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Research article

GENOTYPIC CORRELATION BETWEEN *SALMONELLA* ENTERITIDIS ISOLATES FROM BROILER BREEDERS AND HATCHERY FLOCKS

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In this study, *Salmonella* Enteritidis strains isolated from dust and environmental materials from different flocks located in Turkey's Western Black Sea region were examined by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). A total of 59 *S*. Enteritidis strains isolated from broiler breeder and hatchery flocks, and one *S*. Enteritidis strain isolated from a stool sample of a farm worker were examined. PFGE analysis revealed two major PFGE groups and nine different macro restriction profiles. It was determined that 85% (51/60) of the strains were close to each other and comprised Group I. All *S*. Enteritidis strains had the same sequence type (ST): ST11. Isolation of strains with a single genotype suggests that there may be a cross transmission between the flocks.

Keywords: multilocus sequence typing, phylogeny, pulsed field gel electrophoresis, *Salmonella*

INTRODUCTION

Salmonella enterica serovars are one of the main causes of foodborne infections in humans [1,2]. Intestinal colonization of chickens by *Salmonella* and excretion through feces leads to gastroenteritis in humans, especially by entering the food chain [3]. The most important food sources of *Salmonella* infections are meat, milk and eggs obtained from chickens [4].

Salmonella contamination has been a continuous problem since the 1900s, especially in the poultry industry. For this reason, *Salmonella* control programs are being implemented in many countries [5,6]. Although there are more than 2.500 *Salmonella* serovars, *Salmonella* Enteritidis is the most common serovar in *Salmonella* infections of humans [7,8]. Studies show that *S*. Enteritidis strains can be transmitted to humans from chickens and eggs [9]. In 2017, *Salmonella* was found in 3.31% of broiler flocks while the isolation rate from fresh broiler meat served as prepared food was 4.85% [10]. In Turkey, the isolation rate of *S*. Enteritidis in broiler chickens was 9% [11].

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Molecular typing is an important tool for identifying the sources of outbreaks and for epidemiological research. Pulsed Field Gel Electrophoresis (PFGE) is a form of restriction fragment length polymorphism analysis, which has been considered as the gold standard method for molecular typing of *Salmonella* serovars [12-16]. Multilocus sequence typing (MLST) is another discriminatory subtyping method based on determining nucleotide sequences in a series of housekeeping, ribosomal, and/or virulence-associated genes in bacteria [17-20]. Although MLST is expensive, it is used for typing clinically important bacterial pathogens, including *Neisseria meningitidis, Staphylococcus aureus, Yersinia pestis, Streptococcus pneumoniae, Vibrio cholerae, Campylobacter jejuni*, and *Salmonella* spp. [21,22]. Recently, Multiple Locus Variable Number Tandem Repeat (VNTR) Analysis (MLVA) was performed in order to discriminate the isolates which have same PFGE subtypes [23,24]. Beside this, clustered regularly interspaced short palindromic repeat (CRISPR) typing is also a usable method for subtyping *Salmonella* serovars [25-27]. Whole genome sequencing (WGS) which is used in combination with PFGE for the phylogenetic analyses of highly similar clonal isolates is also a useful approach for determining all genetic variations. All these techniques could be used for the characterization of the pathogens in order to understand their clonal relations [20,28].

In Turkey, there are few studies that investigate the molecular characterization of *S*. Enteritidis strains by PFGE and MLST methods [29-31]. Therefore, this study aimed to characterize and assess the genetic relationships of *S*. Enteritidis serovars isolated from broiler breeder farms and hatchery flocks located in different cities in Turkey's Western Black Sea region by both PFGE and MLST analysis.

