Molecular Characterization of *nfsA* and *nfsB* Genes in Furazolidone-Resistant *Salmonella Spp.* Isolated from Poultry Eggs

Abstract

Furazolidone (FZD), a broad-spectrum antibiotic in the nitrofuran class, is banned in many countries due to health concerns. The illegal use of FZD in poultry can lead to drug resistance in bacteria, such as *Salmonella spp.*, which infect both poultry and humans. Contaminated eggs are a primary source of *Salmonella* infection. This study investigated the resistance of *Salmonella* isolates from eggs to FZD to gain crucial insights into the prevalence of resistant strains within the population. To this end, the susceptibility of 22 *Salmonella enterica* isolates from eggshells to FZD was determined using the disk diffusion and minimum inhibitory concentration methods. Then, the mutations in the *nfsA* and *nfsB* genes were examined using the polymerase chain reaction method and sequencing. Results were analyzed using GeneRunner software and BLAST online software. It was found that 27.27% and 9.09% of the isolates had high and medium resistance to FZD, respectively. The minimum inhibitory concentration results were determined to be 32 µg/ml for sensitive isolates, 256 µg/ml for intermediate isolates, and 512 µg/ml for resistant isolates. Sequencing analysis identified six insertion mutations and one transition mutation in the *nfsA* gene of resistant isolates, as well as one silent mutation in the *nfsB* gene of a sensitive isolate. The study highlights substantial resistance to FZD in *Salmonella* isolates from eggs, associated with mutations in the *nfsA* gene. These findings underscore the necessity for monitoring and managing resistance in foodborne pathogens. The significant resistance to FZD and the related mutations in the nfsA gene highlight the critical need for continuous surveillance and research to address the growing issue of antimicrobial resistance, especially in food products.

Keywords: Furazolidone, *nfsA*, *nfsB*, resistance, *Salmonella*

1. Introduction

Furazolidone ((5-nitrofurfurylideneamino)-2-oxazolidinone, FZD) is a synthetic nitrofuran antimicrobial agent widely used in veterinary medicine to treat infections caused by *Escherichia coli*, *Salmonella spp.*, and *Shigella spp.*, as well as to enhance animal production, particularly in pigs, poultry, and fish (1- 2). In 1995, the European Union enforced a complete ban on the use of four major nitrofurans, including FZD, furaltadone, nitrofurazone, and nitrofurantoin. This action was taken due to concerns regarding the carcinogenic and mutagenic potential of drug residues and their adverse effects on human health (3). In addition to concerns about the harmful effects of nitrofurans on human health, the overuse and long-term use of antibiotics in veterinary can cause the development of the drug-resistant bacteria in the treatment of infections. These bacteria can then be transmitted to humans through the consumption of animal-source foods (4). Nitrofurans exert their effects by generating reactive oxygen species (ROS) within cells. Reduced by flavin-containing nitroreductases, nitrofurans form nitroaryl anion free radicals. These radicals react with molecular oxygen to produce superoxide anions, hydrogen peroxide, and hydroxyl radicals. Highly reactive, these ROS molecules damage cellular components, including lipids, DNA, and membranes. ROS generation is more pronounced in bacterial and protozoal cells compared to mammalian cells, making nitrofurans selectively toxic to these pathogens. The resulting ROS-induced damage leads to cell death through lipid peroxidation and DNA mutagenesis (5). Nitrofuran compounds are prodrugs activated in *E. coli* by nitroreductase enzymes. There are two classes of nitroreductases: oxygen-insensitive (type I), encoded by the *nfsA* and *nfsB* genes, and oxygen-sensitive (type II). Type I enzymes, NfsA and NfsB, catalyze a stepwise two-electron reduction of the nitro moiety into reactive nitroso and hydroxylamino derivatives (6). Evaluating nitrofuran resistance between susceptible and resistant strains of *E. coli* has revealed that their differing abilities to reduce these compounds account for the variation between them. FZD resistance in *E. coli* is associated with the inactivation of nitroreductase present in *E. coli* as a result of the sequential inactivation of the *nfsA* and *nfsB* nitroreductase genes (7). *Salmonella* is a gram-negative bacterium of the Enterobacteriaceae family and the causative agent of salmonellosis, which is an important cause of food poisoning in humans (8). The outbreak of *Salmonella* infections is commonly associated with the consumption of contaminated foods, such as meat and eggs, which are identified as the important transmission factors for human salmonellosis (9- 10). Most *Salmonella* isolates

