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Detection and Molecular Characterization of Salmonella Enterica Serovar Enteritidis in Household Chicken Eggs: A Case Study From Erzurum, Türkiye

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Aile Tipi Tavuk Yumurtalarında Salmonella Enterica Serovar Enteritidis'in Tespiti ve Moleküler Karakterizasyonu: Erzurum İli, Türkiye'den Bir Vaka Çalışması

ABSTRACT

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) is the most predominant serovar in poultry and eggs, and it poses a significant threat to public health worldwide as it is a leading cause of salmonellosis in humans, which is transmitted through the consumption of contaminated poultry products like eggs. The objective of this study was to evaluate the presence of *S.* Enteritidis in household chicken eggs collected from Erzurum Province, Türkiye. A total of 168 household chicken eggs were collected from 168 small family poultry farms. *Salmonella spp.* was not isolated from any of the examined egg internal contents; however, successful isolation and identification of *Salmonella spp.* were achieved in 4 of the shell samples. Further characterization of the identified *Salmonella spp.* isolates was performed at the serovar level using 16S rDNA sequence analysis, and all 4 isolates were identified as *S.* Enteritidis. In conclusion, this study highlights the inherent risk of *S.* Enteritidis contamination in household chicken eggs and emphasizes the vital significance of implementing stringent food safety measures to safeguard consumer well-being and ensure the protection of public health.

Keywords: Chicken, food safety, molecular, *Salmonella* Enteritidis, 16S rDNA

ÖZ

Salmonella enterica serovar Enteritidis (*S.* Enteritidis), kümes hayvanları ve yumurtalarda en yaygın serovardır. Dünya genelinde halk sağlığı için önemli bir tehdit oluşturmaktadır çünkü insanlarda salmonellozun önde gelen nedenidir. Kontamine tavuk ürünleri, özellikle yumurtaların tüketimi yoluyla insanlara bulaşır. Bu çalışmanın amacı, Türkiye'nin Erzurum ilinden toplanan evcil tavuk yumurtalarında *S.* Enteritidis'in varlığını değerlendirmektir. Toplam 168 evcil tavuk yumurtası, 168 adet küçük aile tipi tavuk çiftliğinden toplandı. *Salmonella* spp., incelenen hiçbir yumurta içeriğinden izole edilmedi. Ancak *Salmonella spp*.'nin başarılı izolasyonu ve tanısı 4 kabuk örneğinde elde edildi. Tanımlanan *Salmonella spp*. izolatlarının serovar düzeyinde daha fazla karakterizasyonu için 16S rDNA sekans analizi kullanıldı. Tüm izolatlar *S.* Enteritidis olarak tanımlandı. Sonuç olarak, bu çalışma ev tipi tavuk yumurtalarında *S.* Enteritidis kontaminasyon riskini ortaya koymakta, tüketicinin sağlığını korumak ve halk sağlığını güvence altına almak için sıkı gıda güvenliği önlemlerinin uygulanmasının hayati önemini vurgulamaktadır.

Anahtar Kelimeler: Tavuk, gıda güvenliği, moleküler, *Salmonella* Enteritidis, 16S rDNA

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INTRODUCTION

Salmonella enterica serovar Enteritidis (*S*. Enteritidis) is a prominent serovar commonly found in poultry and eggs, representing a significant public health concern worldwide.[1] Consumption of contaminated eggs, has been identified as a primary mode of transmission for salmonellosis in humans.[2] Traditional microbiological methods for detecting Salmonella in eggs and egg products typically require around five days.[3] However, these methods are time-consuming, labor-intensive, and expensive. They involve the isolation and cultivation of bacteria followed by biochemical and serological identification, which can be prone to false-negative results or misidentification. Additionally, the prolonged time required for analysis hinders timely intervention and control measures. On the other hand, Polymerase Chain Reaction (PCR) provides highly specific and sensitive tools for confirming the presence of *S.* Enteritidis. PCR targets specific regions of the bacterial genome and amplifies them, allowing for rapid detection and identification. This method has been widely utilized in various studies for its ability to detect *S*. Enteritidis with high accuracy.[4,5]

