

Retracted Article: Phenotypic and molecular characterization of *Salmonella* Enteritidis isolates

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Abstract: *Salmonella* spp. is the most frequently isolated foodborne pathogens causing human and animal diseases. The aim of this study was to investigate antimicrobial susceptibility profiles and the molecular typing of 200 *Salmonella* Enteritidis strains isolated from the patients' stools between 2016 and 2019 in Turkey. The isolates were examined for antibiotic susceptibility patterns (21 antimicrobial agents) by Kirby-Bauer disc diffusion method or molecular typing by Pulsed-field gel electrophoresis (PFGE) and plasmid profiling. Although all isolates were susceptible to four antibiotics (sulfamethoxazole/trimethoprim, chloramphenicol, streptomycin and trimethoprim), all were resistant to 15 different antibiotics. In the PFGE study performed with *XbaI* enzyme, all isolates were found to be related to each other according to similarity rates of 85% and above. There were two major clones, clone A and B. Clone A was divided into 6 pulsotypes (A1-A2-A3-A4-A5-A6) and clone B was divided into 3 pulsotypes (B1-B2-B3). Clone A had 87% similarity and Clone B had 90% similarity. The clustering rate was 86% (172/200). All isolates harboured 1-4 plasmid ranging in size from 2.5 to 57 kb and showed 6 plasmid profiles (P1-P6). All isolates carried the 57 kb plasmid individually or in combination with other plasmids. Most of the isolates, 136 (68%) had P2 profile. Our findings indicate that the majority of all isolates were clonally related and had cross contamination problems. In this study, the importance of molecular typing methods in order to take more effective protection and control measures against *Salmonella* has been demonstrated and proposed to use such methods.

Keywords: Antimicrobial resistance, PFGE, plasmid profiling, *Salmonella* Enteritidis.

Salmonella Enteritidis izolatlarının fenotipik ve moleküler karakterizasyonu

Özet: *Salmonella* spp. insan ve hayvan hastalıklarına neden olan en yaygın gıda kaynaklı patojendir. Bu çalışmanın amacı, Türkiye'de 2016 ve 2019 yılları arasında hasta dışkılarından izole edilen 200 *Salmonella* Enteritidis izolatının antimikrobiyal duyarlılık profillerini ve moleküler tiplerini araştırmaktır. İzolatlar Kirby-Bauer disk difüzyon yöntemi ile antibiyotik duyarlılık profilleri (21 antibiyotik) ve pulsed-field jel elektroforezi (PFGE) ve plazmid profili ile moleküler tiplene açısından incelendi. Toplam 200 izolat hem 4 farklı antimikrobiyal ajana (sülfametoksazol / trimetoprim, kloramfenikol, streptomisin ve trimetoprim) duyarlı hem de 15 farklı antimikrobiyal ajana dirençliydi. *XbaI* enzimi ile yapılan PFGE çalışmasında, tüm izolatların %85 ve üzeri benzerlik oranlarına göre birbirleriyle ilişkili olduğu bulundu. İki ana klon vardı, klon A ve B. Klon A, 6 pulsotipe (A1-A2-A3-A4-A5-A6) ve klon B, 3 pulsotipe (B1-B2-B3) ayrıldı. Klon A ve B, sırasıyla %87 ve %90 benzerliğe sahipti. Kümeleme oranı %86 (172/200) idi. Tüm izolatlar, 2,5 ila 57 kb arasında değişen 1-4 plazmid barındırdı ve 6 plazmid profili (P1-P6) gösterdi. Tüm izolatlar 57 kb plazmidi tek veya diğer plazmidlerle birlikte taşıdı. İzolatların çoğu (n= 136, %68) P2 profiline sahipti. Bulgularımız, 200 *S. Enteritidis* izolatlarının çoğunun klonal olarak ilişkili olduğunu ve çapraz kontaminasyon problemleri olduğunu göstermektedir. Bu çalışmada, Salmonellosis'e karşı daha etkin korunma ve kontrol önlemlerinin alınmasında moleküler tiplene yöntemlerinin önemi gösterilmiş ve bu yöntemlerin kullanılması önerilmiştir.