exhibit resistance to numerous antimicrobials and disinfectants commonly used in medical and poultry practices (11). The wide use of antibiotics for veterinary prevention and treatment has made poultry a major reservoir of antimicrobial-resistant *Salmonella*. Most infections caused by antibiotic-resistant *Salmonella* result from consuming contaminated food of animal origin. The emergence of antibiotic-resistant *Salmonella spp*. limits the therapeutic options available for treating *Salmonella* infections (12). Although *Salmonella spp.* is naturally sensitive to nitrofurans, recent studies in Iran have indicated a decrease in sensitivity among isolates of human and non-human origin in *Salmonella spp*. (13-14). The studies by Amiri, Fazlara, and Alawi report that the banned FZD antibiotic is still being used in Iran's poultry industry. The illegal use of FZD can be attributed to factors such as limited regulatory enforcement, lack of awareness about its risks in food production, and its availability and affordability (15-17). Therefore, this study aimed to investigate the resistance of *Salmonella* isolates from eggs to FZD, a banned antibiotic, and to analyze the genetic mutations associated with this resistance. By examining the sensitivity of these isolates and identifying mutations in the *nfsA* and *nfsB* genes, the study sought to elucidate the prevalence and genetic basis of FZD resistance. This research contributes to the broader understanding and management of antibiotic resistance in bacteria that affect both poultry and humans.

2. Materials and Methods

2.1. Isolation and Identification of Bacteria

In this study, 500 eggs from various brands were collected from 20 supermarkets in Lahijan City, Gilan Province, northern Iran, between January and October 2017. A total of 20 to 30 eggs were purchased from each supermarket, and the eggshells and yolks were examined for *Salmonella* contamination.

Egg surfaces were sampled using a swab technique. The swabs were directly inoculated into 4 mL of Buffered Peptone Water (BPW) for pre-enrichment and incubated at 37°C for 18–24 hours. For internal contents, eggs were immersed in 70% ethanol for 2 minutes to prevent shell contamination. The eggs were then aseptically cracked, and the contents were transferred to a sterile container. The mixed egg contents were inoculated into BPW and incubated at 37°C for 18–24 hours. The mixture was then thoroughly mixed, and 1 mL of it was inoculated into 4 mL of BPW for further processing. Then, 100 µL of each sample was transferred to Rappaport Vassiliadis *Salmonella* enrichment broth and incubated at 42°C for 24 h. *Salmonella spp*. was isolated using XLD

agar and brilliant green agar (18- 19). Presumptive *Salmonella* colonies from each selective medium were subcultured on nutrient agar and confirmed by biochemical tests.

To identify the organism biochemically, several tests were performed following the procedures outlined in Bergey's Manual of Determinative Bacteriology. Colonies with typical *Salmonella* morphology were selected for biochemical confirmation using tests such as triple sugar iron agar, Urease, Simmons' citrate agar, Indole, lysine iron agar, methyl red, and Voges-Proskauer tests (20). Then, all isolates were serotyped by agglutination with standard antisera to identify flagellar and somatic antigens based on the Kaufman-White table (21). The *Salmonella*-positive strains were cultured on nutrient agar for antimicrobial susceptibility testing.

2.2. Antibiotic Susceptibility Testing

The Kirby-Bauer disc diffusion method was used to evaluate antimicrobial susceptibility in *Salmonella* isolates on Mueller-Hinton agar, following the 2017 recommendations set by the Clinical and Laboratory Standards Institute (CLSI) (22). The antibiotic discs used were FZD (FR, 100 µg), ampicillin (AM, 10 µg), amikacin (AN, 30 µg), gentamicin (GM, 10 µg), nalidixic acid (NA, 30 µg), ciprofloxacin (CP, 5 µg), imipenem (IPM, 10 µg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CRO, 30 µg), and ceftazidime (CAZ, 30 µg). Biochemically confirmed isolates were transferred to sterilized tubes with 5 mL of tryptic soy broth (TSB) and incubated at 37°C until a 0.5 McFarland turbidity standard was reached. The bacteria were cultured on Mueller-Hinton agar plates, and then antibiotic discs were used. *Salmonella* Typhimurium ATCC 14028 was employed as the quality control for drug susceptibility testing.

The minimum inhibitory concentration (MIC) of FZD was determined using the broth dilution method, according to CLSI guidelines (22). The test was repeated three times to enhance the precision of the assay. In this regard, 96-well microtiter plates were utilized. Each well received 100 µl of Mueller-Hinton broth, and serial dilutions of FZD (ranging from 4096 to 2 µg/mL) were prepared in the wells. Subsequently, 100 µl of a bacterial suspension (10^6 cfu/mL) was added to each well. Positive and negative control wells were used to validate the test results. The plates were incubated at 37°C for 24 h, and bacterial growth in the wells was subsequently examined. The MIC was recorded as the lowest antibiotic concentration that inhibited *Salmonella* growth.

To determine the minimum bactericidal concentration (MBC), wells with no bacterial growth were

transferred to Mueller-Hinton agar plates. After 48 h of incubation at 37°C, the plates were examined for bacterial growth. The MBC was defined as the lowest concentration at which no growth of the *Salmonella* isolate was observed.