Genotyping methods play a pivotal role in characterizing the genetic diversity and relatedness of *S.* Enteritidis isolates.[6] These techniques enable the differentiation and classification of bacterial strains based on their genetic profiles, facilitating epidemiological investigations and providing insights into the transmission dynamics of the pathogen. One commonly used genotyping approach involves the amplification and sequencing of the 16S rDNA gene region. The 16S rDNA gene region is highly conserved among bacteria, making it an ideal target for genotyping studies.[7] The gene region contains both conserved regions that allow for primer design and variable regions that provide discriminatory power for distinguishing between different *S.* Enteritidis strains.[8]

The number of laying hens in Türkiye has reached a staggering 124 million, positioning the country as the 8th largest producer of hen eggs worldwide, with a production volume of 1.2 million tons.[9] The registration of backyard poultry flocks in Türkiye is currently a voluntary practice undertaken by various hobby and ornamental poultry associations. As a result, there is a lack of precise information regarding the number of owners, flock sizes, locations, and management practices. Moreover, with the growing prevalence of backyard poultry farming in urban areas, regional authorities often face difficulties in establishing a comprehensive legal framework to address the situation effectively. Municipalities throughout the country are encountering challenges in regulating backyard poultry farming within residential areas due to the absence of enforceable legislation in Türkiye that delineates the guidelines for raising poultry in such settings.[10] Erzurum Province, located in Türkiye, is renowned for its widespread adoption of house-type poultry farming, which highlights the importance of conducting comprehensive surveillance and characterization of *S.* Enteritidis in this area. The objective of this study was to evaluate the presence of *S.* Enteritidis in household chicken eggs collected from Erzurum Province, Türkiye. Moreover, PCR and genotyping techniques were employed for the confirmation, identification, and categorization of isolates by conducting 16S-rDNA gene sequencing.

MATERIALS AND METHODS

Animals and Experimental Design

The Atatürk University Local Ethical Committee approved the study with a protocol (Date: 19/10/2022). A total of 168 household chicken eggs were collected from 168 small family poultry farms in Erzurum province, Türkiye, between January and March 2023 (Figure 1). These farms primarily produce eggs for their own consumption. The characteristics of the poultry farms included having less than 40 animals, with the majority of chicks hatching through the natural brooding process. The animals were allowed to roam freely and interact with neighboring habitations, and no prophylaxis or veterinarian controls were implemented for the animals. The sampling achieved directly from the depository. The eggs were collected in sterile containers and transported to the laboratory under refrigerated conditions and processed on the same day.

Figure 1. The geographic location of Erzurum province, Türkiye.

Isolation and Detection of Salmonella

The egg samples collected were subjected to separate culture processes for both the eggshell and egg content, following the method described earlier.[11] To initiate the process, sterile swabs were moistened by dipping them in sterile peptone water and then applied to the entire eggshell. Subsequently, the swab samples were transferred into 4 mL of peptone water. Next, the eggs underwent sterilization by being fully immersed in 2% tincture iodine for 1 minute. Following sterilization, the eggs were transferred into sterile bags, where they were broken and

mixed with 50 mL of peptone water. The egg yolk was thoroughly mixed until it achieved complete dispersion and homogeneity. Subsequently, a 1 mL sample was extracted from the mixture and transferred into 4 mL of peptone water.

The samples of eggshell and egg content were incubated at a temperature of 37°C for a duration of 24 hours. Following the incubation period, 100 µL of each peptone water sample was extracted and transferred into Rappaport Vassiliadis broth, which was then incubated at a temperature of 42°C for another 24 hours. Subsequently, loopfuls of both cultures were streaked onto selective agar plates, including Brilliant Green agar (BG), Xylose Lysine Deoxycholate agar (XLD), and Salmonella-Shigella agar (SS). The agar plates were then incubated at 37°C for 24 hours. Presumptive colonies were selected from each plate and subsequently subcultured in TSI slant medium and various differential culture media, including MR-VP broth, urea agar, SIM agar, and Simmon's citrate agar. The subcultures were then incubated at 37°C for 24 hours. A single colony of Salmonella spp. isolates was suspended in 500 µL of phosphate-buffered saline. Meanwhile, a colony was placed in TSB supplemented with 15% glycerin and stored at -20°C for subsequent analysis.