Anahtar sözcükler: Antimikrobiyal direnç, PFGE, plazmid profili, *Salmonella* Enteritidis.

Introduction

Salmonella is Gram-negative facultative anaerobic bacteria which belongs to *Enterobacteriaceae* family and a group of bacteria living in the intestinal tracts of many different domestic animals including birds, cattle (1, 11, 13). Humans usually become infected with *Salmonella* by eating foods contaminated with animals' feces (1, 11, 39). Salmonellosis is an infectious disease of humans and animals caused by these bacteria which are capable of having foodborne zoonosis importance (18). *Salmonella* spp. especially *S. enterica* subsp. *enterica* serovar Enteritidis (*S. Enteritidis*) and *S. enterica* subsp. *enterica* serovar Typhimurium (*S. Typhimurium*) that are known as non-typhoidal *Salmonella* serotypes, continues to be the most frequent cause of bacterial foodborne disease outbreaks (17-19, 44). In addition, products of animal origin, environmental contamination and indirect transmission through food and water are other causes of *Salmonella* outbreaks (4, 38). *Salmonella* infection develops in adults with contaminated foods, and newborns and children are more likely to develop infections with cross-contamination (34).

Foodborne diarrheal diseases caused by the significant ones like non-typhoidal *Salmonella* are an important cause of morbidity and mortality, and thus has emerged as a significant and growing public health and economic problem worldwide, especially industrialized countries (14, 27). *Salmonella* infections have a worldwide distribution and range clinically from the common self-limited uncomplicated gastroenteritis to enteric fever (9).

Antibiotics inhibit the growth of *Salmonella* spp., reducing the economic losses and public health problems (14, 18). However, multidrug-resistant *Salmonella* is increasing due to misuse and overuse of antibiotics in human and animals, which make it difficult to eliminate from its reservoir hosts (44). Multidrug-resistant *Salmonella* isolates have been associated with a considerable number of outbreaks worldwide (20, 44). The presence of antibiotic-resistant *Salmonella* in the human food chain requires the development of new-antibiotic to prevent the pathogens in reservoirs, including cattle, birds (25). The identification of *Salmonella* serotypes is important to monitor common source outbreak or origins, relationships among different isolates and to control future outbreaks of infectious diseases that transmit from animals to humans (4, 9, 11-13). The information to be obtained from typing methods such as Pulsed field gel electrophoresis (PFGE) and plasmid profile analysis (PPA) is very useful in preventing and controlling the spread of disease in animals and public (36). PPA is a molecular method used for subtyping (41). PFGE is also another molecular typing method used for

typing outbreaks. This method provides information on the source and transmission pattern of the microorganism (19, 30). PFGE, which is used to determine the clonal and phylogenetical relationships between strains, is known to have high discrimination power (2, 4). Combined with PPA and PFGE provides a powerful discriminatory tool for the epidemiological analysis of *S. Enteritidis* isolates (29, 43). Most cases of Salmonellosis in humans are associated with the consumption of food contaminated with antibiotic-resistant *S. Enteritidis* from animals. Hence, it is possible to have a relationship between humans and animals regarding genotyping of antibiotic-resistant *S. Enteritidis*. This study aimed to investigate the antimicrobial susceptibility profiles and the molecular typing of 200 *S. Enteritidis* strains isolated from the patients admitted to the hospital by using PFGE and PPA and clarify the possible transmission rates and clonal relationships among these isolates.

Materials and Methods

Samples: *S. Enteritidis* isolates (n=200) were used in this study. Fecal samples of 200 patients who were sent to a public hospital microbiology laboratory (Cankiri province of Turkey) between 2016 and 2019 were examined. The study was performed by using *S. Enteritidis* obtained from the culture collection made from the fecal samples given by the patients who came to the outpatient clinic.

