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Prevalence of Salmonella Contamination and Phenotypic Antibiotic Resistance Patterns to Tetracyclines and Sulfonamides in Eggs from Selected Iranian Brands

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Abstract

Objective: This study aimed to determine the prevalence of Salmonella contamination in eggs from selected Iranian brands and to investigate the phenotypic antibiotic resistance profiles of the isolated strains against tetracycline and sulfonamide families. In addition, the biofilm-forming ability of the isolates was evaluated as a virulence and survival factor.
Methods: A total of 830 egg samples were collected from various Iranian brands. Isolation and identification of Salmonella were performed using selective media (including XLD and MacConkey agar) and standard biochemical tests. Antibiotic resistance of 22 confirmed isolates was assessed by the disk diffusion method according to CLSI 2019 guidelines. Finally, biofilm-forming ability was evaluated using a microtiter plate assay.
Results: Out of all samples, 22 multidrug-resistant (MDR) Salmonella isolates were recovered. The phenotypic antibiogram revealed a very high level of resistance: 90% of the isolates exhibited resistance to all tetracycline-family antibiotics, including tetracycline and doxycycline. These strains also showed resistance to sulfonamides and nitrofurantoin. Molecular analysis attributed this resistance mainly to the presence of the tetC (81%) and sul2 (95.5%) genes. Moreover, the isolates demonstrated significant biofilm-forming ability, indicating their high potential for persistence and cross-contamination.
Conclusion: Eggs distributed in the Iranian market may serve as potential sources of MDR Salmonella strains. The widespread phenotypic resistance to tetracyclines and sulfonamides—likely resulting from antibiotic misuse in the poultry industry—poses a serious public health concern. Therefore, strict surveillance along the production chain, rigorous enforcement of hygiene regulations, and prudent antibiotic stewardship are essential to prevent the transmission of resistant bacteria to humans.

Keywords: Salmonella, egg, antibiotic resistance, tetracycline, biofilm.

Introduction

Salmonella is recognized as a water- and foodborne pathogenic bacterium capable of infecting both humans and animals, often resulting in severe illness and mortality. The severity of Salmonella infections in humans

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varies depending on the serotype involved and the host's health status [1]. Children under five years of age, the elderly, and immunocompromised individuals are more susceptible to Salmonella infection than healthy individuals. Nearly all Salmonella strains are pathogenic because they can invade, replicate, and survive within human host cells, thereby potentially causing fatal disease [2]. The primary factors contributing to Salmonella pathogenicity include consumption of raw or undercooked foods and inadequate sanitary control during food processing. Intracellular survival and replication within host eukaryotic cells are critical to the pathogenesis of Salmonella enterica infection. Several host-related and environmental factors—such as poor hygiene, corticosteroid administration, excessive antibiotic use, alteration of the normal intestinal microbiota, disruption of nonspecific host defenses (e.g., gastric acidity, intestinal motility, salivary lysozyme, and lactoferrin in the gastrointestinal tract)—can influence the bacterium's capacity to cause disease [3]. Salmonella is one of the leading causes of gastroenteritis in humans and animals, which can enter the host through three primary routes: consumption of contaminated cooked foods (such as poultry, cereals, eggs, and milk), exposure to contaminated manure, and ingestion of raw fruits and vegetables [4]. Salmonellosis, caused by the Gram-negative bacterium Salmonella, remains one of the most important zoonotic diseases worldwide and continues to pose a major global public health concern. This pathogen infects a wide range of hosts and accounts for millions of gastrointestinal infections annually [4]. Eggs and egg-derived products, particularly within the poultry production chain, have been identified as major vehicles for the transmission of paratyphoid Salmonella serotypes, including S. enteritidis and S. typhimurium, to humans. Contamination can occur both vertically (from the infected hen to the egg) and horizontally (via environmental sources, fecal matter, or contaminated equipment contacting the eggshell) [5]. Therefore, determining the prevalence of this bacterium in market eggs is an essential step in risk assessment and food safety management [6]. The extensive use of antibiotics in veterinary medicine—especially in the poultry industry—has created intense selective pressure, leading to the emergence of multidrug-resistant (MDR) strains. These strains can transfer their resistance genes to human pathogens via mobile genetic elements, thereby reducing the efficacy of antimicrobial therapies [7]. Among the antibiotics commonly used in veterinary and food production settings, tetracyclines and sulfonamides remain particularly important due to their long history of use. Global studies have reported that resistance to these antibiotics is highly prevalent among Salmonella isolates transmitted through food sources [8].