MATERIALS AND METHODS

Bacterial strains

In this study 59 *S*. Enteritidis strains were randomly selected from 461 *S*. Enteritidis strains isolated between 2015-2019 from 13 poultry farms in the Western Black Sea region of Turkey. These included 6 broiler breeder and 7 hatchery flocks. One human isolate of *S*. Enteritidis, obtained from the stool sample of a farm worker, was also included. All poultry samples were isolated from dust and litter materials collected from different farms. *Salmonella* strains were isolated and serotyped according to the ISO6579 and Kauffmann White Scheme [32], respectively. To show the evolutionary relationship between *S*. Enteritidis strains, two commonly isolated serovars, *S*. Kentucky (n=2), *S*. Typhimurium (n=1), and two rarely isolated serovars *S*. Albany, (n=1) and *S*. Tennessee (n=1) were included as the out group.

PFGE analysis

Genotyping of the isolates was performed according to the CDC protocol (www.cdc.gov/pulsenet). The bacteria were grown on nutrient agar (Oxoid) at 37°C under

aerobic conditions for 16-18 hours. After incubation, the bacteria were suspended in cell suspension buffer (0.01M Phosphate-Buffered Saline and pH 7.4). Cell suspensions were adjusted according to the manufacturer's recommendations for the spectrophotometer. 400 µl of the cell suspensions were mixed gently with 20 µl proteinase K (20 mg/ml stock, Thermo Scientific). 400 µl of 1% SeaKem Gold agarose melted in TE (10 mM Tris, 1 mM EDTA, pH 8.0) were added to the cell suspensions and mixed gently by pipetting three times. The mixture was dispensed immediately into the wells of reusable plug molds and left for 10 min at room temperature to solidify. The plugs were then transferred to 50 ml tubes containing 5 ml Cell Lysis Buffer (50 mM Tris, 50 mM EDTA, pH 8.0 with 1% sarcosyl and 25 µl Proteinase K) and incubated in a 54-55°C shaker water bath for 25-30 min with vigorous agitation. In the washing steps, plugs were removed from the water bath, the lysis buffer was poured off, and 10-15 ml of pre-heated (54- 55°C) sterile Ultra-Pure Water were added before incubation for 10-15 min with vigorous agitation twice. The same procedures were performed with TE buffer four times. After washing, the plugs were digested with *Xba*I(50U/µl, Thermo Scientific) restriction enzyme at 37°C for 2 hours before the plug slices were loaded into the wells of SKG (1%) agarose gel. The electrophoresis conditions on CHEF-DR III were initial switch time 2.2 s, final switch time 63.8 s, and 6V/cm for 19 h in 0.5X TBE.

Phylogenetic analysis

The band patterns of the strains were evaluated by GelCompar II 6.6.11 Gel Electrophoresis Software (Applied Maths, Sint- Matenslatem, Belgium) according to the PFGE gel image. Similarities of PFGE band patterns were calculated by using Dice coefficient with 1.0% tolerance. The dendrogram was performed by the unweighted pair-group method with arithmetic mean (UPGMA).

MLST analysis

Nucleic acid extraction was performed using the boiling method [33]. Multilocus sequence typing was performed using the protocol described at http://mlst.warwick.ac.uk/mlst/dbs/Senterica. Seven housekeeping genes (*aroC* (826 bp), *dnaN* (833 bp), *hemD* (666 bp), *hisD* (894 bp), *purE*(510 bp), *sucA* (643 bp), and *thrA* (852 bp)) were amplified by polymerase chain reaction (PCR) using primer sequences downloaded from the MLST database. The PCR conditions were as follows: 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 min [34]. The PCR amplicons were sequenced using the ABI 3500 genetic analyzer system. The CLC Main Workbench v.8.0.1 sequence analysis program (Qiagen, USA) was used for alignment, and editing forward and reverse sequences. For each isolate, the seven housekeeping gene sequences were uploaded to the MLST database for comparison and analysis to determine the sequence type. The phylogenetic tree was drawn by MEGA v.7.0.20 program on a single DNA sequence (3336 bp) which had been obtained by combining 7 housekeeping gene sequences for each strain.