2.3. DNA Extraction, Polymerase Chain Reaction and Sequencing of *nfsA* and *nfsB* Genes

The DNA was extracted from the isolated strains using a boiling method following incubation on Luria Bertani agar at 37°C for 24 h. Colonies from each sample were suspended in 250 µL of sterile distilled water, vortexed for uniform turbidity, boiled for 10 min, and centrifuged at 6000g for 7 min. The supernatants were collected and stored for subsequent PCR analysis (23). PCR products were electrophoresed on a 2% agarose gel and visualized under UV light. The primers were designed using Gene runner software for the *nfsA* (878bp) and *nfsB* (843bp) genes (Table 1). The PCR test was performed using 7.5 µL of Master mixed, 1 µL of genomic DNA, 1 µL of each primer, and 4.5 µL of sterile nuclease free water in a final volume of 15 µL. The amplification of the *nfsA* and *nfsB* genes involved an initial denaturation for 10 min at 95°C, followed by 30 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C for *nfsA* and 58°C for *nfsB*, extension for 90 sec at 72°C, and a final extension for 5 min at 72°C. The samples were sent to the Codon Company for sequencing while maintaining the cold chain, after ensuring the formation of single-band PCR products. Then, the results were analyzed for identifying the mutations in the *nfsA* and *nfsB* genes in resistant samples using GeneRunner software and BLAST online software. Statistical analyses were conducted using SPSS 25 software, employing descriptive methods such as the mann-whitney U test and chi-Square test. A p-value of less than 0.05 was considered statistically significant.

Table 1. Primers Used for *nfsA* and *nfsB* Gene

Primer	Sequence 5´-3´	Target	T (°C)	Amplicon size (bp)
nfsA- F	CTGGCGCTTGCTCTGCTATC	*nfsA*	60	878 bp
nfsA-R	CTTTAATCAGGGTGCGACGG	*nfsA*		

nfsB-F	TTCCGTCAGTGTGGTTTCAAG	*nfsB*	58	843 bp
nfsB-R	ATCACCGTCTCGCTACTCAAC	*nfsB*		

3. Results

In this study, the shells and yolks of 500 eggs were examined for *Salmonella* infection, and 22 eggshells were observed to be infected with *Salmonella spp.* The serotypes of the isolates were determined, and the results indicated that the distribution of *Salmonella* serotypes (Figure 1) included *Salmonella* Infantis (11 cases, 50%), *S.* Enteritidis (three cases, 13.63%), *S.* Typhimurium (three cases, 13.63%), and *S. Havana*, *S.* Blegdam, *S.* Kottbus, *S.* Moero, and *S.* Paratyphi A (one case for each, 4.5%).

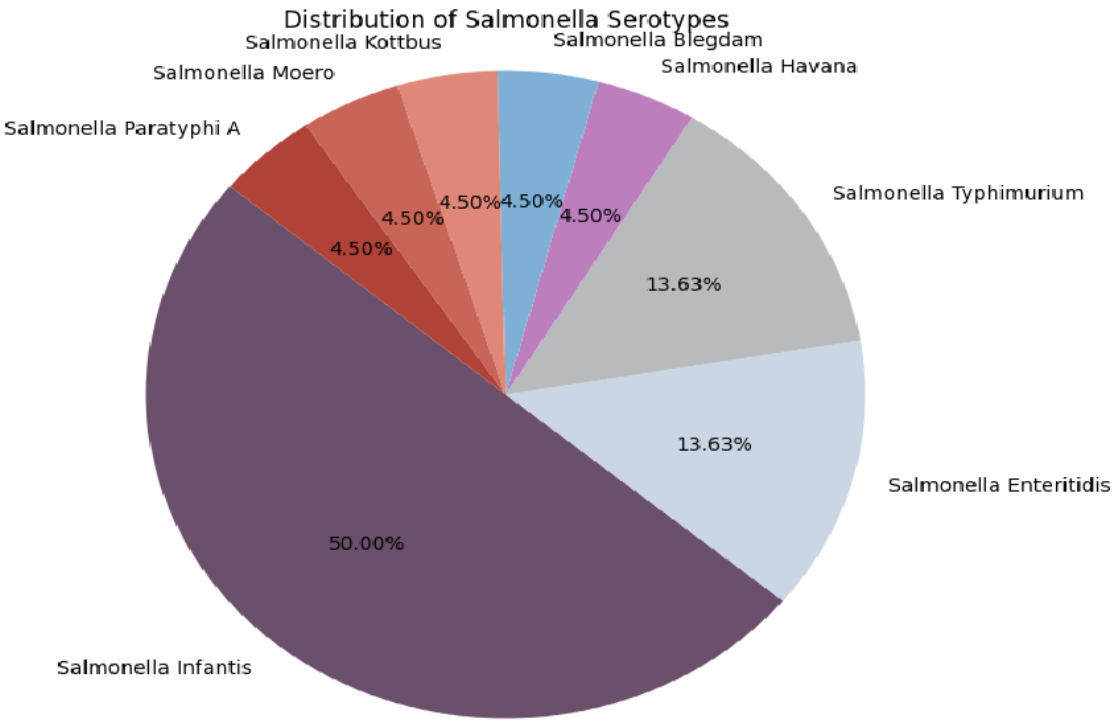

Figure 1. Distribution of *Salmonella* serotypes

3.1. Antibiotic Susceptibility

The antibiotic susceptibility profile of the 22 *Salmonella* isolates is illustrated in Figure 2. Among the isolates, 27.27% exhibited high resistance and 9.09% showed moderate resistance to FZD. Imipenem exhibited the highest resistance rate (77.27%),