***Salmonella* Isolation and Identification:** The samples were inoculated onto Eosin Methylene Blue (EMB) agar and *Salmonella* Shigella (SS) agar (Becton Dickinson, GmbH, Heidelberg, Germany) and into selenite F broth for enrichment (BBL Selenite F Broth, Becton Dickinson and Co., Sparks, MD, USA). They were incubated solid agars for overnight and selenite F broth for 8 hours under appropriate conditions. A single colony picked up and identified as *S. Enteritidis* using IMVIC test (citrate, methyl red, Voges-Proskauer, citrate, ornithine, urea, indole, Kligler iron agar media). Conventional methods (Triple Sugar Iron Agar, Simon's Citrate Agar, Urea Agar and nutrient broth.; Oxoid, Hampshire, UK) and Phoenix 100 (Becton Dickinson and Co., Sparks, MD, USA) automated system were used to identify lactose negative bacterial colonies that grow on EMB and SS agars and in selenite F medium. Serotypes of bacteria identified as *Salmonella* were determined by O and H antigens (Difco, Sparks, MD, USA). Somatic O antigens and flagellar H antigens were determined by slide agglutination using specific antisera. After the identification of the antigens, the name of strains was determined by using the Kauffman-White scheme (23, 24). Serological confirmation and serogroup of microorganisms identified as *S. Enteritidis* were

determined by using species-specific anti-sera (Difco Shigella Antisera Poly, Sparks, MD, USA).

Antimicrobial susceptibility test: Mueller-Hinton agar (Oxoid, Hampshire, UK) was used for antibiotic susceptibility testing. Kirby-Bauer disc diffusion method was used for antibiotic susceptibility according to the Clinical Laboratory Standards Institute guidelines (6) for the following antimicrobial agents (Oxoid, UK): Ampicillin (AMP: 10 µg), cephalothin (KF: 30 µg), gentamicin (CN: 10 µg), amoxicillin-clavulanic acid (AMC: 30 µg), cefuroxime sodium (CXM: 30 µg), cefoperazone (CFP: 30 µg), cefotaxime (CTX: 30 µg), ceftizoxime (ZOX: 30 µg), ceftriaxone (CRO: 30 µg), ceftazidime (CAZ: 30 µg), suphamethoxazole/trimethoprim (SXT: 25 µg), chloramphenicol (C: 30 µg), tetracycline (TE: 10 µg), kanamycin (K: 30 µg), nalidixic acid (NA: 30 µg), ciprofloxacin (CIP: 5 µg), sulphonamides (S3: 300 µg), streptomycin (S10: 10 µg), trimethoprim (W: 5 µg), cefpodoxime (CPD: 10 µg), and amikacin (AK: 30 µg). *Escherichia coli* ATCC 25922 was used as a quality control strain in all tests. The plates were incubated for 24 hours at 35-37 °C. The diameters of the inhibition zones formed around the discs were measured in millimeters and evaluated as susceptible, intermediate, or resistant, according to the CLSI, 2012 (6).

Plasmid analysis: Plasmid DNA was isolated by alkaline lysis methods of Kado and Liu (15) and separated in 0.7% agarose gel (Serva, Heidelberg, Germany) prepared with 0.5xTris-Boric acid-EDTA buffer at 110 V for 3 h at room temperature and stained with ethidium bromide (0.5 µg). Plasmid size was determined by comparison with *E. coli* V517 (5.7, 7.2, 5.6, 5.1, 3.9, 3.0, 2.7, 2.1 kb) and *S. Enteritidis* strains (5.7, 5.8, 4.8 kb) and supercoiled DNA ladder (1 kb gene ruler DNA ladder fermentas, Lithuania).

Pulsed field gel electrophoresis: PFGE analysis was performed using *Xba*I according to the protocol described by Durmaz et al. (8). Briefly, single colony cultivation was performed on trypticase soy agar (Merck, Germany) from the bacteria that were identified as *S. Enteritidis* incubated at 37 °C for 20-24 hours under aerobic conditions. After overnight incubation, the purity was checked, and a single colony of each isolate was again passaged to SS agar (Merck, Germany) and incubated under the same condition.