RESULTS

The PFGE analysis revealed two major PFGE groups (I, II) among *S*. Enteritidis strains, five different clusters (A, B, C, D, E), and 34 different PFGE profiles with the exclusion test group. The analysis of the 60 *S*. Enteritidis strains showed 9 different macro restriction profiles (P1-P9) with common band patterns (Fig.1). All *S*. Enteritidis strains were in the same PFGE cluster (A) among them 15 strains differed by two or three bands from this pattern. On the other hand, other *Salmonella* serovars used for exclusion were in different clusters (B-E).

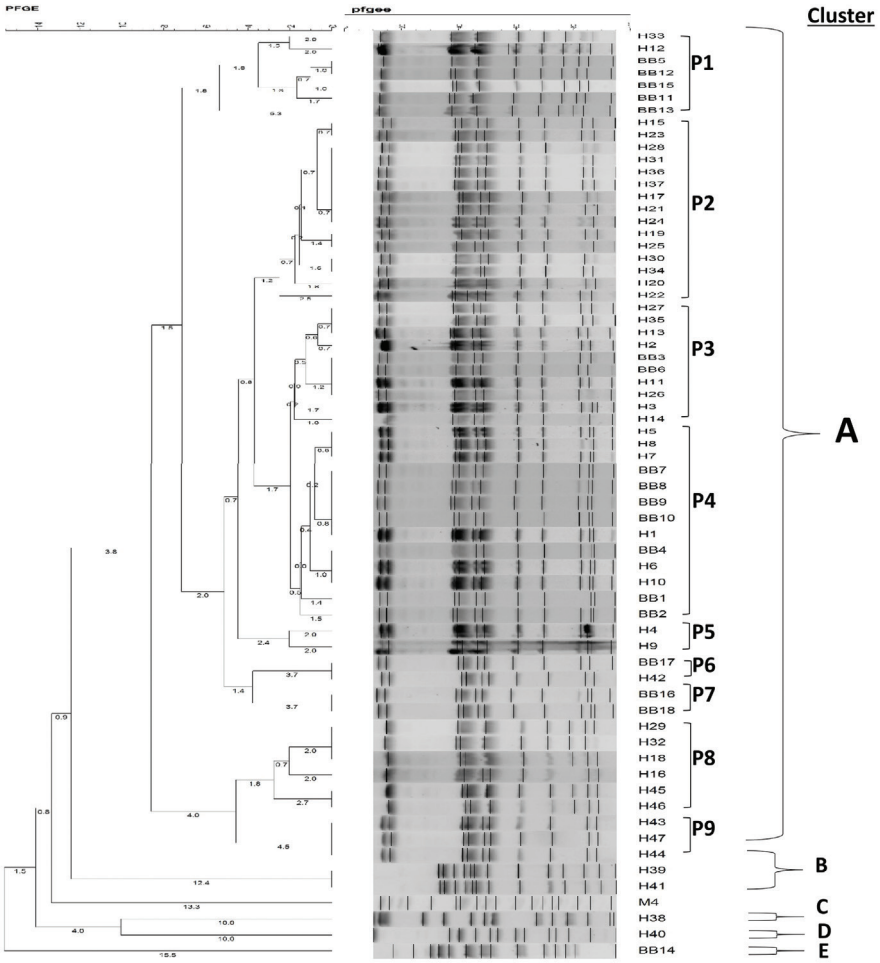

Figure 1. Dendrogram of PFGE showing the relationship between *S*. Enteritidis isolates and the exclusion group (H; isolate from hatchery flocks, BB; isolate from broiler breeder flocks, M4; marker)

Group I consisted of 7 macro restriction profiles (P1-P7) (n=51) and Group II consisted of 2 macro restriction profiles (P8-P9) (n=9) with a high level of genetic similarity (>90%). Although these strains were obtained from different flocks, they had highly similarity PFGE patterns according to the Tenover criteria [35]. Clusters B-E consisted of the exclusion group including *S*. Kentucky, *S*. Typhimurium, *S*. Albany, and *S*. Tennessee. In cluster B, two *S*. Kentucky strains from hatchery flocks had different patterns while clusters C, D, and cluster E had one isolate each in different PFGE profiles.

