Analysis of an Outbreak of *Salmonella* Enteritidis by Repetitive-Sequence-Based PCR and Pulsed-Field Gel Electrophoresis

Abdullah Kilic¹, Orhan Bedir¹, Nafiz Kocak², Belkis Levent¹, Can Polat Eyigun⁴, Omer Faruk Tekbas⁵, Levent Gorenek⁶, Orhan Baylan¹ and A. Celal Basustaoglu¹

## Abstract

**Objective** The aim of this study was to investigate a large food-borne outbreak associated with eggs contaminated by *Salmonella* Enteritidis in a military unit using pulse field gel electrophoresis (PFGE) and the Repetitive-sequence-based PCR (rep-PCR) employing the DiversiLab system.

**Materials and Methods** In mid-January 2008, a food-borne outbreak associated with *S*. Enteritidis occurred in a military unit located in the city centre of Isparta. A total of 2,469 patients were registered to six hospitals with gastrointestinal disease symptoms such as diarrhea and abdominal pain. Of those registered, 445 were hospitalized. *S*. Enteritidis was isolated from 276 stool samples and a blood sample of the hospitalized patients and from a food item. The PFGE patterns after *XbaI* digestion and rep-PCR profiles produced by the DiversiLab system were determined for eight randomly selected stool isolates, one blood isolate and one food isolate.

**Results** The PFGE patterns of all isolates were identical. The Rep-PCR profiles produced by using the DiversiLab system showed that all isolates were indistinguishable. The PFGE and rep-PCR interpretations were concordant for the *S*. Enteritidis isolates. All stool isolates, one blood isolate and one food isolate were susceptible to all antibiotics tested.

**Conclusion** This data suggest that the DiversiLab system may be a reasonable alternative to PFGE for investigation and control of *S*. Enteritidis outbreaks, since it is easy to use, rapid and does not require highly skilled operators.

**Key words:** *S*. Enteritidis, outbreak, PFGE, rep-PCR, DiversiLab system

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## Introduction

*Salmonella* bacteria are one of the most important pathogens causing human illness. Foodborne diseases have been estimated to cause 200,000 deaths each year (1). More than 2,500 serovars of *Salmonella enterica* have been reported from all over the world. *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S*. Enteritidis) is the most common serovar of *Salmonella* isolated from humans, animals, food and feed in the past 10 years, particularly in many developed countries (2).

*S*. Enteritidis caused 29,762 illnesses, 2,904 hospitalizations, and 79 deaths in the United States from 1985 to 1999 (3). In Europe, *S*. Enteritidis emerged as the most often reported serovar since the middle of the 1980s (4). In ten Turkish provinces, *S*. Enteritidis was also found as the most prevalent serovar isolated from various clinical sam-

¹Department of Microbiology and Clinical Microbiology, Gulhane Military Medical Academy and School of Medicine, Ankara, Turkey, ²Isparta Military Hospital, Isparta, Turkey, ³Communicable Diseases Research Department, Refik Saydam National Hygiene Center, Ankara, Turkey, ⁴Department of Infectious Diseases, Gulhane Military Medical Academy and School of Medicine, Ankara, Turkey and ⁵Department of Public Health, Gulhane Military Medical Academy and School of Medicine, Ankara, Turkey

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Correspondence to Dr. Abdullah Kilic, abkilic@gata.edu.tr
S. Enteritidis is also one of the major food-borne pathogens associated with outbreaks (6). S. Enteritidis infections in an outbreak are usually acquired by ingestion of contaminated food items, such as eggs, milk, vegetables and meat or through animal contact (7, 8). Numerous phenotypic and genotyping methods have been used to identify food-borne disease outbreak caused by S. Enteritidis, including phage typing, serotyping, antibiogram, pulsed-field gel electrophoresis (PFGE), ribotyping, random amplified polymorphic DNA, and multilocus sequence typing, etc. (2). The PFGE has been proven to be useful for the discrimination and epidemiological characterization of S. Enteritidis strains and is currently known to be the gold standard. Due to the high genetic homogeneity of S. Enteritidis, the combination of several methods has been suggested for the epidemiological analysis of unrelated and related S. Enteritidis outbreak strains (2, 9, 10).