followed by ciprofloxacin (68.18%), ceftazidime (63.63%), nalidixic acid (59.09%), and ceftriaxone (54.54%). In contrast, gentamicin demonstrated the highest sensitivity, with a rate of 95.95%. The MIC results for FZD were reported to be 32, 256, and 512 µg/ml for sensitive, intermediate, and resistant isolates, respectively (Table 2). The MBC ranged from 32 to 4096 µg/ml, with the highest and lowest values observed (Figure 3). Since there is no cut-off point for FZD in the CLSI guideline, the data were reported based on the MIC results and the diameter of the growth inhibition zone.

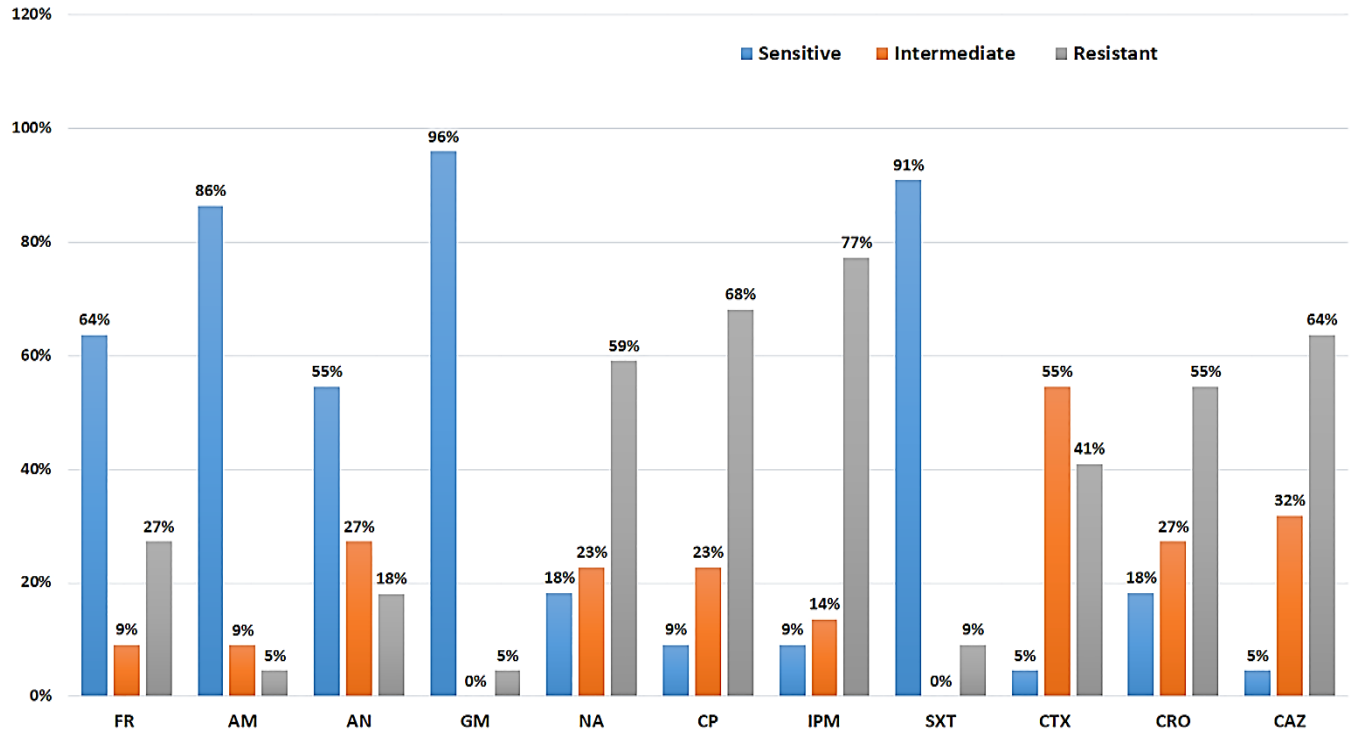

Figure 2. Antibiotic resistance results of *Salmonella* isolates using disk diffusion antibiotic sensitivity test (the Kirby-Bauer test). Abbreviations are as follows: furazolidone (FR), ampicillin (AM), amikacin (AN), gentamicin (GM), nalidixic acid (NA), ciprofloxacin (CP), imipenem (IPM), trimethoprim/sulfamethoxazole (SXT), cefotaxime (CTX), ceftriaxone (CRO), and ceftazidime (CAZ).

