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Study of resistance to cephalosporin compounds in *Salmonella spp.* and *Salmonella* Typhimurium isolated from turkeys

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A B S T R A C T

The high consumption of antimicrobial agents in livestock is a global problem that can increase the antibiotic resistance of human and animal bacteria such as *Salmonella* serotypes. This study used cloacal swab to isolate *Salmonella spp.* from turkeys in Zabol, Iran. The isolated bacteria were evaluated by polymerase chain reaction to confirm the bacteria and to identify some resistance genes. Disk diffusion test on Mueller-Hinton agar was used to determine the susceptibility of the isolated bacteria to cephalosporin compounds. A total of 33 *Salmonella spp.* Including 19 *Salmonella* Typhimurium were identified by polymerase chain reaction. Multiple drug resistance (MDR) to cephalosporin compound was observed in *Salmonella* serotypes. Antibiotic resistance profiles of *Salmonella* serotypes were not statistically associated with $bla_{CTX\text{-}M\text{-}1}$ and *int*1 resistance genes ($p > 0.05$). *S.* Typhimurium and other *Salmonella* serotypes did not show significantly different antibiotic susceptibility to the tested antibiotics ($p = 0.536$). According to the results, there is a high prevalence of resistance to cephalosporin compounds among *Salmonella* spp. and *S.* Typhimurium in Zabol, Iran.

Keywords: *Cephalosporins, Resistance genes, Salmonella.*

1 Introduction

Some serotypes of *Salmonella spp.* are considered important pathogens for the poultry production industry. *S. Pullorum* and *S. Gallinarum* are host-specific and cause severe economic losses in chicks and poults. Motile *Salmonellae*, like *S.* Typhimurium, are responsible for poultry paratyphoid infection and cause human foodborne diseases. Adult turkeys do not show the clinical signs of *Salmonella* and serve as asymptomatic carriers. *Salmonella*-infected poultry are the most important reservoirs of *Salmonella* that can transmit the pathogen to humans through the food chain (1). The most important *Salmonella* reservoirs are domestic animals, poultry, and pigs (2). The distribution of *Salmonella* serotypes from poultry sources varies geographically and changes over time.

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Reports indicate that the prevalence of *S.* Typhimurium is increasing (3). Carrier animals probably play a major role in spreading infection among the herds and act as the source of infection for humans and food contamination (4). Antibacterial compounds are used in poultry farming for various purposes, including clinical purposes. Only a few countries currently use antimicrobial agents for growth-promoting purposes (5). Uncontrolled and long-term use of antibiotics in animals has led to increased antibiotic resistance (5-7). The increasing consumption of these compounds leads to the development of strains of microorganisms resistant to various mechanisms (8-11). One way of developing antimicrobial resistance is to produce antibiotic-degrading enzymes such as beta-lactamases (12, 13). Beta-lactamases are encoded by extended-spectrum beta-lactamase (ESBL) genes such as $bla_{CTX\text{-}M}$ (12). ESBLs are often encoded on large plasmids, which can be exchanged between the bacterial species and the strains (14). ESBL-producing strains mainly include Klebsiella pneumonia and E. coli (15). Clinical cases of resistance to β-lactams have been observed across the world due to excessively administering β-lactam antibiotics for treating infections associated with *Enterobacteriaceae* (16).

The prevalence of resistance genes varies across geographic regions and appears to be closely associated with the rate and type of antibiotics used in those regions (17). Although many studies have been conducted on integrons and ESBL genes in bacteria isolated from humans and animals, there has yet to be a report on the level of cephalosporin resistance and the prevalence of *S.* Typhimurium resistance genes. Thus, this study investigated cephalosporin resistance and determined the prevalence of some resistance genes in *S.* Typhimurium isolated from turkeys in the Zabol region, north of Sistan and Baluchestan Province, Iran.

2 Materials and Methods

2.1 Samples

These bacteria were isolated through cloacal swab preparation from randomly selected turkeys in Zabol, Iran. These turkeys were kept in villages as backyard flocks. Cloacal swabs were prepared in Selenit-F and were transferred to the Laboratory of Microbiology of the University of Zabol. For Salmonella isolation, the primary enrichment of samples in selenite-F at 37 °C for 24 hours was followed by subculture on MacConkey and Salmonella-Shigella agar. The organisms were identified by bacteriological methods (18).

