacteriological and serological tests also *Salmonella* Kentucky were found in 53%, 67%, and 29% in broiler's, breeder's, and duck's litters respectively [26]. In a study on Texas Gulf coast, it was revealed that waterfowl during the fall hunting season have a minimal risk of *Salmonella* infection [12, 27].

A limitation of this study was lack of sampling of waterfowls for detection of *Salmonella*. Future studies could investigate *Salmonella* infection in other birds in the Sistan region and compare results with chicken of selected sample. According to our investigations, no research has been published on the effects of proximity with waterfowl on chicken *Salmonella* infection to date. Determining the underlying cause of this relationship requires further investigations.

Indiscriminate use of antibacterial agents can lead to increasing resistant strains. Resistant gens mostly located on plasmids which can transmitted to other bacteria and can easily transfer to human through the food chain [28] By identifying the types of gens responsible for resistant more effective approaches maybe develop to reduce antimicrobial resistance and improve efficiency of antibiotics.

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Data availability The datasets generated during the current study are not publicly available due to respect for people's privacy but are available from the corresponding author on reasonable request.

Declarations

Ethics approval This is an observational study. The protocol of the present research was reviewed and approved by vice chancellor for academic affairs of the Veterinary Faculty of Zabol University and the vice chancellor has confirmed that no ethical approval is required.

Consent to participate This is an observational descriptive study and Informed consent is not applicable in this research.

Consent for publication This manuscript does not include any individual data or sensitive personal information; therefore, consent to publication is not applicable in this case.

Competing interests The authors declare no competing interests.

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