In a study conducted in Indonesia [9], researchers investigated antibiotic residues in chicken meat and eggs obtained from traditional markets. A total of 24 chicken meat samples and 24 egg samples were collected from eight traditional markets. The samples were screened for residues of penicillin, aminoglycosides, macrolides, and tetracyclines. The bioassay employed bacterial indicator strains, including Bacillus stearothermophilus, Bacillus cereus, and Bacillus subtilis. The results indicated that 8.33% (2 out of 24) of chicken meat samples contained tetracycline residues, while 75% (18 out of 24) of egg samples were positive for penicillin residues. Residues of aminoglycoside and tetracycline antibiotics were detected in 12.5% (3 out of 24) of egg samples each. Another study conducted in South Africa [10] examined antibiotic prescription practices and antimicrobial resistance in Salmonella isolates originating from the broiler production chain. Data on antibiotic use were collected through a structured questionnaire covering 21 antimicrobial agents, while antimicrobial resistance (AMR) testing was performed for 18 antibiotics commonly used against Salmonella in humans. Salmonella isolates were identified and tested for antimicrobial resistance using modified ISO 6579 protocols and an invA PCR. The pathogen was detected in 23% of tested samples (261 out of 1,135 samples, including carcasses, products, and environmental specimens). Over 80% (145 out of 181) of the reported antimicrobial prescriptions within the National Broiler Production Value Chain (NBPVC) had been administered without veterinary supervision. The most frequently used antibiotics, both therapeutically and prophylactically, included enrofloxacin (63% and 24%), tetracyclines (58% and 33%), and erythromycin (50% and 17%), respectively. A study conducted in Sudan [11] investigated the presence of tetracycline residues in eggs. A total of 180 egg samples were randomly collected from 18 retail outlets. The microbiological inhibition method, using Bacillus subtilis as the test organism, was applied to screen for the presence of antibiotic residues. Ninety egg samples that

tested positive in the microbiological assay were further analyzed by high-performance liquid chromatography (HPLC) to detect and quantify oxytetracycline residues. The microbiological inhibition test revealed that 50% of the examined samples contained detectable antibiotic residues, with positive results reported for 34 (18.9%), 28 (15.6%), and 28 (6.6%) samples across different antibiotic categories. Therefore, assessing the phenotypic resistance patterns of Salmonella isolates from eggs to these antibiotic families provides insight not only into animal health status but also into the direct risks associated with human food consumption [12]. The emergence of antibiotic-resistant Salmonella strains represents a major public health concern worldwide. In addition, Salmonella's ability to form biofilms is an important virulence factor that enhances its survival and persistence across diverse environments, including food processing surfaces [13]. Biofilm formation increases the bacterium’s tolerance to environmental stressors such as disinfectants and antibiotics. Accordingly, the present study aimed to determine the prevalence of Salmonella contamination in eggs from selected Iranian brands and to characterize the phenotypic antibiotic resistance patterns of the isolated strains against the tetracycline and sulfonamide families. Furthermore, the pathogenic potential of these isolates was evaluated by assessing their biofilm-forming ability, providing a comprehensive overview of the health risks they pose.

2. Materials and Methods

Experimental Details: This study consisted of several sequential stages, including sampling, sample preparation, isolation and identification of Salmonella, storage of isolates, antibiotic susceptibility testing, and biofilm formation assay.

Sampling Design: A total of 830 egg samples from 36 commercial Iranian brands, each with a clearly labeled expiration date, were collected from northern regions of Iran. The samples were transported under aseptic conditions to the microbiology laboratory for Salmonella isolation and analysis.

Study Period: Although the exact sampling period is not specified, antibiotic resistance testing was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines, 2019 edition, ensuring that the evaluation methods reflected up-to-date and standardized antimicrobial susceptibility testing procedures.