The colonies were collected with a plastic loop and suspended in 4 ml of Cell Suspension Buffer (CSB; 100 mM Tris-HCL, 100 mM EDTA, pH 8.0). The cell suspension was centrifuged at 13000 rpm for 2 minutes at 4°C. The supernatant was discarded after centrifugation. 1 ml cold CSB was added to the pellet again and vortexed for a short time. The bacterial density was adjusted to 1

absorbance at 590 nm using a spectrophotometer (UV/Vis. Spectrophotometer, Shimadzu-1280, Japan). 2% low-melting agarose (LMA, Gibco BRL, Paisley, UK) was prepared in CSB buffer and the mixture containing CSB, LMA and 10% Sodium Dodecyl Sulfate (SDS, Merck, Germany) was transferred to plug molds (10mm x 5mm x 1.5mm, Sigma-Aldrich, Germany). The molds were allowed to stand for 10 minutes at +4 °C until the agarose solidifies for quality DNA preparation. The agaroses containing the bacteria were removed from the plug mold and transferred into Cell Lysis Solution 1 (CLS-1, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 2.5 mg/ml lysozyme, 1.5 mg / ml proteinase K) and incubated for 1 hour at 37 °C. Then, the plugs were incubated in Cell Lysis Solution-2 (CLS-2, 0.5 M EDTA, 1% sarkosyl, 400 µg/ml proteinase K) at 55 °C for 2 hours. After incubation, the plugs were treated 3 times successively with sterile ultrapure water (Reagent Grade Type 1) and TE buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 7.6).

Each of the agarose containing DNA was transferred into 100 µl *Xba*I buffer (1x) and incubated in a shaking water bath at 37 °C for 10 minutes. The restriction buffer was removed and fresh buffer (100 µl) containing *Xba*I restriction enzyme (Promega Corporation, WI, USA) was added to each plug in the tubes. Incubation was performed at 37 °C for 2 hours. 1% agarose (pulsed-field certified agarose, Sigma-Aldrich, Germany) was prepared in 100 ml of 0.5xTBE (44.5 mM Trisma Base, 44.5 mM Boric acid, 1 mM EDTA, pH 8.0) solution and restricted DNA plugs were loaded on each tooth of tooth comb (USA Scientific, USA). The agarose solidified on the teeth was placed in a PFGE chamber containing 1900-2000 ml 0.5x TBE buffer. PFGE was performed via CHEF-DR® II system (Bio-Rad, Hercules, CA, USA). The electrophoresis condition was set as follows: Initial switch time; 2.3 s; final switch time, 65 s, run time, 19 h; gradient, 6V/cm²; angle 120°; temperature, 14 °C. After electrophoresis, the gel was transferred into ultra-pure water containing ethidium bromide (5 µg/ml) and incubated to be stained for 20 minutes and visualized under UV light.

The DNA band images were photographed using Digi Genius Imaging System (Syngene, UK). Band profiles were analyzed using Gene Directory software (Syngene, Cambridge, UK). First of all, normalization was performed with the help of three control strains in each image. Clustering analysis was performed by creating a dendrogram of PFGE profiles. The relationship between the strains was determined according to the "Dice" similarity coefficient. Using the criteria developed by Tenover et al. (40), isolates were evaluated as indistinguishable, closely related, possibly related, or different.

Results

The *S. Enteritidis* isolates were isolated from clinical specimens (200 fecal samples) in this study. There were the multi-resistant *S. Enteritidis* (100%) which are resistant to a large diversity of antimicrobial agents (Figure 1). A total of 200 isolates were susceptible to four antimicrobial agents (SXT, C, S10 and W). The antibiotic resistance profiles were similar among *S. Enteritidis* isolates which are resistant to more antibiotics. The susceptibility and resistance patterns of the strains were found to be similar and were mainly divided into 3 groups as RI, RII and RIII (Table 1).

