Original Article

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

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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 that has gained significant traction in the veterinary field. It is employed 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 imposed a comprehensive prohibition on the utilization of four significant nitrofurans, namely 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). Concerns have been raised regarding the potentially deleterious effects of nitrofurans on human health. Moreover, the excessive and prolonged utilization of antibiotics in veterinary medicine has been demonstrated to induce the emergence of antibiotic-resistant bacteria in the context of infection treatment. The transmission of these bacteria to humans can occur through the consumption of animal-source foods (4). Nitrofurans have been demonstrated to exert their effects by generating reactive oxygen species (ROS) within cells. Flavin-containing nitroreductases catalyze the reduction of nitrofurans, resulting in the formation of nitroaryl anion free radicals. These radicals react with molecular oxygen to produce superoxide anions, hydrogen peroxide, and hydroxyl radicals. These ROS molecules possess high reactivity, which causes damage to 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 resultant ROS-induced damage leads to cell death through lipid peroxidation and DNA mutagenesis (5). Nitrofuran compounds are prodrugs that are activated in E. coli by nitroreductase enzymes. Two distinct classes of nitroreductases have been identified: oxygen-insensitive (type I), which is 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). A comparative analysis of nitrofuran resistance between susceptible and resistant strains of E. coli has revealed that their differential capacities to reduce these compounds underlie the observed variations. The phenomenon of FZD resistance in E. coli has been demonstrated to be associated with the inactivation of nitroreductase, a protein that is present in E. coli. This association has been observed to result from 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 a significant 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). The majority of Salmonella isolates demonstrate resistance to a wide range of antimicrobials and disinfectants frequently utilized in medical and poultry settings (11). The extensive utilization of antibiotics for both veterinary prevention and treatment has led to the emergence of antimicrobial-resistant Salmonella in poultry populations. The majority of infections caused by antibiotic-resistant Salmonella are associated with the consumption of contaminated food of animal origin. The emergence of antibiotic-resistant Salmonella spp. has led to significant limitations in the therapeutic options available for treating Salmonella infections (12). Although Salmonella spp. is inherently susceptible to nitrofurans, recent studies conducted in Iran have revealed a decline in susceptibility among isolates of both human and non-human origin (13, 14). The studies conducted by Amiri, Fazlara, and Alawi reveal that the utilization of the banned FZD antibiotic persists within Iran's poultry industry. The illicit use of FZD can be ascribed to several factors, including but not limited to: limited regulatory enforcement, a paucity of awareness regarding its risks in food production, and its availability and affordability (15-17). The objective of this study was to investigate the resistance of Salmonella isolates from eggs to FZD, a banned antibiotic, and to analyze the genetic mutations associated with this resistance. The objective of the present study was to elucidate the prevalence and genetic basis of FZD resistance. To this end, the sensitivity of the isolates was examined, and mutations in the nfsA and nfsB genes were identified. 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, a total of 500 eggs from various brands were procured from 20 supermarkets in Lahijan City, Gilan Province, northern Iran, between January and October 2017. A total of 20 to 30 eggs were procured from each supermarket, and the eggshells and yolks were examined for Salmonella contamination. Samples of egg surfaces were obtained through the implementation of a swab technique. The swabs were then directly inoculated into 4 mL of Buffered Peptone Water (BPW) for pre-enrichment and incubated at 37°C for 18–24 hours. To ensure the absence of contamination from the shell, the eggs were immersed in 70% ethanol for a duration of two minutes. Subsequently, the eggs were meticulously cracked under aseptic conditions, and the contents were meticulously transferred to a sterile container. The mixture of egg components was then inoculated into BPW and subjected to incubation at 37°C for a period of 18–24 hours. Subsequently, the mixture was thoroughly mixed, and 1 mL of it was inoculated into 4 mL of BPW for further processing. Subsequently, 100 microliters of each specimen was transferred to Rappaport Vassiliadis Salmonella enrichment broth and incubated at 42°C for 24 hours. The isolation of Salmonella spp. was conducted 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. In order to identify the organism biochemically, a series of tests were conducted in accordance with the procedures delineated in Bergey's Manual of Determinative Bacteriology. Colonies exhibiting characteristic 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). Subsequently, the isolates were serotyped by agglutination with standard antisera to identify flagellar and somatic antigens. This procedure was based on the Kaufman-White table (21). The Salmonella-positive strains were cultivated on nutrient agar for the purpose of conducting antimicrobial susceptibility testing.