Sample Preparation and Isolation of Salmonella

A total of 830 egg samples from 36 commercial Iranian brands, each with a clearly labeled expiration date, were collected from northern regions of Iran. After transportation to the laboratory, egg yolks were separated from the albumen, and the internal contents were thoroughly homogenized. The homogenized samples were stored at 4 °C until microbiological analysis was performed. For Salmonella isolation, aliquots of the homogenized yolk and albumen were inoculated onto selective and enrichment media, including MacConkey agar (Merck, Germany) and Xylose Lysine Deoxycholate (XLD) agar. The plates were incubated at 37 °C for 24 hours. These procedures were conducted according to standard Salmonella detection protocols, such as ISO 6579 [14].

Identification of Salmonella: Suspicious colonies—characterized by colorless appearance on MacConkey agar and black or pink colonies on XLD agar—were subjected to microscopic and biochemical identification tests, as summarized in Table 1.

Table 1. Biochemical and Diagnostic Tests Used for the Identification of Salmonella

Diagnostic Test	Purpose and Culture Medium Used
Gram Staining	Observation of Gram-negative bacilli
Triple Sugar Iron (TSI) Agar	Evaluation of carbohydrate fermentation, gas production, and hydrogen sulfide (H_2S) formation
Sulfur–Indole–Motility (SIM) Agar	Determination of H_2S production, motility, and indole formation
Simmons Citrate Agar	Assessment of the ability to utilize citrate as the sole carbon source
Urease Test	Determination of the ability to hydrolyze urea
Carbohydrate Fermentation Tests	Evaluation of glucose, lactose, and arabinose fermentation in phenol red medium
Voges–Proskauer (VP) Test	Differentiation of glucose fermentation pathways

[15]

Confirmation and Preservation of Isolates
Isolates exhibiting all characteristic biochemical features of Salmonella—including Gram-negative morphology, hydrogen sulfide (H_2S) production, and negative urease activity—were identified as Salmonella and preserved for subsequent analyses. Final confirmation of Salmonella identity can be achieved through molecular methods such as polymerase chain reaction (PCR) targeting the invA gene, a specific genetic marker for the Salmonella genus [16].

Storage of Isolates: Confirmed Salmonella isolates were stored in liquid broth supplemented with 30% glycerol at −70 °C to ensure long-term viability and stability for further testing.

Antibiotic Susceptibility Testing
The antibiotic susceptibility of 22 selected Salmonella isolates was determined using the standard disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2019) guidelines [17].

Preparation of Bacterial Suspension: Bacterial suspensions were prepared from 24-hour cultures of each isolate in sterile physiological saline. The turbidity of the suspension was adjusted to match the 0.5 McFarland standard.

Inoculation and Incubation: A uniform bacterial lawn was prepared on Mueller–Hinton agar using a sterile cotton swab. Commercial antibiotic disks (Padtan Teb Co., Iran) were placed on the inoculated plates, which were then incubated at 37 °C for 24 hours.

Antibiotics Tested: This study evaluated resistance to tetracycline- and sulfonamide-family antibiotics, as listed in Table 2 [18]. These antibiotics were selected for their widespread use in veterinary practice, particularly in the poultry industry, and for their relevance to food safety and public health.

Table 2. Antibiotics Used for Resistance Testing within the Tetracycline and Sulfonamide Families

Antibiotic Group	Antibiotic Disks Used
Tetracyclines	Chlortetracycline, Tetracycline, Doxycycline, Oxytetracycline
Sulfonamides	Sulfonamide disks or combined formulations such as Sulfamethoxazole/Trimethoprim (used when single sulfonamide disks are not available in the laboratory supply list to ensure comprehensive coverage)

[18]