2.2 DNA extraction of bacteria

The boiling method was used to extract the DNA of bacteria. First, pure *Salmonella* colonies grown on eosin methylene blue agar or MacConkey agar media (Merck, Germany) were removed by a sterilized needle and inoculated in test tubes containing 5 ml of Luria-Bertani broth medium. After 18-26 hours of incubation at 37 °C, the tubes were centrifuged at 4800-5000 rpm at room temperature for five minutes. After decanting the supernatant, the pellet was resuspended in 1 ml of double-distilled water, transferred to a 1.5 ml microtube, and centrifuged at 4800-5000 rpm at room temperature for five minutes. Then, 200 µl of double-distilled water was added to the precipitate and the microtubes were placed in a thermomixer (Eppendorf, Germany) at 95 °C for 10 minutes. In the next stage, the microtubes were centrifuged at 15000 rpm for 10 minutes, and the supernatant containing DNA was finally transferred to 200 µl microtubes and stored at -20 °C until use (19).

2.3 Polymerase chain reaction (PCR)

Specific primers of *invA* and *fliC* genes in multiplex PCR were used to identify *Salmonella spp.* and *S.* Typhimurium, respectively (20). Bacteria that were negative in terms of *the invA* gene were removed, and the rest of the samples underwent a second round of PCR to identify $bla_{CTX\text{-}M\text{-}1}$ (21) and *int*1 (22) genes using specific primers. *S. Typhimurium ATCC 14028 was used as a positive control and double-distilled water instead of DNA as the negative control*. Pishgam Company, Tehran, Iran, provided the primers used in this study. The nucleotide sequences of the primers used to identify *invA*, *fliC*, *int*1, and $bla_{CTX\text{-}M\text{-}1}$ genes are shown in Table 1. PCR was performed at a volume of 25 µl containing 12 µl of 2X Master Mix (2X PCR Master Mix Red; Pishgam, Iran), three µl of DNA, one µl of forward primers, one µl of reverse primers, and eight µl of sterilized distilled water (6 µl in multiplex PCR for *invA* and *fliC* genes). The applications used in the thermocycler (Eppendorf, Germany) to replicate the *invA*, *fliC*, *int*1, and $bla_{CTX\text{-}M\text{-}1}$ genes are shown in Table 2. Notably, 1.5% agarose was used for electrophoresis of PCR products and ethidium bromide (CinnaGen, Iran) was used to stain the genes loaded on the gel.

Table 1. Nucleotide sequence of primers used to detect invA, fliC, int1 and blaCTX-M-1 genes

.Target gene	Sequences (5'-3')	PCR product size (bp)	References
invA	*GTGAAATTATCGCCACGTTCGGGCAA* *TCATCGCACCGTCAAAGGAACC*	284	Jamshidi et al. 2009
fliC	*CGGTGTTGCCCAGGTTGGTAAT* *ACTCTTGCTGGCGGTGCGACTT*	559	Jamshidi et al. 2009
int1	*CAGTGGACATAAGCCTGTTC* *CCCGAGGCATAGACTGTA*	160	Li et al. 2012
$bla_{CTX-M-1}$	*ATGTGCAGYACCAGTAARGT* *TGGGTRAARTARGTSACCAGA*	593	Pagani et al. 2003

Table 2. The steps used in thermocycler device for replication of invA, fliC, int1, and blaCTX-M-1 genes

Step	Temperature and time span			Number of cycles
	invA/fliC	*int1*	$bla_{CTX-M-1}$	
Initial Denaturation	95°C 5 min	94°C 5 min	94°C 7min	1 Cycle
Denaturation	94°C 1 min	94°C 30 sec	94°C 50 sec	
Annealing	56°C 30 sec	55°C 30 sec	50°C 40 sec	35 Cycle
Extension	72°C 30 sec	72°C 1 min	72°C 1min	
Final Extension	72°C 10 min	72°C 10 min	72°C 5min	1 Cycle

2.4 Determining the susceptibility to cephalosporins

The disk diffusion method on Muller-Hinton agar medium was adopted to determine the susceptibility of *Salmonella* strains and *S.* Typhimurium to cephalosporin compounds. First, 4-6 ml of sterilized saline 0.9% was added to sterile tubes. A sterilized needle was then used to dissolve some bacterial colonies in the saline solution to obtain tube turbidity equal to 0.5 McFarland. These tubes were finally used for bacterial culture in the Mueller Hinton agar medium. The antibiotic disks used in this study were procured from Padtan Teb Company, Tehran, Iran and contained cefazolin (30 µg), cefalotin (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), cefixime (5 µg), and cefepime (30 µg). After placing antimicrobial disks on the culture media, the plates were incubated at 37 °C for 24 hours. The plates were then removed from the incubator, and the diameters of the zones created around the disks were precisely measured. Based on the Clinical and Laboratory Standards Institute guidelines (23), the antibiograms of the isolated bacteria were classified as susceptible, intermediate, and resistant to antibiotics.