Table 2. Relationship Between Disk Diffusion, Minimum Inhibitory, Concentration, and Nitroreductase Gene Mutations

N	Fur(mm)	MIC Fur (µg/ml)	nfsA	nfsB
3	22(S)	32	wt	wt
4	15(I)	256	wt	wt
5	24(S)	32	wt	A to G wobble →GTA to GTG

7	22(S)	32	wt	wt
9	10(R)	512	GGGGACT insertion → Frame shift	wt
10	0(R)	512	GGGGACT insertion → Frame shift	wt
11	0(R)	512	C insertion → Frame shift	wt
12	18(S)	32	wt	wt
13	10(R)	512	C insertion → Frame shift	wt
15	15(I)	256	GGGGACT insertion → Frame shift	wt
16	23(S)	32	wt	wt
19	11(R)	512	C to T transition → Pro to ser	wt
20	0(R)	512	A insertion → Frame shift	wt

n, number of isolates; Fur (mm), furazolidone disk diffusion; S, susceptible; I, intermediate; R, resistance;MIC, minimum inhibitory concentration(µg/ml); nfsA, mutations found in nfsA gene; nfsB, mutations found in nfsB gene; wt, without mutation.

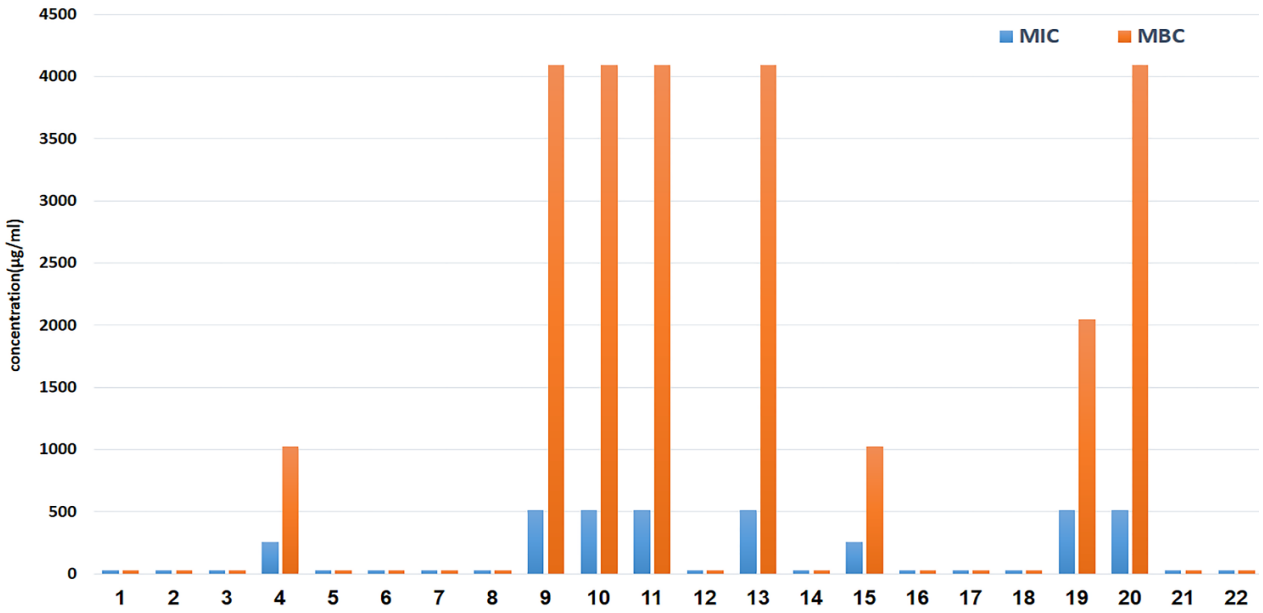

Figure 3. Minimum inhibitory concentration and minimum bactericidal concentration of furazolidone against *Salmonella* isolates

3.2. Polymerase Chain Reaction and Sequencing

The *nfsA* and *nfsB* genes in 13 *Salmonella* isolates, including resistant, intermediate, and some FZD-sensitive isolates, were amplified using polymerase chain reaction. The PCR products exhibited approximate sizes of 878bp for *nfsA* and 843bp for *nfsB*. The nucleotide sequence of the amplified *nfsA* and *nfsB* genes was determined (Figure 4). The results showed that among the resistant isolates, seven had mutations in the *nfsA* gene (Figure 5). Of them, three isolates had the same GGGGACT insertion mutation at nucleotide position 366-372 (Figure 6). Moreover, one isolate had an A insertion at nucleotide position 457 (Figure 6), and another had C to T transition mutation at nucleotide position 112 (Figure 6). C insertion mutations at nucleotide position 451 were observed in the other isolates (Figure 6). Only one of the sequenced isolates, sensitive to FZD, had a mutation in the *nfsB* gene as an A to G wobble at nucleotide position 165, which changed the GTA codon to GTG. In the other isolates, no mutations were observed in the *nfsA* and *nfsB* genes. The mean MIC difference between the mutated and wild groups for the *nfsA* gene was 406.095 µg/ml, with a p-value of 0.00190. This indicates a significant increase in MIC (µg/ml) in the mutated group compared to the wild group.