Interpretation of Results

The diameters of the inhibition zones around each antibiotic disk were measured, and the results were interpreted and reported as sensitive (S), resistant (R), or intermediate (I) according to the CLSI 2019 guidelines. Biofilm formation, as a virulence factor, was evaluated in all 22 Salmonella isolates under laboratory conditions using the microtiter plate assay [19]. A suspension equivalent to 0.5 McFarland standard was prepared in trypticase soy broth (TSB) supplemented with 2% glucose, and 200 µL of this suspension was transferred into the wells of sterile 96-well polystyrene microplates. The plates were incubated at 37 °C for 96 hours. After incubation, the wells were gently washed with phosphate-buffered saline (PBS), fixed with 95% methanol, and stained with 1% methylene blue. The formed biofilm was then solubilized with 33% glacial acetic acid, and its optical density was measured at 570 nm using an ELISA reader. This method, employing crystal violet staining, is also widely used for the quantitative assessment of biofilm formation [20]. Based on the objectives of the study, the implicit hypotheses included the following:

1. Eggs from selected Iranian brands are contaminated with Salmonella.
2. Salmonella isolates recovered from these eggs exhibit high phenotypic resistance to antibiotics belonging to the tetracycline and sulfonamide families.
3. The observed phenotypic resistance is associated with specific resistance genes, such as tetC and sul2.
4. The isolated Salmonella strains can form biofilms.

References for Methodology

The methodological references used in this study included the CLSI 2019 guidelines for antibiotic susceptibility testing, as well as the application of standard culture media such as MacConkey agar, Xylose Lysine Deoxycholate (XLD) agar, Mueller–Hinton agar, and Trypticase Soy Broth (TSB). Standard biochemical reagents, including methanol, methylene blue, and glacial acetic acid, were also employed [21]. Moreover, the procedures for Salmonella isolation and identification followed established international protocols, including ISO 6579 standards [22].

Data Analysis

Experimental data were statistically analyzed using SPSS software. The relative frequency of contamination, the percentage of antibiotic resistance, and the prevalence of resistance genes were calculated and compared across the different egg brands.

3. Results and Discussion

The primary objective of this study was to determine the prevalence of Salmonella contamination in eggs from selected Iranian brands. Among the 830 egg samples analyzed, 22 multidrug-resistant (MDR) Salmonella isolates were recovered. The isolation of pathogenic strains from such a widely consumed food source underscores the need for rigorous hygienic controls and continuous monitoring throughout the production and distribution chain. Salmonella identification based on phenotypic tests—including Gram-negative bacilli morphology, a yellow/red pattern in Triple Sugar Iron (TSI) agar with H_2S production, positive MR and Simmons citrate tests, and a negative urease reaction—was entirely consistent with the characteristic microbiological profile of the genus. Table 3 summarizes the study's key findings.

Table 3. Summary of Key Findings

Finding	Percentage / Number	Description
Number of egg samples	830	Collected from various Iranian brands
Number of MDR Salmonella isolates	22	Out of the total samples
Phenotypic resistance to tetracyclines	90%	Including oxytetracycline, doxycycline, chlortetracycline, and tetracycline
Phenotypic resistance to sulfonamides and nitrofurantoin	—	All isolates exhibited resistance
Presence of the tetC gene	81%	Associated with tetracycline resistance
Presence of the sul2 gene	95.5%	Associated with sulfonamide resistance
Biofilm-forming ability	Significant	Indicates high potential for persistence and cross-contamination

Source: Study findings

The findings of this study, which indicate the isolation of pathogenic Salmonella strains from a highly consumed food source such as eggs, reaffirm the importance of hygienic controls and continuous surveillance throughout the production and distribution chain. The reported prevalence of Salmonella contamination in eggs varies considerably across global and regional studies, ranging from 0 to 13.3% in Europe, and from 0.6% in Birjand to 19% in Talesh, Iran [23]. Other studies have also reported variable contamination rates, including 5.6% in China [24], 2.7% in Ethiopia [25], 5.5% in Ghana [26], and 4.85% in Iraq [27]. Such regional variations highlight the importance of localized epidemiological assessments and reflect major differences in hygienic practices and poultry farm management systems [28].

The isolation of 22 resistant strains from the analyzed samples suggests the presence of an active, potentially hazardous Salmonella reservoir within the production chain of Iranian egg brands. The phenotypic resistance profiles of these 22 Salmonella isolates represent a serious public health concern. Ninety percent of the isolates exhibited resistance to all tetracycline family antibiotics, including oxytetracycline, doxycycline, chlortetracycline, and tetracycline. According to disk diffusion test results, 18 of 20 tested isolates demonstrated resistance to the antibiotics tested (Figure 1). The high level of resistance observed is most likely a consequence of the widespread and uncontrolled use of these antibiotics in the poultry industry, primarily driven by their low cost and easy accessibility [29].