DNA Extraction

A commercial ready-made nucleic acid isolation kit (Qiagen, Hilden, Germany) was utilized for DNA extraction. The DNA extraction process was carried out on Salmonella spp. isolates suspended in PBS, which were in the culture stage. The resulting nucleic acids were subsequently stored at -20°C for analysis. Molecular identification and phylogenetic analysis of the isolated Salmonella spp. isolates were performed using 16S rDNA analysis.

PCR Amplification and DNA Sequence Analysis

Salmonella spp. isolated in culture were subjected to DNA sequence analysis. To distinguish each Salmonella spp. isolate based on the order of isolation, a unique "chicken egg" (CE) code was assigned to each isolate. Prior to sequence analysis, genomic DNA extraction was performed using the cador pathogen mini kit (Qiagen, Hilden, Germany). The quantity and purity of the extracted nucleic acids were assessed using Thermo Scientific Nanodrop 2000. Subsequently, the 16S rDNA gene region was amplified for DNA sequencing using 27F and 1492R primers, as described earlier.[12] PCR reactions were conducted in 0.2 ml tubes with a final volume of 35 µL. Each reaction contained 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTP mix, 0.3 µM of each primer, 2 U of Taq DNA polymerase, and 3 µL of template DNA. The remaining volume was made up to 35 µL using sterile distilled water. The PCR cycling conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 57°C for 45 seconds, and elongation at 72°C for 1 minute. A final elongation step was performed at 72°C for 5 minutes. The DNA sequence analysis was carried out on the obtained amplicons to determine the nucleotide sequence information.

DNA sequencing was performed using the Oxford Nanopore Technologies® MinION™ Mk1C device. The Rapid Barcoding Kit 96 (SQK-RBK110.96) was utilized for sequence analysis. DNA samples were measured using the Qubit 1X dsDNA BR (Broad Range) Assay Kit. Each DNA sample was extracted and transferred to a sterile microcentrifuge tube with a volume of 1.5 ml, and the volume was adjusted to 9 µl with sterile distilled water. Barcodes were added to the prepared DNA samples separately, with 1 µl for each. For barcoding, heat treatment was applied at 30°C for 2 minutes and at 80°C for 2 minutes. Following the heat treatment, all barcoded DNAs were combined in the same microcentrifuge tube, and library preparation was conducted according to the kit protocol.

After library preparation, loading was performed onto the MinION Flow Cell (R9.4.1), which was attached to the Oxford Nanopore Technologies® MinION™ Mk1C device, and sequencing was conducted. The sequencing process involved obtaining a minimum of 300 reads for each barcode, and the resulting Fastq and Fast5 files were analyzed using Geneious Prime 2022.1.1. From the analysis, the consensus sequence data obtained was subjected to NCBI BLAST analysis. The genetic relationships between the obtained 16S rRNA genes and representative strains of Salmonella spp. were inferred using the MEGA X software, employing the maximum likelihood method based on Kimura 2-parameter model with 1000 bootstrap.

RESULTS

The analysis revealed that *Salmonella spp*. was not isolated from the internal contents of the egg samples. However, *Salmonella spp*. was found to be isolated from only 2.3% (4/168) of the outer surface samples of the examined eggs (Figure 2).

The CE1 DNA sample showed 100% similarity with *S*. Enteritidis strain SEO, identified by the Sequence ID CP033090.1 in the NCBI Blast search. The CE2 DNA sample exhibited 99.93% similarity with *S*. Enteritidis strain NCM 61 (Sequence ID: CP032851.1), *S*. Enteritidis strain SEE2 (Sequence ID: CP011791.1), and *S*. Enteritidis strain SEE1 (Sequence ID: CP011790.1). The analysis of the CE3 labeled strain's 16S rDNA identified it as an isolate that shared the