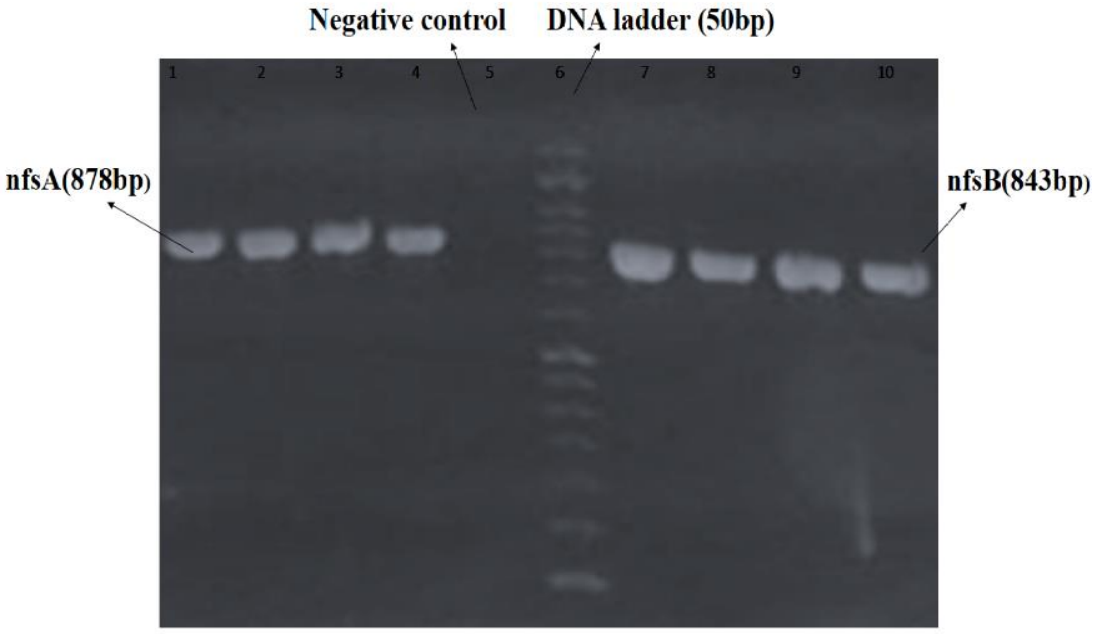

Figure 4. Electrophoresis results of PCR products carrying nfsA and nfsB genes on a 2% agarose gel. Lanes 1-4 show 878 bp PCR amplicons of nfsA. Lane 6 contains the DM1160 DNA Ladder (50-1,500 bp). Lanes 7-10 show 843 bp PCR amplicons of nfsB

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Figure 5. Mutations revealed in sequence alignment of the *nfsA* gene. This image presents a sequence alignment, highlighting specific regions where mutations have occurred in the *nfsA* gene.

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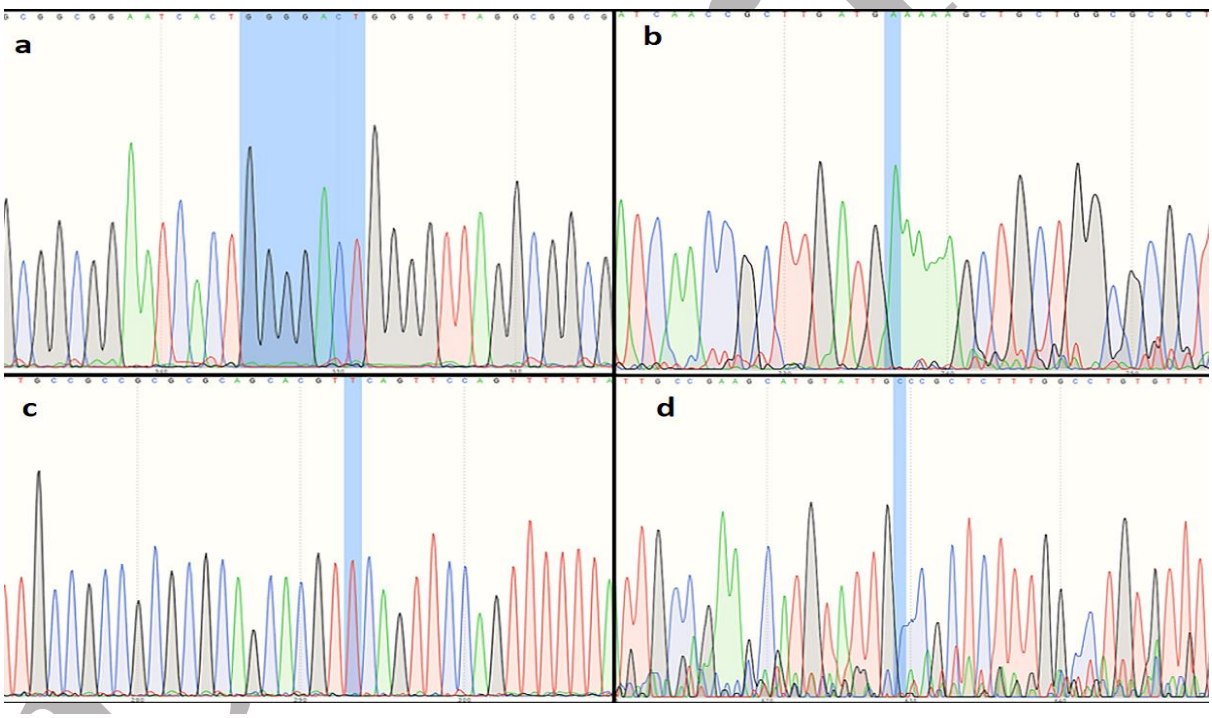