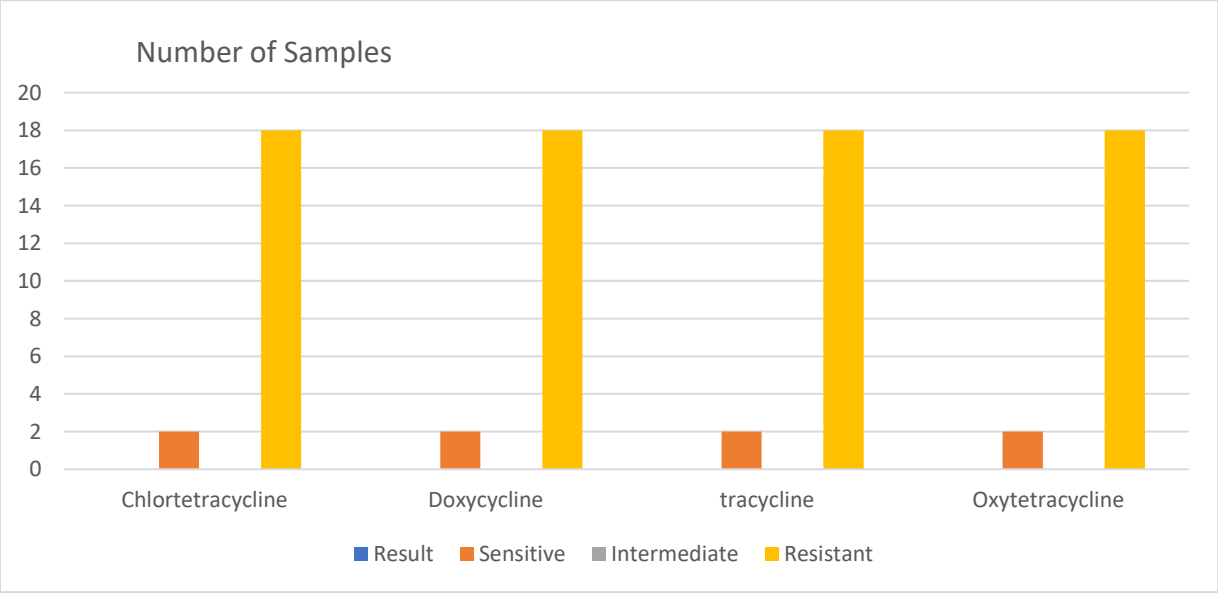
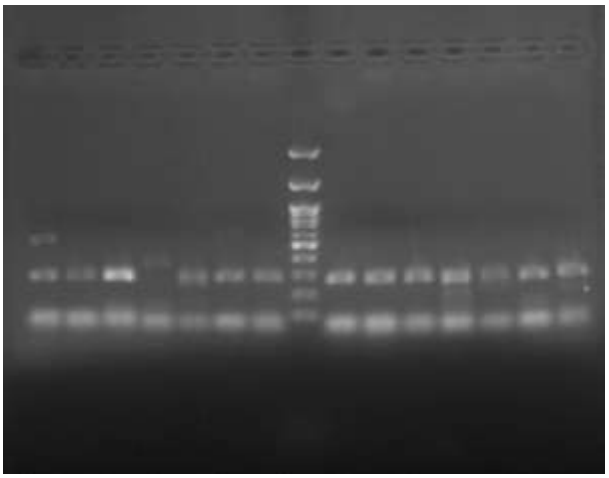