PCR-based methods are rapid, simpler, easy to perform and less costly than PFGE (11). Repetitive-sequence-based PCR method (rep-PCR) has been used to subtype a variety of bacteria with much success for over 10 years (12). This method uses primers that target non-coding repetitive sequences interspersed throughout the bacterial and fungal genome. A semi-automated rep-PCR assay system was commercially changed to the DiversiLab system which allows simplistic data elaboration, archiving fingerprint patterns, and reporting (13, 14).

The aim of this study was to investigate a large food-borne outbreak associated with eggs contaminated by S. Enteritidis in a military unit using PFGE and rep-PCR employing the DiversiLab system.

**Materials and Methods**

**Definition of the outbreak**

A large food-borne outbreak occurred in a military unit located in the city centre of Isparta. During the time of outbreak 6,418 soldiers were staying in this military unit. There were two kitchens to cook meals. The food-borne outbreak was based in one of the kitchens. According to the history of food consumption, all ill soldiers ate meatballs, lentil soup, mashed potatoes with egg, leek, and small cakes with syrup at 19:00 dinner time on Friday, January 12, 2008. A case was defined as having had meals in the military unit, and had developed diarrhea in addition to at least one of the gastrointestinal symptoms including fever (>38.5°C), vomiting, nausea, headache or abdominal pain.

**Microbiological analysis**

Stool samples were collected from 445 hospitalized soldiers and using processed standard methods with some modification in six different hospitals. Briefly, stool samples were inoculated on Selenit F broth (Merck, Darmstadt, Germany). After 6-8 hours of incubation, the samples were immediately subcultured onto Salmonella-Shigella agar (Merck) and MacConkey agar (Merck) plates. All plates were incubated for up to 3 days at 35 to 37°C. After incubation, colonies were identified on the basis of biochemical tests and confirmed as Salmonella spp. by the BD Phoenix Automated Microbiology System (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) (7, 15). Serotyping of S. Enteritidis strains was determined according to the Kauffmann-White method by using agglutination with specific antisera (Statens Serum Institut, Copenhagen, Denmark) according to the manufacturer’s instructions (16). Antibiotic susceptibility testing for S. Enteritidis strains was performed by using the BD Phoenix Automated Microbiology System (Becton Dickinson Diagnostic Systems). When suspected bacteremia, patients’ blood samples were processed from two different venous vessels with the Bactec 9240 non-radiometric blood culture system (Becton Dickinson, Detroit, MI, USA). If the two blood cultures become positive, they were sub-cultured onto MacConkey (Merck) and 5% sheep blood agar plates (Merck). Various food and environmental samples (kitchen items, sinks, and taps) were also collected for laboratory analysis to identify the causal agents and then cultured with methods as described above (7, 15).

**Pulse field gel electrophoresis method**

S. Enteritidis cells were grown overnight on brain heart infusion broth (Merck). Bacterial cells suspension consisting of approximately 2×10^9 cells/mL were mixed with an equal volume of 2% low-melting-point agarose at 50°C. The cell-agarose suspension was pipetted into a block mold and was allowed to solidify at 4°C. The cells were lysed at 50°C overnight with gentle shaking in a lysis buffer (100 μg of proteinase K per mL and 1% lauroylsarcosine in 0.5 M EDTA). The blocks were washed three times in TE buffer (10 mM Trizma base, 1 mM EDTA) at 4°C for 10 minutes and were stored at 4°C. An agarose block was equilibrated in restriction enzyme buffer for 30 minutes first and then digested with 40 U of XbaI for 4 hours according to the manufacturer’s instructions. The restricted blocks were washed in TE buffer for 60 minutes at 37°C and then stored at 4°C. The blocks were loaded into the wells of a 1% agarose gel. Electrophoresis was performed in 0.53 TBE buffer (44.5 mM Trizma base, 44.5 mM boric acid, 1 mM EDTA) by the contour-clamped homogeneous electric field method with a CHEF-DRII drive module (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom). To achieve optimal separation of the fragments, XbaI-digested DNA was electrophoresed with a run time of 18 hours under 2.2 to 63.8 seconds linear ramped pulse times. The gels were stained with ethidium bromide (1 μg/mL) for 40 to 60 minutes (17). Banding patterns were photographed under UV transillumination and analyzed with the aid of Quantity One software (Biorad/USA). Loci were scored for the presence or absence of a band. For dendrogram construction, a 2% difference in band position was tolerated. The percent simi-
Pulsed-field gel electrophoresis patterns of *Xba*I-digested genomic DNA of *Salmonella* Enteritidis isolates. Lane 1, *Salmonella* Enteritidis clinical isolates recovered at Gulhane Military Medical Academy and confirmed at Refik Saydam National Hygiene Center; Lane 2, food isolate; Lane 3, blood isolate; Lanes 4 to 11, stool isolates; Lane 12, repeated food isolate. Molecular size (SM) is given in kilobases.