Molecular typing of the *S. Enteritidis* isolates were analyzed by using molecular techniques, PFGE and PPA. In the PFGE study performed for genotypic typing of *S. Enteritidis*, PFGE gel images were obtained after cutting *Salmonella* DNA with *Xba*I enzyme with restriction endonuclease activity. In the next step, dendrogram analysis was performed from gel images of *Salmonella* isolates showing PFGE band profiles. After the band profile analysis, PFGE profile dendrograms were established and the relationships between strains were determined. Cluster analysis was evaluated with the Dice

similarity coefficient and UPGMA (Unweighted Pair Group Method Average) relationship rule parameters. When the dendrogram of 200 *Salmonella* strains were examined; according to the criteria of Tenover et al. (40), *S. Enteritidis* were found to be related to each other according to similarity rates of 85% and above. There were two major clones, but strains were divided into clones A and B. Clone A was divided into 6 pulsotypes (A1-A2-A3-A4-A5-A6) and clone B was divided into 3 pulsotypes (B1-B2-B3). Clone A had 87% similarity and Clone B had 90% similarity. The clustering rate was 86% (172/200) according to Tenover criteria (40). Clone A contained 172 strains and clone B contained 28 strains (Figure 1).

In this study, all of the *S. Enteritidis* isolates (n=200) were observed to have plasmid. It was determined that isolates showed 6 plasmid profiles (P1-P6) (Table 2). The 200 isolates carrying plasmids were found to carry 1-4 plasmids, ranging in size from 2.5 to 57 kb (Table 2). All isolates carried the 57 kb plasmid individually or in combination with other plasmids. Fourteen of the isolates carried at least one plasmid (57 kb). Most of the isolates (n = 136, 68%) had P2 profile.

Table 1. Antibiotic resistance profiles for *S. Enteritidis* isolates

Resistance phenotype	Resistance pattern	Isolate number	%
RI	AMP, KF, CN, AMC, CXM, CFP, CTX, ZOX, CRO, CAZ, TE, K, NA, CIP, S3, CPO, AK	28	14
RII	AMP, KF, CN, AMC, CXM, CTX, ZOX, CRO, CAZ, TE, K, NA, CIP, S3, CPO, AK	121	60.5
RIII	AMP, KF, CN, AMC, CXM, ZOX, CRO, CAZ, TE, K, NA, CIP, S3, CPO, AK	51	25.5

AMP: Ampicillin; KF: Cephalothin; CN: Gentamicin (CN); AMC: Amoxycillin-clavulanic acid; CXM: Cefuroxime sodium; CFP: Cefoperazone; CTX: Cefotaxime; ZOX: Ceftizoxime; CRO: Ceftriaxone; CAZ: Ceftazidime; SXT: Suphamethoxazole/trimethoprim; C: Chloramphenicol; TE: Tetraacycline; K: Kanamycin; NA: Nalidixic acid; CIP: Ciprofloxacin; S3: sulphonamides; S10: Streptomycin; W: Trimethoprim; CPD: Cefpodoxime; AK: Amikacin.

Table 2. Plasmid patterns of *S. Enteritidis* isolates.

Plasmid profiles	Plasmids (kb)	Number of <i>S. Enteritidis</i> isolates (%)
P1	57	14 (7)
P2	57, 40, 3.0	136 (68)
P3	57, 40, 6.5, 4.5	29 (14.5)
P4	57, 5.8, 4.8	7 (3.5)
P5	57, 6.5, 4.5	7 (3.5)
P6	57, 2.5	7 (3.5)