2.2. Antibiotic Susceptibility Testing

The Kirby-Bauer disc diffusion method was employed to assess the antimicrobial susceptibility of Salmonella isolates on Mueller-Hinton agar, in accordance with the 2017 guidelines established by the Clinical and Laboratory Standards Institute (CLSI) (22). The antibiotic discs utilized in this study included 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), and trimethoprim/sulfamethoxazole (SXT, 1.The concentrations of the antibiotics utilized in this study ranged from 25/23.75 µg to 30 µg, with the specific concentrations being cefotaxime (CTX, 30 µg), ceftriaxone (CRO, 30 µg), and ceftazidime (CAZ, 30 µg). Isolates that were biochemically confirmed were subsequently transferred to sterilized tubes containing 5 mL of tryptic soy broth (TSB). The tubes were then incubated at 37°C until a turbidity standard of 0.5 McFarland was reached. The bacterial samples were cultivated on Mueller-Hinton agar plates, and antibiotic discs were utilized for the purpose of identification. Salmonella Typhimurium ATCC 14028 was utilized as the quality control standard for the evaluation of drug susceptibility. The minimum inhibitory concentration (MIC) of FZD was determined using the broth dilution method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (22). The assay was repeated on three separate occasions to enhance its precision. 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 (106 CFU/mL) was added to each well. The validation of the test results was achieved through the implementation of positive and negative control wells. The plates were then subjected to an incubation temperature of 37°C for a period of 24 hours, after which an examination of the bacterial growth in the wells was conducted. The MIC was defined as the lowest antibiotic concentration capable of inhibiting Salmonella growth. The minimum bactericidal concentration (MBC) was determined by transferring wells with no bacterial growth to Mueller-Hinton agar plates. Following a 48-hour incubation period at a temperature of 37°C, the plates were subjected to a thorough examination to ascertain the presence of any 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 an incubation period on Luria Bertani agar at 37°C for 24 hours. Colonies from each sample were suspended in 250 microliters of sterile distilled water, vortexed for uniform turbidity, boiled for 10 minutes, and subjected to a centrifugation process at 6,000g for 7 minutes. The isolates were subsequently collected and stored for subsequent PCR analysis (23). The PCR products were subsequently subjected to electrophoresis on a 2% agarose gel, and visualization was performed under UV light. The primers were designed using Gene Runner software for the nsfA (878 base pairs [bp]) and nsfB (843 bp) genes (Table 1). The PCR test was performed using 7.5 microliters of Master Mix, 1 microliter of genomic DNA, 1 microliter of each primer, and 4.5 microliters of sterile nuclease-free water, yielding a final volume of 15 microliters. The amplification of the nfsA and nfsB genes involved an initial denaturation step for 10 minutes at 95°C, followed by 30 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C for nfsA and 58°C for nfsB, extension for 90 seconds at 72°C, and a final extension step for 5 minutes at 72°C. The samples were then dispatched to the Codon Company for sequencing, with meticulous attention to maintaining the cold chain. This process was undertaken only after the formation of single-band PCR products had been thoroughly confirmed. Subsequently, the results were subjected to analysis to identify mutations in the nfsA and nfsB genes in resistant samples. This analysis utilized both 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 designated as statistically significant.