The single human (BB15) isolate was in cluster A with a very similar banding pattern to the chicken flock strains.

Among all isolates, minimum similarity was 66% according to the similarity matrix created using the unweighted pair group method using arithmetic averages (UPGMA) with dendrograms of the PFGE band patterns.

MLST showed that *S*. Enteritidis strains were ST11, with identical alleles at all loci: *aroC* allele type 5, *dna*N allele type 2, *hemD* allele type 3, *hisD* allele type 7, *purE* allele type 6, *sucA* allele type 6, and *thrA* allele type 11. In the exclusion group *S*. Albany, *S*. Kentucky, *S*. Tennessee and *S*. Typhimurium strains were found to be ST1583, ST314, ST319, and ST36, respectively. In the phylogenetic tree of the MLST analysis, all *S*. Enteritidis strains were in the same branch with 100% similarity (Fig.2).

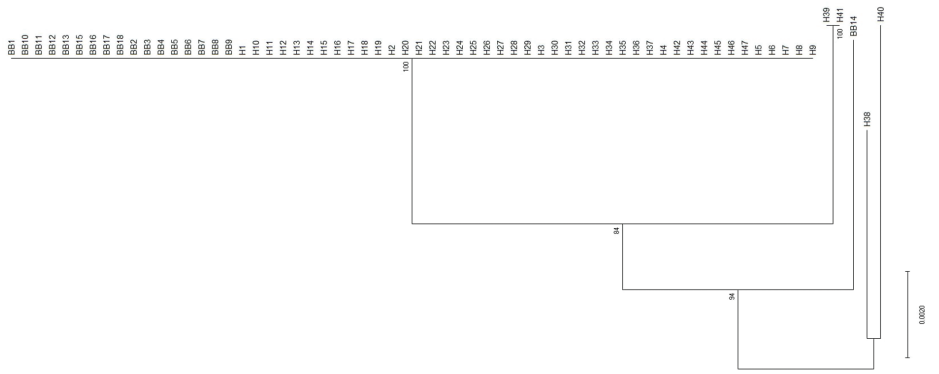

Figure 2. Evolutionary relationships of taxa
The evolutionary history was inferred using the Neighbor-Joining method [47]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (50 replicates) are shown next to the branches [48]. The evolutionary distances were computed using the Tamura 3-parameter method [49]. Evolutionary analyses were conducted in MEGA 7 [50].

DISCUSSION

In this study, PFGE and MLST analyses were used to determine the clonal relationship between the *S*. Enteritidis strains isolated from boiler breeder and hatchery flocks

located in Turkey's Western Black Sea region. Among *S*. Enteritidis strains two major PFGE groups, five different clusters and 34 different PFGE profiles with the exclusion test group were determined. All *S*. Enteritidis strains were in the same PFGE cluster (A) and had same sequence type (ST11).

Among the *Salmonella* serovars, *S*. Enteritidis is the most commonly reported serovar in humans worldwide. Human infections caused by *S*. Enteritidis are associated with food obtained from animals [9,36]. Williams et al. [37] reported that contamination may spread through a hatchery because individual eggs are contaminated by infected litter, dust, or equipment found in the production site [37]. Other studies have shown that the transport vehicles may play a critical role in transferring *Salmonella* between the flocks [38,39]. The strain isolated from a farm worker in this study had a very similar band pattern and was in the same cluster as strains isolated from the chicken flocks (n= 59). Two *S*. Enteritidis strains from a chicken flock and one from a worker isolated from the same poultry farm, and the PFGE patterns of these three *S*. Enteritidis strains were highly similar (>99%). This result may indicate possible chicken-human transmission between the flocks. However, for a correct comparison comprehensive studies including more human strains should be performed.