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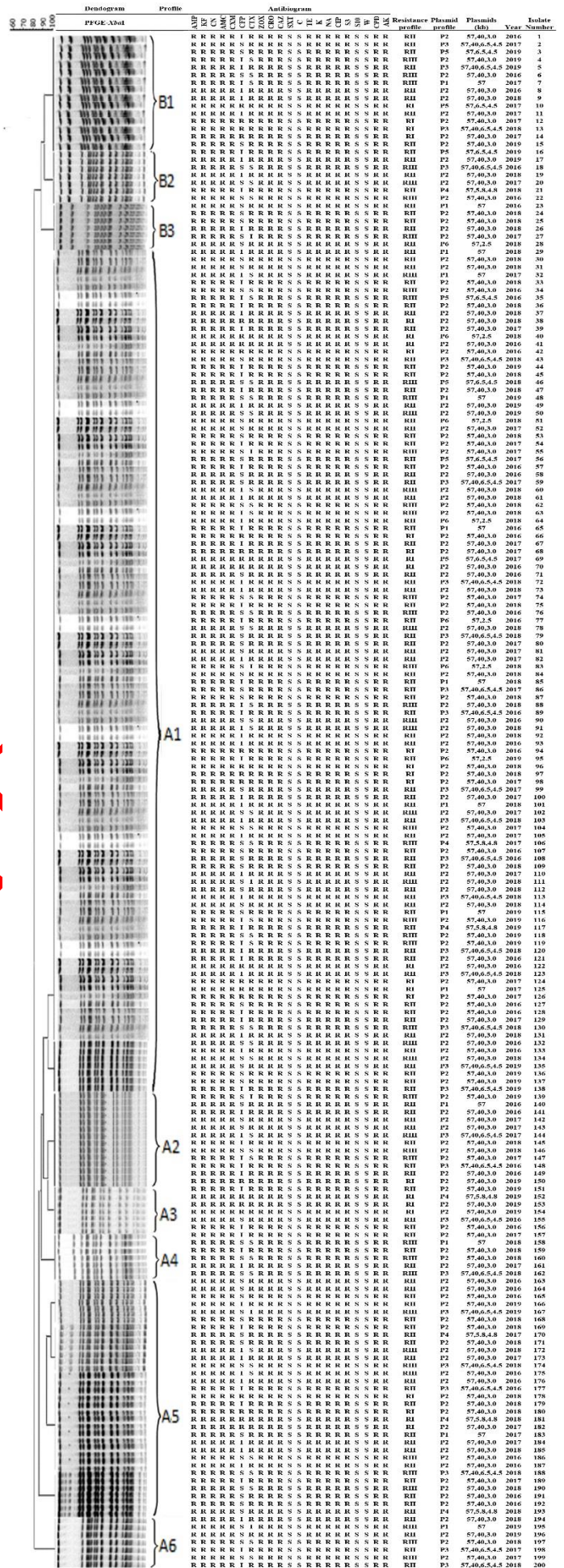


Figure 1. XbaI PFGE patterns, plasmid patterns and antibiotic resistant profiles of *S. Enteritidis* isolates.

Discussion and Conclusion

Salmonella infection in humans and animals continues to be a public health problem in Turkey, as it is in almost every region of the world (4, 17, 22). Phage typing and genotyping methods are accepted as the gold standard value for differentiation and subtyping of *Salmonella* isolates an epidemiological method (28, 35, 45). Genotyping methods such as PFGE, PPA, ribotyping, which rely on the comparison of multiple electrophoresis banding patterns, have been used for monitoring the epidemic evolution of *S. Enteritidis* strains (1, 37). *Salmonella* phage typing is a method that can be performed in several reference centers in the world and is not implemented in Turkey. Therefore, our limited knowledge about phage types and molecular characteristics of isolated *Salmonella* serotypes in Turkey is based on a few specific studies and there is insufficient information about phage types and molecular characteristics of multi-drug resistant (MDR) *S. Enteritidis* strains (1, 21, 43).

S. Enteritidis is the most common *Salmonella* serotype isolated from the human in Turkey (10). From the past to the present, the incidence of *S. Enteritidis* has been gradually increasing in humans (3). The occurrence of antibiotic-resistant strains of *Salmonella* spp. has become a serious health problem worldwide (5, 7). High rates of resistance against a large number of antibiotics in *S. Enteritidis* isolates from humans and animals in Turkey have been reported previously (3, 10, 12, 13, 21, 26, 33, 42). In this study, there were the multi-resistant *S. Enteritidis* (100%) and all isolates were susceptible to four antimicrobial agents (SXT, C, S10 and W). The antibiotic resistance profiles were similar among *S. Enteritidis* isolates which are resistant to more antibiotics. Erdem et al. (10) reported that there was resistance or decreased susceptibility to CIP in *Salmonella enterica* strains (n = 620) isolated from clinical samples in 10 provinces of Turkey between 2000 and 2002 years. After about 17 years, all of the isolates were resistant to CIP in this study. These results show that increasing incidence of multi-drug resistant *Salmonella* strains represent a risk to public health.