3. Results

In the present study, a total of 500 eggs were examined for the presence of Salmonella infection. Of these, 22 exhibited signs of infection, and subsequent investigation revealed that these 22 eggs were infected with Salmonella spp. The serotypes of the isolates were identified through further analysis, 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%).

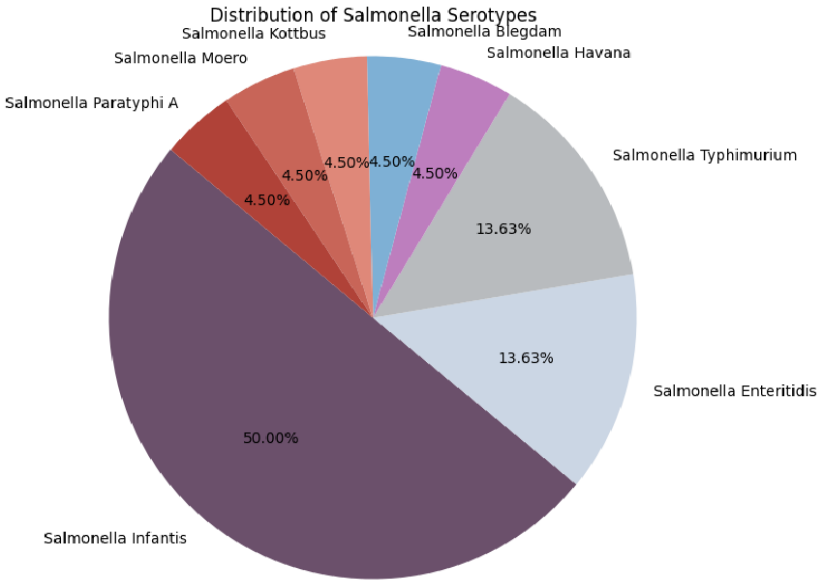

Figure 1. Distribution of *Salmonella* serotypes

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*		

3.1. 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. The highest resistance rate was exhibited by imipenem (77.27%), followed by ciprofloxacin (68.18%), ceftazidime (63.63%), nalidixic acid (59.09%), and ceftriaxone (54.54%). Conversely, gentamicin exhibited the highest degree of 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 median MBC ranged from 32 to 4096 µg/ml, with the highest and lowest values observed (Figure 3). As the CLSI guideline does not establish a cut-off point for FZD, the data were reported based on the MIC results and the diameter of the growth inhibition zone.

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 (PCR). The products of the polymerase chain reaction (PCR) exhibited approximate sizes of 878 base pairs (bp) for the nfsA gene and 843 bp for the nfsB gene. The nucleotide sequence of the amplified nfsA and nfsB genes was subsequently determined (Figure 4). The results demonstrated that among the resistant isolates, seven had mutations in the nfsA gene (Figure 5). Of the isolates examined, three exhibited a GGGGACT insertion mutation at the nucleotide position 366-372 (Figure 6). Furthermore, one isolate exhibited an A insertion at the nucleotide position 457 (Figure 6), while another isolate manifested a C to T transition mutation at the nucleotide position 112 (Figure 6). C insertion mutations at the nucleotide position 451 were observed in the other isolates (Figure 6). However, among the sequenced isolates demonstrating sensitivity to FZD, only one isolate exhibited a mutation in the nfsB gene, specifically an A to G wobble at nucleotide position 165. This mutation resulted in a change from the GTA codon to the GTG codon. 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 finding suggests a substantial increase in MIC (µg/ml) in the mutated group compared to the wild group.