2.5 Statistical analysis

Data was analyzed using SPSS 26 software. Fisher's exact test was applied to compare the presence of genes between *S.* Typhimurium and non-*S.* Typhimurium bacteria. Moreover, the likelihood-ratio chi-square statistic was conducted to investigate the relationship between the presence of genes and the susceptibility to the studied antibiotics. Moreover, the average diameter of the inhibition zone by cephalosporin compounds was compared between *S.* Typhimurium and non-*S.* Typhimurium isolates using the between-subject test of repeated measures ANOVA. The

significance level was set at $p < 0.05$. IBM SPSS Statistics Version 26 was used for data analysis.

3 Results

In this study, 33 *Salmonella spp.*, including 19 *Salmonella* Typhimurium, was identified by polymerase chain reaction (Figure 1). As shown in Table 3 and Table 4, the antibiotics resistance profiles for $bla_{CTX\text{-}M\text{-}1}$ and non-$bla_{CTX\text{-}M\text{-}1}$ genes, and *int*1 and non- *int*1 genes was not significant for *Salmonella* serotypes ($p > 0.05$). Detected *int*1 and $bla_{CTX\text{-}M\text{-}1}$ genes by PCR are shown in Figure 2 and Figure 3. The results showed multiple drug resistance (MDR) in 15.15% (5 out of 33) of *Salmonella* serotypes. The *int*1 and $bla_{CTX\text{-}M\text{-}1}$ gene frequencies for *Salmonella spp.* were 96.96% (32 out of 33) and 75.75% (25 out of 33), respectively. A statistically significant difference was not seen in the prevalence of *int*1 (p = 0.424) and $bla_{CTX\text{-}M\text{-}1}$ (p = 0.238) genes between *S.* Typhimurium and other *Salmonella* serotypes.