In this study, molecular typing of the *S. Enteritidis* isolates was analyzed by molecular techniques, PFGE and PPA. All strains were found to be related to each other according to similarity rates of 85% and above. There were two major clones, divided into clones A and B. Clone A had 87% similarity and Clone B had 90% similarity. The clustering rate was 86% (172/200). In a study, PGFE profiles of *S. Enteritidis* strains (n = 23) isolated from clinical samples from 7 provinces of Turkey between 2004 and 2010 have been determined using *Xba*I restriction enzyme. The findings demonstrated that 4 different PFGE profiles (type 1, 4, 9, and 10) were found among serotype

S. Enteritidis and PFGE types had similarities below 85% and above 95% in strains within the same type (26). Us et al. (43) reported that a total of 122 *S. Enteritidis* strains (epidemic, n = 13; sporadic, n = 109) (103 stool, 16 blood and one bile, one urine and one cerebrospinal fluid) isolated from 10 different provinces of Turkey after 2000 year were investigated for PFGE profile. The PFGE analysis showed 11 different patterns (a to k) and PFGE pattern a and b consisted of 53 strains (43.4%) and 42 strains (34.4%), respectively, after digestion with macrorestriction enzyme *Xba*I. In mid-January 2008 (Isparta province of Turkey), after a large foodborne outbreak associated with eggs contaminated by *S. Enteritidis* in a military unit, *S. Enteritidis* was isolated from 276 stool samples and a blood sample of the hospitalized patients and a food item. Authors have reported that the PFGE patterns after *Xba*I digestion of these isolates were identical (19). Numerous studies in different countries reported that *S. Enteritidis* isolates were genetically similar and clonally highly related (16, 29-32). These data are consistent with our results of PFGE profile after digestion of with *Xba*I enzyme.

The plasmids carrying antibiotic resistance genes have an important role in transferring those genes to other strains or other species (25). In this study, all of the *S. Enteritidis* isolates (n = 200) were observed to have plasmid. The isolates carrying plasmids were found to carry 1-4 plasmids, ranging in size from 2.5 to 57 kb. In a study conducted by Aktaş et al. (1) in Istanbul between 2001 and 2004, plasmid profiling analysis of pediatrics *S. Enteritidis* isolates showed six plasmid profiles, ranging in size from 2.5 MDa to 38 MDa. High rate (92%) of the isolates harboured the same plasmid of 38 MDa. In another study in Ankara, although *S. Enteritidis* isolates harbored 1-4 plasmids with sizes ranging from 2.0 to 100 kb, 85 (69.7%) of isolates harbored the 57 kb plasmid (43). In these studies, the plasmid size harboured by *S. Enteritidis* isolates are similar to those found in our study.

In the present study, the occurrence of multi-resistance observed in 200 (100%) of the *S. Enteritidis* isolates from clinical human samples is of great public health concern and suggests the successful implementation of surveillance and monitoring of antimicrobial use and continuing education on prudent antimicrobial agent use. The high level of resistance observed in the isolates was likely to the fact antibiotics were overused to the patients. While no correlation was found between resistance profiles and genotypic profiles, it was determined that isolates showing multiple resistance to antibiotics were distributed to different PFGE clusters and different plasmid profiles.

In addition, our results revealed that the majority of *S. Enteritidis* isolates in Cankiri province of Turkey is similar PFGE pattern and confirmed the clonal structure

of *S. Enteritidis* strains. Identification and typing of microorganisms at molecular level are very important. Investigating whether epidemiologically related isolates are genetically related may help to find the source and control spread of the epidemic in the community and hospitals (27).

In conclusion; combination of PFGE conducted with the restriction endonuclease *XbaI* and plasmid analysis was useful in detection of clonal relationship among *S. Enteritidis*. The importance of molecular typing methods in order to take more effective protection and control measures has been demonstrated and proposed to use such methods in this study.

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Ethical Statement

The study was approved by the ethic committee of Burdur Mehmet Akif Ersoy University (Ethic approval Code: GO 2020-258).

Conflict of Interest

The authors declared that there is no conflict of interest.

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