The PFGE analysis showed that 73% (44/60) of strains isolated from the chicken flocks belong to the same macro restriction profile (P2, P3, P4, P5, P6 and P7) while homogeneity was mainly observed in the P2 profile among 15 isolates from hatchery flocks from different poultry farms. In addition, the isolates from both hatchery and broiler breeder flocks were in the same PFGE profile even though they were isolated from different poultry farms. The highly consistent PFGE pattern from different flocks suggests that the propagation of *Salmonella* clones through the broiler chain could spread to other poultry commercials in this region of Turkey.

The results revealed that PFGE provides greater discriminatory subtyping than MLST for *Salmonella* Enteritidis serovars. Previous studies focused on the discriminatory power of subtyping methods for *Salmonella* serovars indicated that strains with the same ST in MLST analysis could have different PFGE profiles [22,40]. Harbottle et al. [22] reported that the 81 *Salmonella* Newport isolates in their study had 12 different STs and 43 different PFGE profiles. Fakhr et al. [41] reported that 85 *S*. Typhimurium isolates had the same ST but 50 different PFGE profiles. In the present study, all *S*. Enteritidis strains had same ST in the MLST analysis but 30 different PFGE profiles.

The MLST results revealed that 60 *S*. Enteritidis strains belonged to the predominant *S*. Enteritidis genotype in the MLST database (http://pubmlst.org/), namely ST11 [42,43]. Similarly, Gunel et al. [29] reported ST11 in *S*. Enteritidis strains isolated from food samples in Turkey. In addition, Sarıçam et al. [31] and Acar et al. [30] found ST11 in *S*. Enteritidis strains isolated from poultry and human samples, respectively. On the other hand, in MLST database *S*. Enteritidis has been classified into multiple STs, between which ST11 represents majority of the *S*. Enteritidis strains in different countries [20,44-46]. As in the other studies results of this study also demonstrated

that, MLST is not adequate for epidemiologic investigation of *S*. Enteritidis serovar, since only one MLST type was obtained [16,44,46].

This study has identified a predominant *S*. Enteritidis clone in Turkey's Western Black Sea region. Further investigations into the epidemiology of this serovar in other regions of Turkey can contribute to research into controlling and protecting against *Salmonella* in poultry. Our data also reveal that PFGE characterization of *Salmonella* serovars is critical, not just for surveillance, but to understand transmission of this pathogen between the animals and possible zoonotic transmission.

Authors' contributions

IBM conceived of the study, carried out the PFGE studies, participated in the phylogenetic analysis and drafted the manuscript. HKM participated in the design and coordination of the study and helped to draft the manuscript. SSI carried out the MLST and participated in the phylogenetic analysis. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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GENOTIPSKA KORELACIJA IZMEĐU IZOLATA *SALMONELLA* ENTERIDIS IZ BROJLERSKIH RODITELJSKIH JATA KOKOŠAKA I JATA İZ INKUBATORA

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U studiji je obavljeno ispitivanje izolata sojeva *Salmonella* Enteritidis iz uzoraka prašine i okolne sredine, poreklom od jata iz različitih regiona Turske zapadne obale Crnog mora pri čemu su upotrebljene metode PFGE (pulsna gel elektroforeza) i multilokus tipiziranje sekvencioniranjem (MLST). Ispitano je ukupno 59 *S*. Enteritidis izolovanih sojeva poreklom od brojlerskih roditeljskih jata i iz inkubatora kao i jedan soj *S*. Enteritidis izolovan iz uzorka fecesa jednog od radnika. Analiza metodom PFGE ukalazala je na dve PFGE grupe i devet različitih makro restriktivnih profila. Ustanovljeno je da je bilo 85% (51/60) bliskih sojeva koji su činili Grupu I. Svi *S*. Enteritidis sojevi su imali iste tipove sekvenci (ST): ST11. Izolacija sojeva sa jednim genotipom ukazuje da postoji unakrsna kontaminacija i prenošenje bakterija između jata živine.