4. Discussion

Feed represents a pivotal component of the food chain, and its safety is paramount for the well-being of both humans and animals (24). Foodborne illness represents a significant global health concern, particularly in developing countries, where it is a leading cause of morbidity and mortality (25). Salmonella enterica subsp. enterica is a major cause of

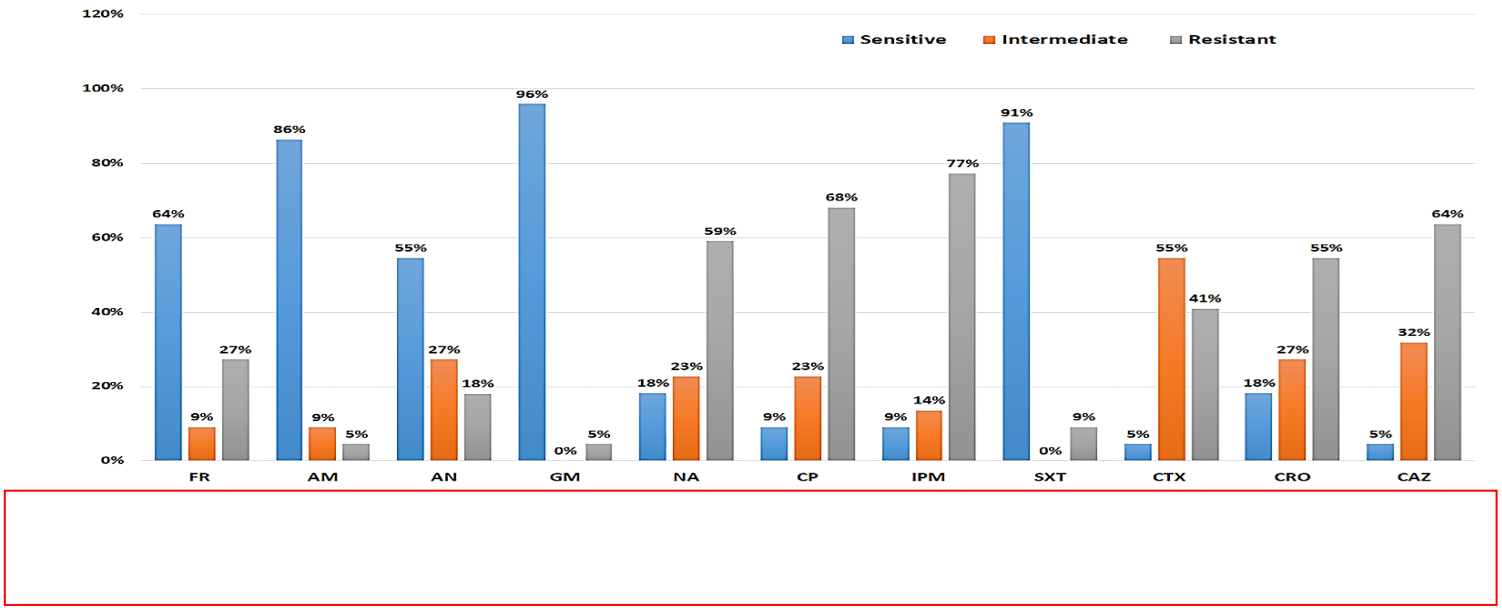

Table 2. Relationship Between Disk Diffusion, Minimum Inhibitory, Concentration, and Nitroreductase Gene Mutations.