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Figure 6. The Sanger sequencing electropherograms of the nfsA gene. (a) GGGGACT insertion mutation at nucleotide position 366-372. (b) A insertion at nucleotide position 457. (c) C to T transition mutation at nucleotide position 112. (d) C insertion mutations at nucleotide position 451

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٣٣٤ **4. Discussion**

٣٣٥ Feed is a critical part of the food chain, and its safety ٣٣٦ is essential for the health of both humans and animals ٣٣٧ (24). Foodborne illness is a major problem, especially ٣٣٨ in developing countries, and is a leading cause of ٣٣٩ morbidity and mortality in the world (25). *Salmonella* ٣٤٠ *enterica* subsp. enterica is a major cause of infectious ٣٤١ gastroenteritis and one of the most significant

foodborne pathogens worldwide (26). Since infected meat and eggs are the most important sources of salmonellosis transmission to humans, identification and control of *Salmonella* is crucial for the public health (27). In Azirpour's study, four out of 110 egg samples (3.63%) were found to be infected with *Salmonella* (28). In the study by Bahramianfard et al., 8.7% of poultry samples and 6.3% of eggs were contaminated with *Salmonella* species, while 2.3% of poultry samples and 1.3% of eggs were contaminated with *S.* Enteritidis (29). Shahbazi et al. identified *Salmonella* species in 11 out of 80 poultry product samples (13.75%), including four eggs and seven meat samples (30). In the present study, 22 out of 500 eggs (4.4%) were found to be infected with *Salmonella spp*.

The Food and Drug Administration approved the use of antibiotics as animal additives without a prescription, a practice later adopted by European Union countries with their own regulations. Unfortunately, the misuse of these compounds has led to the development and spread of antimicrobial resistance, now a significant public health concern (31). Improper use of antibiotics, especially unapproved antibiotics such as nitrofurans, in the treatment of poultry salmonellosis increases food poisoning caused by *Salmonella.* This misuse leads to the amplification of the drug-resistant *Salmonella* and its transition to humans (32). FZD belongs to the nitrofuran family and is one of the broad-spectrum antibiotics, which is widely used in medicine and veterinary (33). Although the use of these antibiotics and other nitrofurans is not approved in the poultry industry based on the instructions of the Veterinary Organization of Iran due to their carcinogenic and mutagenic effects on human health, their illegal use persists in the country (34). Recent studies have demonstrated an increase in *Salmonella* resistance to nitrofurans and the emergence of the resistant strains in Iran. In this regard, Azizpour, Jahantigh, and Raisi reported resistance rates of *Salmonella* isolates to FZD as 63.7%, 73%, and 87.5%, respectively (13- 14, 35). In the study by Sarba et al., 205 chicken samples were analyzed, and *Salmonella* isolates were obtained from the liver, kidney, ovary, and spleen. Among the 39 *Salmonella* isolates, all were found to be resistant to nitrofurantoin (36). In a study by Punchihewage-Don et al., 40.3% of 213 *Salmonella* isolates from chickens in Maryland's Eastern Shore were resistant to nitrofurantoin (37). In this study, 27.27% of the 22 *Salmonella* isolates exhibited high resistance, while 9.09% demonstrated moderate resistance to FZD. The results of this study, along with findings from previous research, demonstrate an increase in the resistance of *Salmonella* isolates to nitrofurans.