Figure 1. Number of Antibiotic-Resistant Bacterial Isolates

The widespread phenotypic resistance to tetracyclines, along with resistance to sulfonamides and nitrofurantoin, classified the isolates as multidrug-resistant (MDR) strains. The presence of such resistance among foodborne pathogens poses a direct threat to the effectiveness of therapeutic regimens in humans infected through the consumption of contaminated eggs. Resistance to tetracyclines and sulfonamides in Salmonella isolates recovered from food sources, including eggs, has been extensively reported in previous studies [30–33]. In the present study, molecular mechanisms of resistance were further investigated, revealing that the observed phenotypic resistance correlated with the presence of specific resistance genes such as tetC (detected in 81% of isolates) and sul2 (detected in 95.5% of isolates). These genes are known to confer resistance to tetracyclines and sulfonamides, respectively, in Salmonella [34]. The findings confirm a strong association between phenotypically observed resistance patterns and their corresponding genetic determinants. Based on PCR amplification and gel electrophoresis results, the tetC gene was detected in 16 out of 20 tested isolates (Figure 1). Considering that resistance genes—particularly in Gram-negative bacteria such as Salmonella—are frequently located on mobile genetic elements (e.g., plasmids), the presence of these genes in egg-derived isolates suggests a potential for horizontal gene transfer to other bacteria within the human gastrointestinal tract, thereby contributing to the establishment of a community-wide resistance reservoir [35]. As illustrated in Figure 1, in most isolates, resistance to tetracycline antibiotics was associated with the tetC gene. Additionally, PCR and electrophoresis results indicated that the sul1 gene was detected in one isolate (Figure 2).



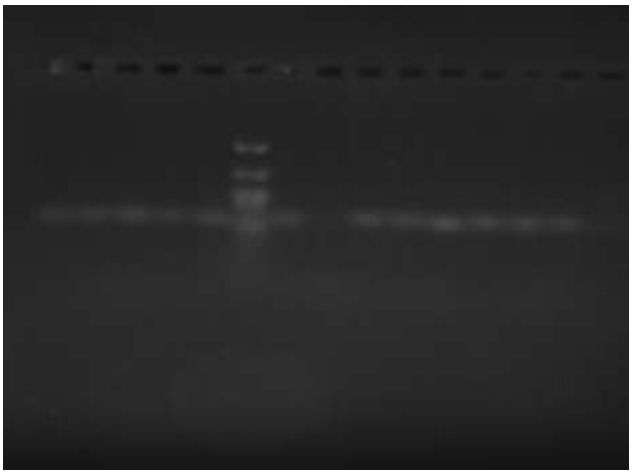


Figure 1. Agarose gel electrophoresis showing the presence or absence of the tetC gene.

Figure 2. Agarose gel electrophoresis showing the presence or absence of the sulI gene.

Given that resistance genes—particularly in Gram-negative bacteria such as Salmonella—are often located on mobile genetic elements (e.g., plasmids), their presence in isolates recovered from eggs suggests the potential for horizontal gene transfer to other bacteria within the human gastrointestinal tract. This process could contribute to the establishment of a broader reservoir of antimicrobial resistance within the community. Therefore, strict monitoring and control of poultry and egg trade, along with prudent antibiotic prescription practices, represent the most critical measures to limit the spread of Salmonella and its antimicrobial resistance.

Furthermore, the isolated strains exhibited a considerable capacity for biofilm formation. Biofilm development by Salmonella serves as an essential survival and persistence strategy under unfavorable environmental conditions [36]. Biofilms enhance bacterial tolerance to various stressors, including disinfectants and antibiotics [37], allowing Salmonella to persist on diverse surfaces—such as food-

processing equipment—and thereby promote cross-contamination [38]. Genes such as csgD and fimA play pivotal roles in biofilm formation, and their expression may be influenced by environmental factors such as temperature [39].

5. Conclusion

This study demonstrated that eggs from selected Iranian brands serve as potential sources of multidrug-resistant (MDR) Salmonella strains. The high and nearly universal (90%) phenotypic resistance to tetracycline-class antibiotics poses a serious threat to consumer health and underscores the urgent need to reevaluate the use of these drugs in the poultry industry. The similarity in resistance gene profiles among different isolates supports the hypothesis of horizontal gene transfer and the dissemination of resistance through the production and consumption chain. Continuous surveillance of protein-based food products is therefore essential to prevent the transmission of MDR bacteria to humans. Future research should focus on identifying specific Salmonella serotypes, elucidating more detailed mechanisms of genetic resistance transfer, and evaluating the effectiveness of control measures in reducing the prevalence and resistance of Salmonella in poultry products.

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