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

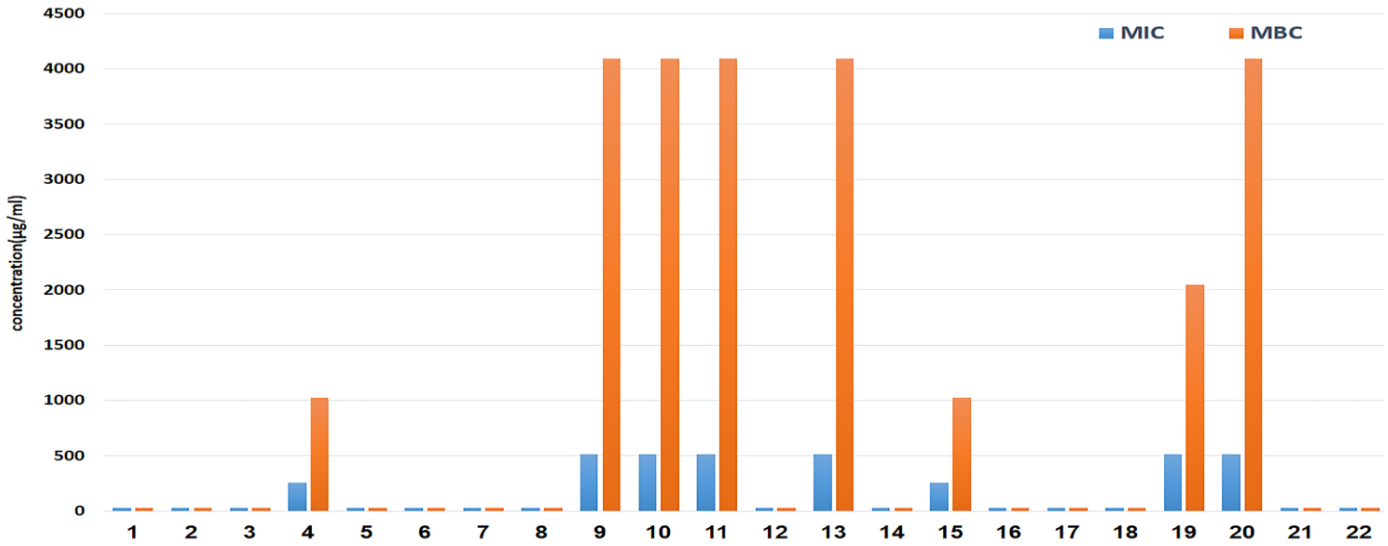

Figure 3. Minimum inhibitory concentration and minimum bactericidal concentration of furazolidone against *Salmonella* isolates.

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a:GGGGACT insertion
Query   540       CACTGGGGACTGGGGTTAGGCGGCGTGTATATCGGCGGCATCCGTAATAATATTGAATCT  599
                  ||||        ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   2994939   CACT-------GGGGTTAGGCGGCGTGTATATCGGCGGCATCCGTAATAATATTGAATCT  2994887

b:C insertion
Query   602       CTATTAAAATTGCCGAAGCATGTATTGCCCGCTCTTTGGCCTGTGTTTGGGATGGCCTGC  661
                  |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct   2805515   CTATTAAAATTGCCGAAGCATGTATTG-CCGCTCTTTGGCCTGTGTTTGGGATGGCCTGC  2805573

c:C to T transition
Query   242       CCGTGACTGACGCGCAGCGAGAGGCCATTATTGCCGCCGCGCGCAGCACGTTCAGTTCCA  301
                  |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct   2805153   CCGTGACTGACGCGCAGCGAGAGGCCATTATTGCCGCCGCGCGCAGCACGTCCAGTTCCA  2805212

d:A insertion
Query   730       TTGATGAAAAAGCTGCTGGCGCGCTATGACGAGCAGTTGGCTGAGTATTATCTGACCCGC  789
                  ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   3209466   TTGATG-AAAAGCTGCTGGCGCGCTATGACGAGCAGTTGGCTGAGTATTATCTGACCCGC  3209408
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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