Bacterial resistance to nitrofurans increases stepwise through the successive mutations in the genes encoding nitroreductase, *nfsA* and *nfsB*, which reduce the reduction ability of nitrofurans. First, mutations occur in the *nfsA* gene, which can subsequently affect mutations in the *nfsB* gene. Overall, mutations in both genes are required to achieve complete resistance (38).

In the present study, the *nfsA* and *nfsB* genes were amplified in all *Salmonella* isolates by PCR reaction and sequenced to evaluate the mechanism of FZD resistance. The results indicated that mutations occurred in the *nfsA* and *nfsB* genes, but no isolates had simultaneous mutations in both genes. Among the sequenced isolates, mutations were detected in the *nfsA* gene in 53.84% of cases. However, mutations in the *nfsA* gene were detected in all resistant isolates, highlighting its significant role in resistance development. On the other hand, the absence of mutations in the *nfsA* gene in FZD-sensitive *Salmonella* isolates suggests a correlation between *nfsA* gene mutations and resistance to this antibiotic. Resistant isolates with frameshift-inducing mutations exhibited higher MIC values, suggesting a direct association between *nfsA* mutations and increased FZD resistance. However, mutations in the *nfsB* gene were observed in only one isolate, despite the intact *nfsA* gene. This particular isolate, which exhibited sensitivity to FZD, displayed an altered codon (GTG) in the *nfsB* gene; yet, the altered and unaltered codons were synonymous, both encoding the valine amino acid. Therefore, there was no change in the structure of the resulting protein, and the protein had its normal function. In other studies, mutants with high resistance to nitrofurans were observed in the *E. coli* isolates, with mutations solely in the *nfsA* gene while retaining the wild-type *nfsB* gene (38). Insertion mutations were found in the nucleotide sequence analysis of resistant isolates with an MIC of 512 µg/ml and intermediate isolates with an MIC of 256 µg/ml, compared to sensitive isolates with an MIC of 32 µg/ml. These mutations change the reading frame of amino acids and the termination codons, yielding different or inactive nitroreductase enzymes. Therefore, the resulting enzyme cannot reduce nitrofuran antibiotics, causing resistance to these antibiotics in mutated isolates. Although studies on the mechanism of resistance to nitrofurans have been limited, especially in *Salmonella*, they have demonstrated mutations in the *nfsA* and *nfsB* genes in resistant isolates. Garcia et al. reported a missense mutation that affected the *nfsA* start codon among high- and medium-resistant *Salmonella* isolates, with only one of these resistant isolates having a frameshift mutation in the *nfsB* gene (38).

Most studies conducted on nitrofuran resistance evaluated *E. coli*. Shanmugan et al. reported that insertion mutations were found in the *nfsA* gene in resistant *E. coli* and *pneumoniae* isolates, causing frameshift mutations in this gene (39). Further, Sandegren et al. demonstrated that deletion and insertion mutations were identified among resistant *E. coli* isolates, leading to premature termination of the protein (40). Wan et al. investigated nitrofurantoin resistance in nine *E. coli* samples exhibiting resistance to this drug. They identified four types of mutations in the nitroreductase genes *nfsA* and *nfsB*: gene interruptions by insertion sequences, frameshift mutations, nonsense mutations, and missense mutations. Each sample harbored alterations in both genes, leading to high levels of nitrofurantoin resistance (MIC ≥ 128 mg/L) (41). In the present study, in addition to the insertion mutation, a transition mutation as C→T was observed in one of the resistant isolates with an MIC of 512 µg/ml, which caused the conversion of the amino acid proline to the amino acid serine. These results probably confirm the key role of mutations in the *nfsA* gene in causing FZD resistance. As mentioned, resistance to nitrofurans occurs in two stages. In the first stage, mutations occur in the *nfsA* gene, which are considered critical for causing resistance. The second stage mutation causes resistance along with the first stage mutation. As reported by Whiteway J et al., a mutation in the *nfsB* gene in *nfsA*$^{+}$ isolates is not sufficient to cause resistance to nitrofurans (42). Therefore, mutations in the *nfsA* gene are more important than those in the *nfsB* gene, and resistance to nitrofurans is more affected by *nfsA* gene changes.

The results indicated that the *Salmonella* isolates separated from the brand eggs obtained from supermarkets in Gilan Province, Iran, were resistant to FZD, Moreover, the isolates were observed to be correlated with changes in genes encoding oxygen-insensitive nitroreductase. Due to the importance of nitrofuran in the treatment of infections, especially urinary tract infections, and the limited studies on nitrofuran resistance in the world, especially in Iran, further studies in this field are recommended.

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Authors' Contribution

HS, LM, and ACh designed the study, and HS performed the experiments. HS, LM, and ACh analyzed the data, and HS wrote the manuscript. All authors read and approved the final manuscript.

Ethics

We hereby declare that all ethical standards were respected in preparing the submitted article.

Conflicts of Interest

The authors declare that they have no competing interests.

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Data Availability

The data generated and/or analyzed during the current study are available from the corresponding author on request.

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