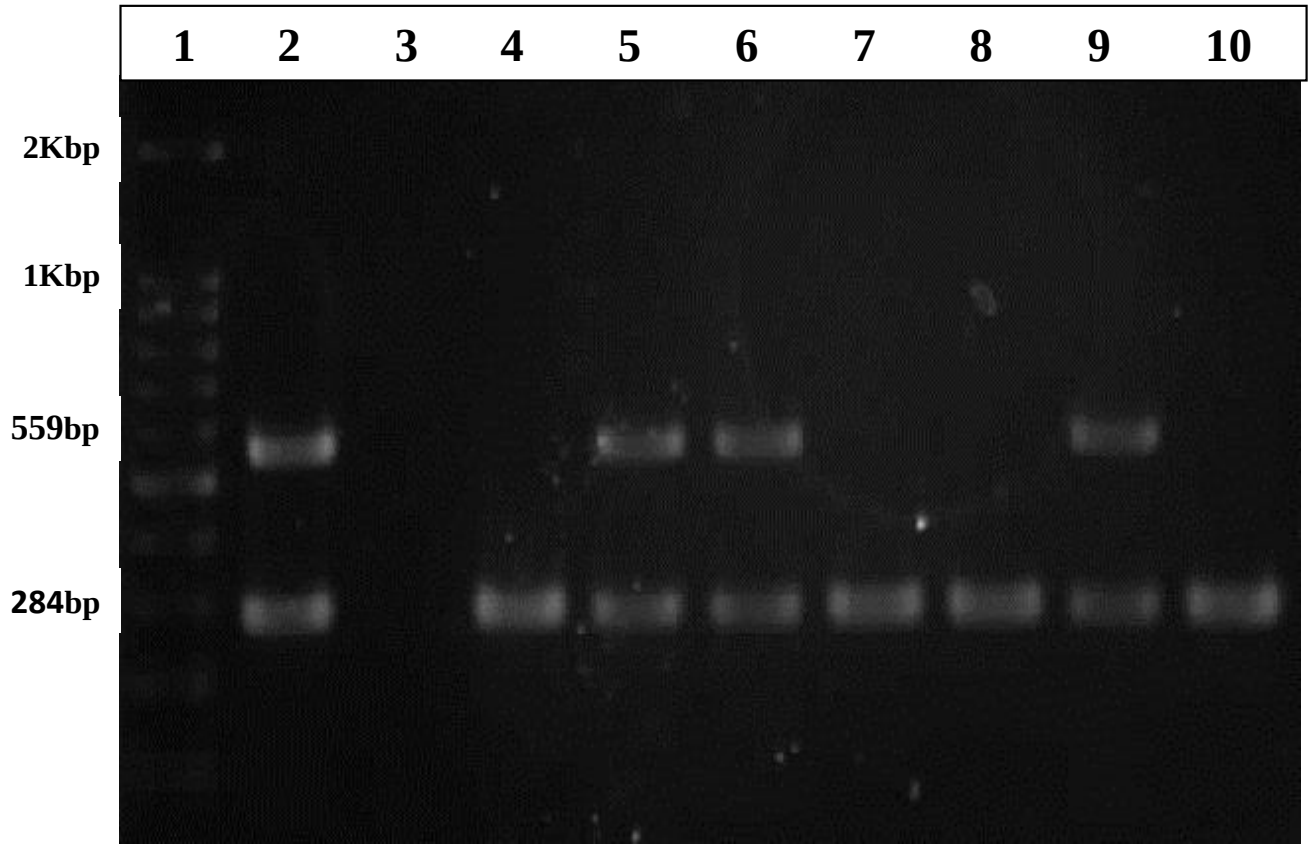

Figure 1. Multiplex PCR test results using primers specific for invA and fliC genes (Column 1: 100 bp DNA ladder, Column 2: positive control (Salmonella Typhimurium), Column 3: negative control, Columns 4–10: Salmonella samples, Columns 5, 6 and 9: S. Typhimurium samples)

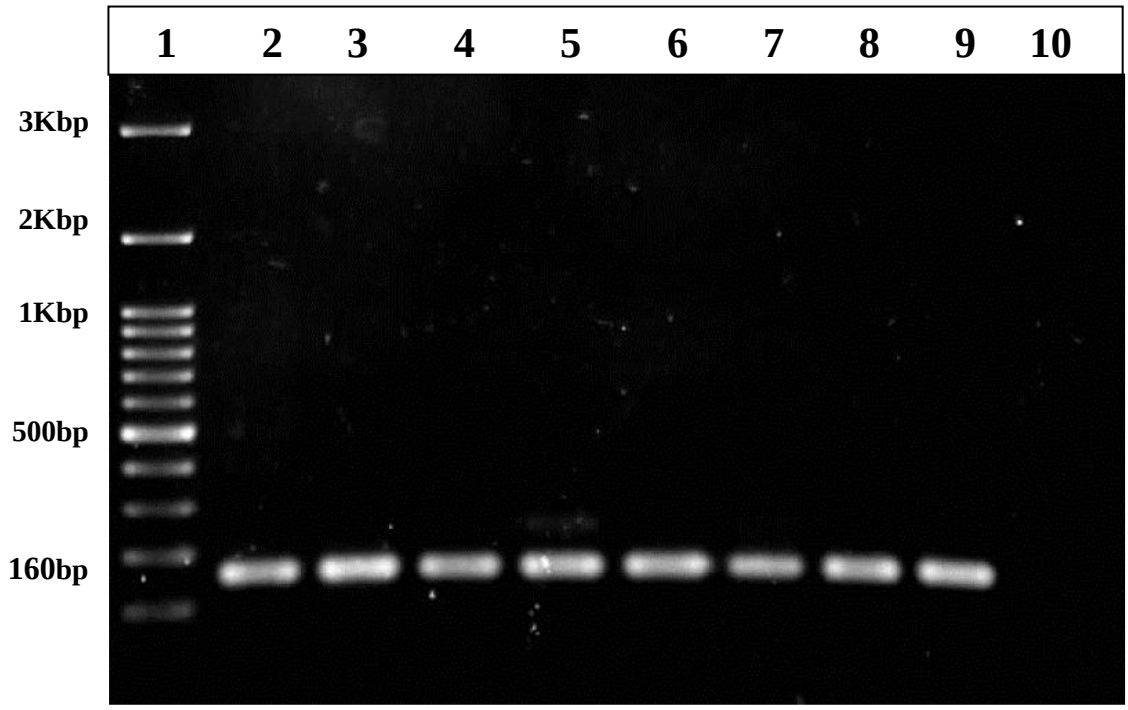

Figure 2. PCR test result for *int1* gene of *Salmonella (*Column 1: 100 bp DNA ladder, Column 2: positive control, Column 10: negative control, Columns 3-9: positive samples)