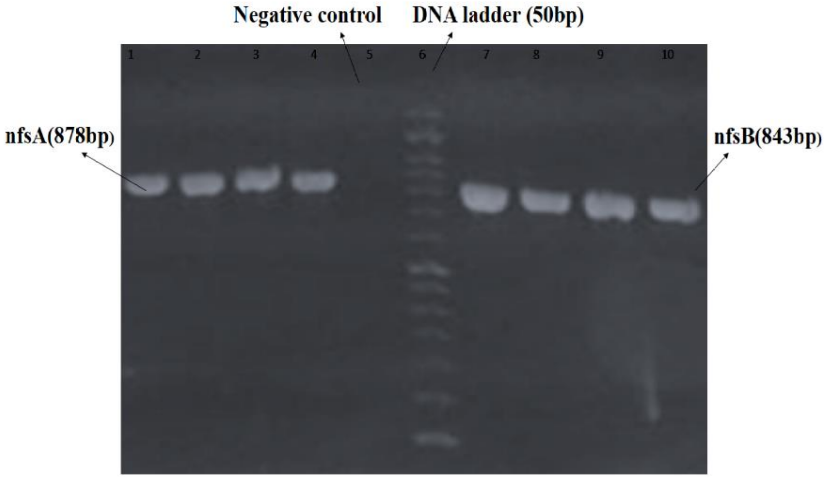

Figure 5. Mutations revealed in sequence alignment of the *nfsA* gene. This image presents a sequence alignment, highlighting specific regions where mutations have occurred in the *nfsA* gene.

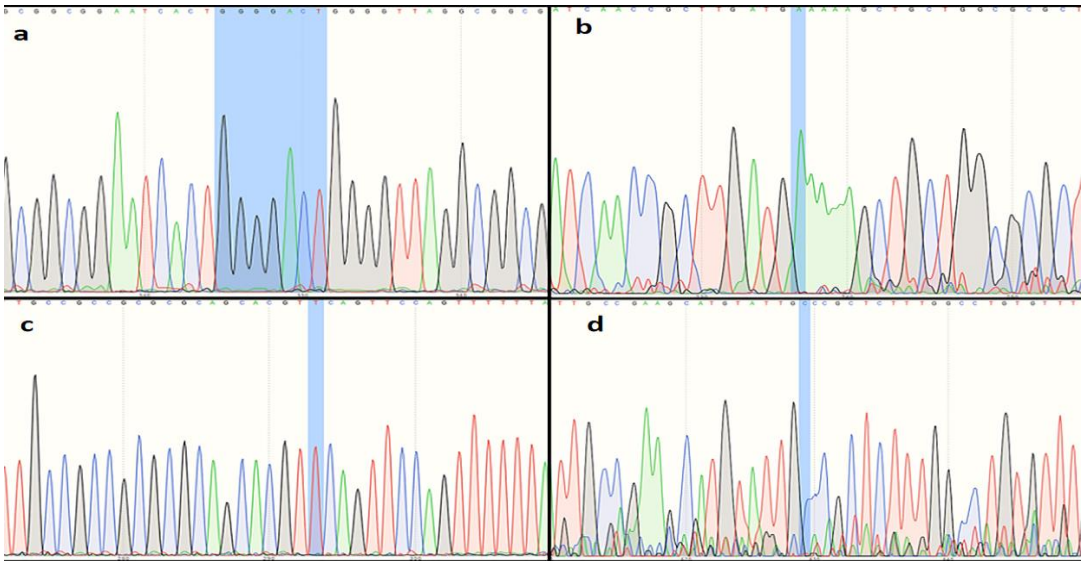

Figure 6. The Sanger sequencing electropherograms of the nfsA gene. (a) GGGGACT insertion mutation at nucleotide position 366-372. (b) A insertion at nucleotide position 457. (c) C to T transition mutation at nucleotide position 112. (d) C insertion mutations at nucleotide position 451.