highest similarity (100%) with a bacterial sequence. *S*. Enteritidis strain MFDS1018147 exhibited 100% similarity to the CP110220.1 gene region, *S*. Enteritidis strain MASJG9 to the OP744581.1 gene region, *S*. Enteritidis strain R17.1476 to the CP100724.1 gene region, *S*. Enteritidis strain R18.1630 to the CP100666.1 gene region, *S*. Enteritidis strain SE006 to the CP099973.1 gene region, *S*. Enteritidis strain PNUSAS034908 to the CP092321.1 gene region, *S*. Enteritidis strain CVM N17S192 to the CP082726.1 gene region, *S*. Enteritidis strain CVM N17S111 to the CP082729.1 gene region, *S*. Enteritidis strain CFSAN051827 to the CP075122.1 gene region, *S*. Enteritidis strain CFSAN051882 to the CP075120.1 gene region, *S*. Enteritidis strain CFSAN022640 to the CP075019.1 gene region, *S*. Enteritidis strain CFSAN008104 to the CP074661.1 gene region, *S*. Enteritidis strain CFSAN051890 to the CP075118.1 gene region, *S*. Enteritidis strain CFSAN026631 to the CP074254.1 gene region, and *S*. Enteritidis strain CFSAN026633 to the CP074252.1 gene region, all with 100% similarity. The analysis of the CE4 isolate revealed a 100% similarity to the CP007245.1 gene region of *S*. Enteritidis strain EC20120008.

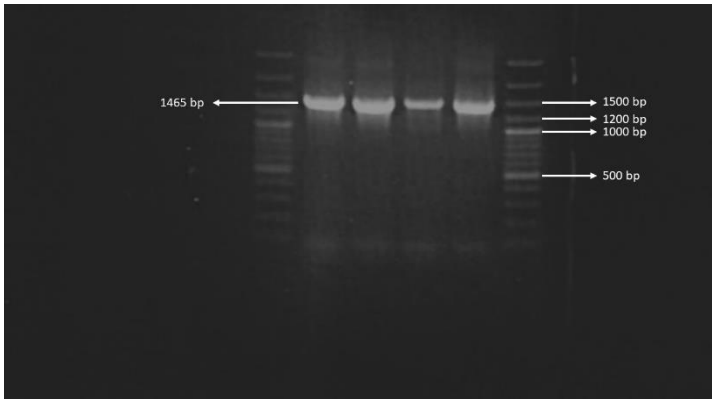

Figure 2. The image of positive S. Enteriditis PCR reactions is shown. In the agarose gel image of S. Enteriditis positive egg samples; the reference indicator (1st place marker (100-3000 bp), second (CE1), third (CE2), fourth (CE3), and fifth (CE4) are 1465bp bands belonging to positive samples.

The neighbor-joining phylogenetic tree generated using Mega X software is shown in Figure 3. The DNA sequences determined in the analysis have been uploaded to the NCBI GeneBank, and accession numbers were obtained for the CE1, CE2, CE3, and CE4 isolates, respectively, as OQ108763.1, OQ108764.2, OQ108765.1, and OQ108766.1.

DISCUSSION

The objective of the present study was to isolate *Salmonella spp*. from both eggshells and eggs, and to perform phylogenetic characterization through 16S rDNA sequence analysis, utilizing egg samples collected from the Erzurum province.

Figure 3. Phylogenetic tree based on the nucleotide sequences of 16S rDNA genes. The tree was constructed by the neighbor-joining method, using the computer program (Mega X software).

The prevalence of *Salmonella spp.* in eggs across European Union countries is reported to be 0.8%,[13] while in France, the prevalence of *Salmonella spp*. on eggshells was found to be 1.05%.[14] In China, 7.1% of *Salmonella spp*. was isolated from eggshells intended for consumption, and 8.3% from the internal contents of the eggs.[15] Notably, a study conducted in Iran reported a detection rate of 1.33% for *Salmonella spp*. on eggshells, whereas *Salmonella spp*. could not be isolated from the egg content.[16] In a study carried out in Poland, *Salmonella spp*. could not be isolated from both the shell samples and the egg content of 1200 eggs.[17] Conversely, in India, *Salmonella spp.* was detected on 6.1% of eggshells and in 1.8% of the egg content.[18] In the presented study, the observed isolation rate from both eggshells and egg content is consistent with findings from similar studies.[13,14]

The contamination of eggs with *Salmonella spp.* can occur through two distinct routes: horizontal transmission, which involves colonization of the digestive tract and subsequent contamination with feces, or vertical transmission, wherein the reproductive organs become infected with *Salmonella spp*.[13] The study's specific isolation from eggshells suggests the occurrence of horizontal transmission.