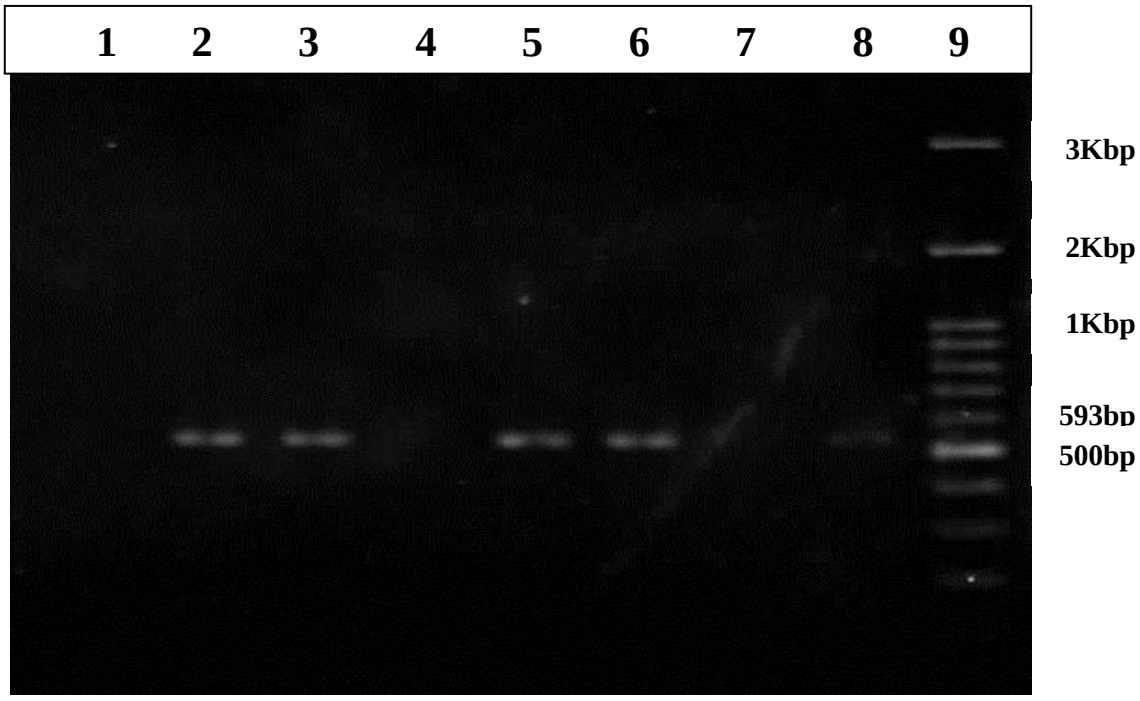

Figure 3. PCR test result for blaCTX-M-1 gene of Salmonella (Column 9: 100 bp DNA ladder, Column 8: positive control, Column 7: negative control, Columns 2, 3, 5, and 6: positive samples, Columns 1 and 4: negative samples)

Table 3. Antibiotic resistance profiles for blaCTX-M-1 and non-blaCTX-M-1 Salmonella isolates

Antibiotic	*bla*$_{CTX-M-1}$ positive isolates			*bla*$_{CTX-M-1}$ negative isolates			*P*-Value
	S[1] (%)	I[2] (%)	R[3] (%)	S (%)	I (%)	R (%)	
Cefazolin	14 (56)	7 (28)	4 (16)	5 (62/5)	3 (37/5)	0 (0)	0.296
Cefalotin	19 (76)	2 (8)	4 (16)	6 (75)	2 (25)	0 (0)	0.178
Cefoxitin	18 (72)	3 (12)	4 (16)	7 (87/5)	0 (0)	1 (12/5)	0.386
Cefteriaxone	18 (72)	7 (28)	0 (0)	7 (87/5)	1(12/5)	0 (0)	0.349
Cefexime	19 (76)	0 (0)	6 (24)	8 (100)	0 (0)	0 (0)	0.053
Cefepime	25 (100)	0 (0)	0 (0)	8 (100)	0 (0)	0 (0)	-[4]

1-Susceptible; 2-Intermediate; 3-Resistant; 4- It was impossible to perform a statistical test.