**Results**

**Definition of the outbreak**

All patients were male. Their ages ranged from 21 to 29 old (median 23 ages). The 2,469 soldiers (38.4% of the all 6,418 person) having gastroenteritis had consumed the same kind of meal of mashed potatoes with egg. Among these soldiers, 445 (18%) were admitted to six different hospitals in Isparta during the outbreak period. The first case was hospitalized on Friday January 12, 2008 within 5 hours of having suspicious meals and then the others continued to visit a hospital in subsequent hours. Incubation period among those who ate the suspected meals varied between 5 and 96 hours. The average length of incubation was 46 hours. The main symptoms reported were diarrhea (100%), abdominal pain (93%), fever (91%), nausea (82%), headache (78%), and vomiting (74%). They were given ciprofloxacin (500 mg bid for 7 days), buscopan and total parenteral nutrition solution. Mortality did not occur among these patients during hospitalization. However, four patients required kidney dialysis due to acute renal failure after dehydration.

**Microbiological analysis**

In this study stool samples collected from 445 (18% of the 2,469 cases) hospitalized soldiers having gastroenteritis were analyzed for *Salmonella* spp. Of them, 276 (11.1% of the 2,469 cases) had laboratory confirmed *S.* Enteritidis. *S.* Enteritidis strain was isolated from one of hospitalized patient’s blood culture samples. All employees working in the kitchen were asymptomatic and their stool sample cultures were negative for *Salmonella* spp. No employee declared having had diarrhea or other gastrointestinal symptoms during the previous three months. All food and environmental samples from the kitchen submitted for microbiological analyses were negative for *Salmonella* spp. except for mashed potatoes with egg. All stool isolates, one blood isolate and one food isolate had the same antibiotic susceptibility pattern and were susceptible to all drugs tested which were ampicillin (≤ 4 μg/mL), amoxicillin/clavulanate (≤ 4/2 μg/mL), cefotaxime (≤ 4 μg/mL), chloramphenicol (≤ 4 μg/mL), trimethoprim/sulfamethoxazole (SXT) (≤ 0.5/95 μg/mL), tetracycline (≤ 2 μg/mL), and ciprofloxacin (≤ 0.5 μg/mL).

**PFGE and rep-PCR using DiversiLab**

The PFGE patterns after digestion with *Xba*I, and Rep-PCR using the DiversiLab system were performed for randomly eight selected stool isolates, one blood and one food isolate. All isolates had the same PFGE pattern (Fig. 1). The DiversiLab system showed that all isolates, were genotypically indistinguishable having >95% similarity (Fig. 2). The PFGE and the DiversiLab system interpretations were concordant for *S.* Enteritidis food-borne outbreak isolates. The DiversiLab system allowed a complete microbial typing analysis in approximately 4 hours compared to 3 days for PFGE in our study.
Eggs are especially known to be a source of S. Enteritidis infections in humans. S. Enteritidis contaminate eggs both external by during passage through the hen cloaca and internal by penetration through the eggshell via microscopic cracks. In the United States, either primarily egg-based or that which contained egg ingredients has been described as the source of 75% of food-borne outbreak with a confirmed food vehicles from 1985 to 2003. Although it is not possible to determine the source of infection for most individual case of salmonellosis, the Centers for Disease Control and Prevention has estimated that 50,000-110,000 cases of S. Enteritidis infection are attributed to eggs each year in the United States (26). Also in European Union, eggs, bakery products and meat products were the most important sources, and in approximately 50% of the food-borne outbreaks exposure took place in private homes or restaurants (24). In Turkey, Tansel et al reported a food-borne outbreak causing S. Enteritidis. An egg-containing omelet was considered to be the source of outbreak based on the epidemiological proof (25). In our study, undercooked mashed potatoes with egg were described as the source of the outbreak. We suggest that food items containing eggs should be cooked thoroughly to avoid of food-borne outbreak caused by S. Enteritidis which is the most common egg-associated pathogen.

