



ORIGINAL ARTICLE

A Comparison of Subtyping Methods for Differentiating *Salmonella enterica* Serovar Enteritidis Isolates Obtained from Food and Human Sources

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Abstract

Purpose: To evaluate the abilities of these subtyping methods, we distinguished *Salmonella* Enteritidis (*S. Enteritidis*) isolated from food products and human clinical samples between 2009 and 2010 in Seoul using five subtyping methods.

Methods: We determined the subtypes of 20 *S. Enteritidis* isolates from food and human sources using phage typing, antimicrobial susceptibility, pulsed-field gel electrophoresis (PFGE), repetitive sequence-based PCR (rep-PCR), and multi-locus sequence typing (MLST).

Results: A total of 20 tested isolates were differentiated into six antimicrobial susceptibility patterns, three different phage types, four different PFGE profiles, seven rep-PCR patterns, and one MLST type. Food isolates were considerably more susceptible to antibiotics than human isolates. We were best able to discriminate among *S. Enteritidis* isolates using rep-PCR, and obtained the highest Simpson's diversity index of 0.82, whereas other methods produced indices that were less than 0.71. PFGE pattern appeared to be more related to antimicrobial resistance and phage types of *S. Enteritidis* isolates than rep-PCR. MLST revealed identical alleles in all isolates at all seven loci examined, indicating no resolution.

Conclusion: The results of this study suggest that rep-PCR provided the best discriminatory power for phenotypically similar *S. Enteritidis* isolates of food and human origins, whereas the discriminatory ability of MLST may be problematic because of the high sequence conservation of the targeted genes.

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1. Introduction

Nontyphoidal *Salmonella enterica* is a major cause of foodborne illness worldwide [1,2]. Most human cases of nontyphoidal *Salmonella* result from the consumption of contaminated foods of animal origin, especially poultry meat and eggs [3–5]. The *S. enterica* serotypes Typhimurium, Enteritidis, and Newport were the most common serotypes identified among human infection pathogens commonly transmitted through food, according to preliminary FoodNet data for 2005 [2].

Salmonella typing technologies are essential for bacterial source tracking and to determine the distribution of pathogens that have been isolated from infected people [6]. Traditional typing methods based on phenotypic traits, such as biotyping, antibiotic susceptibility profiles, serotyping, and phage typing, provide insufficient information for epidemiological purposes [6,7]. Molecular subtyping methods have revolutionized the identification of microbial strains, but most of them have not been internationally standardized [7]. Subtyping methods have been developed based on three main mechanisms of discrimination: (1) restriction analysis of bacterial DNA, (2) polymerase chain reaction (PCR) amplification of particular genetic targets, and (3) the identification of DNA sequence polymorphism at specific loci in the genome [6].

Pulsed-field gel electrophoresis (PFGE) is a form of restriction fragment length polymorphism analysis typing, in which restriction patterns of whole bacterial genomes are analyzed and compared [6,7]. PFGE is used by the PulseNet program, a molecular subtyping network for foodborne bacterial disease surveillance at the U.S. Centers for Disease Control and Prevention (CDC), to identify widespread outbreaks of bacterial foodborne illness [8,9].

Repetitive sequence-based PCR (rep-PCR) is an amplification-based method that utilizes the repeated DNA sequence elements distributed throughout the genomes of many bacterial species [6]. Rep-PCR uses noncoding repetitive sequence primers to produce copies of DNA fragments [10]. Differences in the resulting banding patterns due to differences in the number and size of amplified repetitive elements and can be compared to determine the genetic relatedness of microbial strains [6,10].

In recent years, powerful sequencing facilities and the availability of genome sequences have allowed the development of new typing methods such as multilocus sequence typing (MLST) [11]. In MLST, the variability in a relatively small part of the genome due to mutation or recombination events is investigated through the comparison of nucleotide base changes in multiple genes with conserved sequences, such as housekeeping genes [8,12–14]. MLST is a relatively expensive method that may not be available for use in many clinical laboratories or for routine surveillance [12]. However, it has been developed for a number of

clinically important bacterial pathogens, including *Salmonella* spp., and can be useful as a discriminatory typing method for *Salmonella* spp. [13].