Table 4. Antibiotic resistance profiles for int-1 and non-int-1 Salmonella isolates

Antibiotic	*int*-1 positive isolates			*int*-1 negative isolates			*P*-Value
	S[1] (%)	I[2] (%)	R[3] (%)	S (%)	I (%)	R (%)	
Cefazolin	19 (59)	10 (31)	3 (9)	0 (0)	0 (0)	1 (100)	0.107
Cefalotin	24 (75)	4 (12.5)	4 (12.5)	1 (100)	0 (0)	0 (0)	0.754
Cefoxitin	24 (75)	3 (9)	5 (16)	1 (100)	0 (0)	0 (0)	0.754
Cefteriaxone	25 (78)	7 (22)	0 (0)	0 (0)	1 (100)	0 (0)	0.087
Cefexime	26 (81)	0 (0)	6 (19)	1 (100)	0 (0)	0 (0)	0.523
Cefepime	32 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	-[4]

1-Susceptible; 2-Intermediate; 3-Resistant; 4- It was impossible to perform a statistical test.

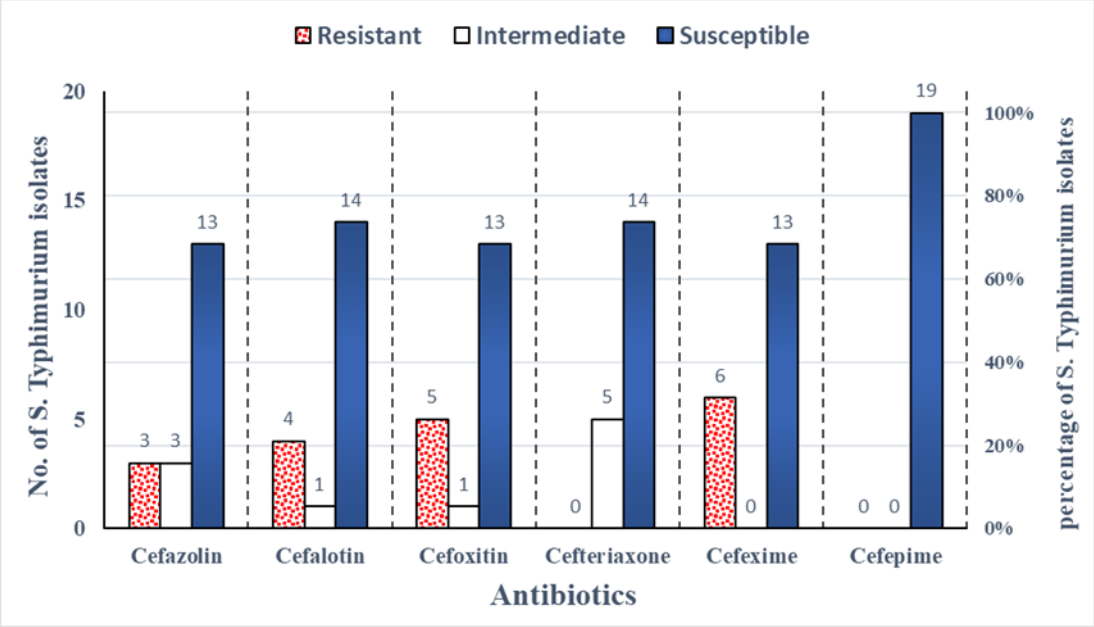

Figure 4. Sensitivity of Salmonella Typhimurium isolates to various cephalosporin compounds

Results of sensitivity analysis of *S.* Typhimurium to cephalosporin compounds are shown in Figure 4. As seen, the rates of susceptibility of *S.* Typhimurium to cefazolin, cefalotin, cefoxitin, ceftriaxone, cefixime and cefepime were 68.4%, 73.7%, 68.4%, 73.7%, 68.4%, and 100%, respectively. The corresponding rates for other *Salmonella* serotypes were 42.9%, 78.6%, 85.7%, 78.6%, 100% and 100%, respectively. The average diameter of the inhibition zone by the tested antibiotics between *S.* Typhimurium and other *Salmonella* serotypes to the tested antibiotics was not significantly different (p = 0.536).