Since its emergence, the description of outbreak in short-time, tracing transmission routes and defining relationship between humans and food items have demonstrated to be very important (10). Because of the genetic homogeneity of S. Enteritidis isolates, a single method cannot be reliable for epidemiological analysis of unrelated and related strains of S. Enteritidis (2,27-29). Therefore, reliable, sensitive and specific molecular epidemiologic methods are needed for the prevention and control of outbreaks caused by S. Enteritidis. Amplified fragment length polymorphism, random amplified polymorphic DNA, single-enzyme ribotyping analysis has been used subtyping for surveillance and investigation of food-borne outbreaks attributable to S. Enteritidis (2,10,30). Each of these methods has limited discriminatory power, poor reproducibility and, in addition difficult to standardize and interlaboratory comparison (10). It was reported that for multi-locus variable-number tandem repeat analysis its is a useful tool for detection and investigation of outbreak caused by S. Enteritidis (2,10,17,27). PFGE method has been proven to be useful in determining the relatedness of S. Enteritidis isolates and investigation of outbreak as currently gold standard (31-34). However, PFGE is mainly a complex and time-consuming procedure and there is reduced comparability of results between different laborato ries. It also exhibits limited discriminatory power for S. Enteritidis (10,17). The Rep-PCR using the DiversiLab system is a genotyping technique that provides good resolution between multiple strains within a single species (35). Recent studies on Rep-PCR using DiversiLab system have been published for bacteria such as Staphylococcus aureus (36), Acinetobacter baumannii (13), vancomycin-resistant entero-

**Figure 2.** The DiversiLabb systems result (dendrogram and gel-like image) for *Salmonella* Enteritidis. Se 1, food isolate; Se 2, blood isolate; Se 3 to 10, stool isolates.

**Discussion**

To our knowledge, this is the first report of *S. Enteritidis* food-borne outbreak from Turkey investigating the source and route by conventional and molecular microbiological methods.

*Salmonella* cause an estimated millions of illnesses and hundreds of deaths annually in many countries (21). *S. Enteritidis* has been one of the most frequently isolated *Salmonella* associated with food-borne outbreak (22). In the United States, a total of 121 *Salmonella* outbreaks occurred in 2006, causing greater than 3,300 illnesses reported to the CDC Foodborne Outbreak Reporting System. The most common outbreak serotypes were *S. Enteritidis* and *S. Typhimurium* (23). A total of 6,860 food-borne outbreaks were reported in European Union in 2004. *Salmonella* was the most frequently reported cause for these outbreaks (73.9% of the reported outbreaks). *S. Enteritidis* were the predominant serotype associated with outbreaks where the serotype was reported (24). Since surveillance studies about food-borne outbreaks are newly established in Turkey, there is not sufficient documentation on *Salmonella* outbreaks. Tansel et al, described a *S. Enteritidis* food-borne outbreak which occurred in a military battalion located at the north-east of Turkey. The causative strain affected 60 soldiers in the battalion, 16 of whom hospitalized in the first month of the study (25). In the present study, 2,469 soldiers (445 of them hospitalized) were affected from the outbreak caused by *S. Enteritidis*. We should be aware that *S. Enteritidis* is the most common *Salmonella* serotype and may cause large food-borne outbreak in Turkey.
coccis (20), *Streptococcus pneumoniae* (37), and *Escherichia coli* (38). In our study, the PFGE and the DiversiLab system interpretations were concordant for food-borne outbreak isolates. The DiversiLab system completed analysis of 10 samples in approximately 4 hours, compared to 3 days for PFGE. The DiversiLab system was shown to be technically simpler and more easily learned than PFGE. In addition the DiversiLab system, provides an option to digitalize gel images that can be archived in the web-based software, providing for easier comparison of samples between international laboratories.

We suggest that eggs containing food items should not be consumed raw or undercooked. Molecular epidemiologic methods can significantly contribute to a more reliable interpretation of food-borne outbreak caused by *S. Enteritidis*. The DiversiLab system may be a reasonable alternative to PFGE for surveillance and outbreak studies for *S. Enteritidis*, since it provides a simple, rapid and highly specific screening method that archives all gel image data.

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### References


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