More than 90% of *Salmonella* species isolated in European countries are reported to be *S.* Enteritidis.[13] Similarly, a study conducted in India found that 86% of the serotypes isolated from eggshells were *S*. Enteritidis.[18] The results of our study conducted in the province of Erzurum indicate that the isolation of only *S.* Enteritidis from eggshells is consistent with the literature.

The findings from our analysis of the DNA samples obtained from different *S.* Enteritidis strains provide valuable insights into the genetic characteristics and relatedness of these isolates. The CE1 and CE2 isolates exhibited significant degrees of similarity, indicating a potential genetic lineage or shared ancestry with the reference strains. These reference strains include SEO (unpublished data), isolated from human feces in China, NCM 61 isolated from chicken meat in China, and SEE2 and SEE1 (unpublished data), both isolated from eggshells. Close genetic similarity was observed between the CE3 isolate from traditional Japanese food (CP110220.1=unpublished) in South Korea, broiler chicken secal content in Pakistan (OP744581=unpublished), human feces (CP100724=unpublished) in Taiwan, chicken (CP099973) in Taiwan,[19] human feces in the USA,[20] chicken meat in the USA,[21] and mouse, goose, duck, domestic pig, and spinach samples in the USA (CP075122, CP075019). Furthermore, these isolates exhibited a close genetic similarity to *S.* Enteritidis isolates CP074254, CP075118, CP074252, CP074661, and CP075120 (unpublished). The remarkable similarity of the CE3 isolate with strains isolated from diverse sources across different geographic locations highlights its significance for public health. Furthermore, the 100% similarity observed between the CE4 isolate and the gene region of *S*. Enteritidis strain EC20120008, isolated from a reptile in Canada by previous report,[22] suggests a potential clonal relationship between these two isolates. These findings enhance our understanding of the genetic characteristics of *S.* Enteritidis strains and provide a foundation for future studies on their epidemiological and pathogenic properties.

Despite the valuable insights provided by our analysis into the *S.* Enteritidis strains, it is important to acknowledge certain limitations that should be considered when interpreting the results. Firstly, the identification of *S.* Enteritidis strains based on genetic similarity through the NCBI Blast search and gene region analysis has its inherent limitations. The method relies on the available reference sequences in the database, and the accuracy of the identification is dependent on the comprehensiveness and quality of the reference database. Incomplete or insufficient representation of *S.* Enteritidis strains may lead to potential misidentification or incomplete characterization. Secondly, the analysis focused on a limited number of samples (CE1, CE2, CE3, and CE4 isolates). While these samples provided valuable information, they may not be fully representative of the overall *S*. Enteritidis population in the region. The genetic diversity and prevalence of different strains may vary, and additional samples from different sources and regions would provide a more comprehensive understanding of the *S*. Enteritidis population dynamics. Furthermore, the study employed specific molecular techniques, such as NCBI Blast search and gene region analysis, to assess genetic similarity and identify strains. While these techniques are widely used and reliable, they have their own limitations. Other genetic analysis methods, such as whole-genome sequencing, could provide more detailed information about the genetic characteristics, virulence factors, and potential antimicrobial resistance profiles of the strains. Future studies incorporating these advanced techniques would enhance the understanding of *S.* Enteritidis diversity and its implications. Lastly, the phylogenetic tree generated using the Mega X software represents a visual representation of the genetic relationships among the analyzed strains. However, the accuracy and reliability of the tree are dependent on the quality of the input data and the chosen methodology. Alternative phylogenetic analysis methods or additional statistical support would strengthen the robustness of the findings.

In conclusion, our analysis demonstrated significant genetic similarities between the tested samples and various strains of *S.* Enteritidis. These findings suggest the presence of closely related strains in the studied region. However, it is important to note that genetic similarity does not necessarily imply identical characteristics in terms of virulence or antimicrobial resistance. Future studies should focus on further characterizing these strains to gain a more comprehensive understanding of their phenotypic traits and their potential impact on public health.

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