infectious gastroenteritis and one of the most significant foodborne pathogens worldwide (26). As infected meat and eggs represent the most significant sources of salmonellosis transmission to humans, the identification and control of Salmonella are of paramount importance for 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 authorized the utilization of antibiotics as animal additives without the necessity of a prescription, a practice that was subsequently adopted by European Union countries in accordance with their own regulatory framework. However, the inappropriate use of these compounds has contributed to the emergence and propagation of antimicrobial resistance, a matter of significant public health concern (31). The improper use of antibiotics, particularly unapproved antibiotics such as nitrofurans, in the treatment of poultry salmonellosis, has been demonstrated to increase the incidence of food poisoning caused by Salmonella. This inappropriate use of antibiotics has been demonstrated to result in the proliferation of drug-resistant Salmonella and its subsequent transmission to humans (32). FZD is classified as a nitrofuran, a member of the broader category of broad-spectrum antibiotics, which are extensively utilized in both medical and veterinary contexts (33). Despite the fact that the Veterinary Organization of Iran has not approved the use of these antibiotics and other nitrofurans in the poultry industry 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 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 demonstrated resistance to nitrofurantoin (37). In the present study, 27.27% of the 22 Salmonella isolates exhibited high resistance, while 9.09% demonstrated moderate resistance to FZD. The findings of this study, when considered in conjunction with the results of previous research, indicate an increase in the resistance of Salmonella isolates to nitrofurans. The development of bacterial resistance to nitrofurans is a gradual process that occurs through successive mutations in the genes encoding nitroreductase, nfsA, and nfsB. These mutations result in a reduction in the reduction ability of nitrofurans, leading to an increase in bacterial resistance. First, mutations occur in the nfsA gene, which can subsequently affect mutations in the nfsB gene. It is evident that mutations in both genes are necessary to attain 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 exhibited 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, underscoring its pivotal role in resistance development. Conversely, 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. Furthermore, 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 demonstrated sensitivity to FZD, exhibited an altered codon (GTG) in the nfsB gene. However, the altered and unaltered codons were synonymous, both encoding the valine amino acid. Consequently, the structure of the resulting protein remained unaltered, and the protein continued to fulfill its customary 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). The analysis of the nucleotide sequence of resistant isolates revealed insertion mutations, 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 alter the reading frame of amino acids and the termination codons, resulting in the production of different or inactive nitroreductase enzymes. Consequently, the resulting enzyme is incapable of reducing nitrofuran antibiotics, leading to the development of resistance in mutated isolates. Despite the paucity of studies on the mechanism of resistance to nitrofurans, particularly in Salmonella, these studies 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). The majority of studies conducted on nitrofuran resistance have focused on E. coli. Shanmugan et al. reported the discovery of insertion mutations in the nfsA gene in resistant E. coli and pneumoniae isolates, leading to frameshift mutations in this gene (39). Furthermore, 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. conducted a study on nitrofurantoin resistance in nine samples of E. coli that exhibited resistance to this pharmaceutical agent. Four distinct types of mutations were identified in the nitroreductase genes nfsA and nfsB: gene interruptions by insertion sequences, frameshift mutations, nonsense mutations, and missense mutations. The analysis revealed that each sample exhibited alterations in both genes, resulting in elevated 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. This transition caused the conversion of the amino acid proline to the amino acid serine. These results likely substantiate the pivotal function of mutations in the nfsA gene in inducing FZD resistance. As previously stated, resistance to nitrofurans manifests in two distinct stages. In the initial stage, mutations arise in the nfsA gene, which are regarded as pivotal for inducing resistance. The second stage mutation has been observed to induce resistance, in conjunction with the initial 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). Consequently, mutations in the nfsA gene are more significant than those in the nfsB gene, and resistance to nitrofurans is more influenced by nfsA gene alterations. The findings of the study indicated that the Salmonella isolates obtained from eggs of the brand in question, which were procured from supermarkets in Gilan Province, Iran, exhibited resistance to FZD. Furthermore, an observation was made of a correlation between the isolates and changes in genes encoding oxygen-insensitive nitroreductase. The significance of nitrofuran in the treatment of infections, particularly urinary tract infections, warrants further investigation, especially in light of the paucity of research on nitrofuran resistance worldwide, particularly in Iran.

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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
It is hereby asserted that the ethical standards that govern the preparation of the submitted article were scrupulously adhered to.

Conflict of Interest
The authors declare that they have no competing interests.

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Data Availability
The data that has been generated and/or analyzed during the present study is available upon request from the corresponding author.

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