4 Discussion

In this study, the resistance rate to cefazolin, cefalotin, cefoxitin and cefixime among the obtained *S.* Typhimurium isolates was 15.8%, 21.1%, 26.3%, and 31.6 respectively. Since no resistance to ceftriaxone and cefepime antibiotics was observed in the collected *S.* Typhimurium isolates, resistance to these agents can be prevented by their proper administration and preventing their uncontrolled use. Due to the complete sensitivity of the bacteria to cefepime (100%), this drug is the most effective for treating *Salmonella* infections. In previous studies, the resistance rate of *Salmonella* to cephalexin within the class of first-generation cephalosporins was observed at 89.2% (18). In a study by Chiu et al. (2010), 164 *Salmonella* isolates were obtained from 1595 cloacal swabs. All collected isolates were susceptible to cefazolin and ceftriaxone (24). In a study conducted by Ghotaslou et al. (2010), the rates of resistance to ceftriaxone, ceftazidime, cefixime, cefotaxime, ceftizoxime, and cefepime among *Enterobacteriaceae* isolates were 33%, 38%, 29%, 20%, 3%, and 5%, respectively (25). Boraei-nezhad et al. (2023) investigated the antimicrobial resistance in Salmonella isolated from village chickens in the Sistan region, Iran. The susceptibility to tetracycline (78%), gentamycin (37%), cefepime (0.0%), and difloxacine (93%) was observed (26). Rahimi et al. (2013) evaluated the occurrence and frequency of antimicrobial resistance and resistance genes in salmonella isolated from broilers in northern Iran. 94% of the isolates resisted nalidixic acid and ciprofloxacin (27). Manzari et al. (2022) examined the drug resistance of Salmonella isolates in Shiraz, Iran. High resistance to nalidixic acid (67.6%), tetracycline (62.9%), and sulfamethoxazole (42.3%) were observed (28).

In a recent study, the frequency of *the int1* gene among *Salmonella* serotypes was 96.96%. Cabrera et al. (2006) observed that 25% of *Salmonella* strains, resistant to antibacterial compounds, had class 1 integrons (29). In a study conducted by Firoozeh et al. (2011), out of 58 *Salmonella* isolates, 43 isolates (74.1%) had multi-drug resistance, and 38 isolates (88.3%) had class 1 integrons (30). In our study, the prevalence of the $bla_{CTX\text{-}M\text{-}1}$ gene among *Salmonella* isolates was 75.75%. In a study conducted by Leinberger et al. (2010), out of 60 Enterobacteriaceae samples, 58 samples (97%) contained ESBL, and 76% had $bla_{CTX\text{-}M}$ (31). In a study by Tajbakhsh et al. (2012), out of 174 *Salmonella* isolates, seven isolates showed resistance to cefotaxime, ceftazidime, and ceftriaxone. Genotypic analysis of the samples showed that 4% had ESBL, and all were carriers of bla_{CTX} and bla_{TEM} genes (32). Based on the results of this study, there is a high prevalence of antimicrobial-resistant genes, especially *int*1, among *Salmonella* isolates in the Sistan region. It can result from excessive use of antibiotics in medicine and veterinary medicine in this region.

According to the antibiogram, multiple antimicrobial resistance to the cephalosporin compound was observed in *Salmonella* serotypes. Based on the results, there is a high prevalence of antimicrobial-resistant genes, especially *int*1, among *Salmonella* bacteria, which might result from the excessive use of antibiotics in medicine and veterinary medicine in the Sistan region, Iran. The issue of resistance to cephalosporin compounds in Salmonella serotypes is a severe problem that can soon lead to the need for antibiotics.

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Conflict of Interest

All authors declare that they have no conflicts of interest.

Author Contributions

MJ, KS, and RED contributed to the study design and data collection. KS performed PCR and antibiogram. MJ, DS, and KS wrote the main manuscript text. KS and DS prepared figures and tables. DS performed statistical analysis.

Data Availability Statement

Data are available from the corresponding author upon reasonable request.

Ethical Considerations

All the experiments were done following the principles for the care and use of laboratory animals. The Ethics Committee of the University of Zabol reviewed and approved the protocol.

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