There are many subtyping methods that have been developed as described above, but only PFGE is often considered the “gold standard” for molecular typing methods of bacterial foodborne pathogens [6,8,15]. In addition, few studies that evaluated the ability of subtyping methods based on different mechanisms of discrimination to differentiate phenotypically similar but epidemiologically unrelated isolates were reported. Therefore, we determined the subtypes *S. Enteritidis* isolates from food and human sources using two phenotypic subtyping methods (phage typing and antimicrobial susceptibility) and three genotypic subtyping methods (PFGE, rep-PCR, and MLST) and compared their ability to distinguish among *S. Enteritidis* isolates.

2. Materials and Methods

2.1. Bacterial isolates

We used a total of 20 *S. Enteritidis* isolates isolated from human fecal samples ($n = 10$) and retail meats ($n = 10$). The 10 retail-meat isolates were from beef ($n = 3$), pork ($n = 2$), and chicken ($n = 5$) that were obtained from retail markets in Seoul, South Korea, in 2009 and 2010. Ten human isolates were randomly selected from the culture collection of the Seoul Research Institute of Public Health and Environment (SIHE; Gwachon, South Korea). These samples were isolated from sporadic diarrheal patients in Seoul, South Korea, between 2009 and 2010. SIHE confirmed that the isolates were *Salmonella* by using VITEK Gram-negative identification cards (bioMérieux, Durham, NC) and serotyped using commercial Difco antisera (BD, Sparks, MD, USA).

2.2. Phage typing

All 20 *S. Enteritidis* isolates were phage typed using the standardized CDC method [16,17]. Phage typing was performed at the Animal, Plant, and Fisheries Quarantine and Inspection Agency (Anyang, South Korea).

2.3. Antibiotic susceptibility test

The antibiotic susceptibilities of *S. Enteritidis* isolates were determined with the disk diffusion method, as recommended by the Clinical and Laboratory Standards Institute [18]. Sensi-Disc Antimicrobial Susceptibility Test Discs (Oxoid, Basingstoke, UK) were used with the following antibiotics: 10 μg ampicillin, 30 μg amikacin, 30 μg chloramphenicol, 30 μg cephalothin, 5 μg ciprofloxacin, 10 μg gentamicin, 10 μg streptomycin, 25 μg sulfamethoxazole/trimethoprim, 30 μg tetracycline, 30 μg cefazolin, 30 μg amoxicillin/clavulanic acid, 30 μg cefepime, 30 μg ceftaxime, 30 μg cefotaxim, 5 μg enrofloxacin, 10 μg norfloxacin, and 10 μg imipenem.

The diameter of inhibition zones was recorded, and samples were scored as sensitive, intermediate, and resistant according to the scoring system recommended by the Clinical and Laboratory Standards Institute.

2.4. PFGE

DNA and agarose were prepared for PFGE as described in previous studies [19,20]. For restriction endonuclease digestion, two 1-mm-thick slices of each plug were incubated at 37 °C for 1.5 h with 30 U of *NotI* enzyme (Takara Bio Inc., Otsu, Shiga, Japan) in 100 mL of the appropriate restriction enzyme buffer. The restriction fragments were separated by electrophoresis in 0.5M Tris borate–EDTA buffer at 14 °C for 18 h using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA, USA) with pulse times between 2.16 and 54.17 seconds. The gels were stained with ethidium bromide, and DNA bands were visualized with UV transillumination (Bio-Rad). *Salmonella* serovar Braenderup ATCC BAA 664 was used as the control strain and digested with 30 U of *XbaI* (Takara Bio Inc.). Interpretation of DNA fingerprint patterns was accomplished using Bionumerics 4.0 software (Applied Maths, Austin, TX, USA). The banding patterns were compared using Dice coefficients with a 1.5% band position tolerance. Patterns with no noticeable differences were considered indistinguishable and were assigned the same PFGE pattern designation.

2.5. Rep-PCR using DiversiLab

S. Enteritidis was cultured on nutrient agar (Difco) for 24 h at 37 °C. DNA from each isolate was extracted using UltraClean Microbial DNA Isolation Kits (MoBio Laboratories, Solana Beach, CA, USA) based on the manufacturer's instructions. Genomic DNA samples were quantified using a NanoDrop 2000 UV spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at 260 nm. For PCR reactions, 2 µL of genomic DNA (approximately 25 ng/µL) was amplified using the DiversiLab *Salmonella* Kit (bioMérieux Inc.). The final mixture (23 µL) contained 0.5 µL (or 2.5 U) of AmpliTaq polymerase (Applied Biosystems, Foster City, CA, USA), 2.5 µL of 10× GeneAMP PCR Buffer I (Applied Biosystems), 2 µL kit-supplied primer mix, and 18 µL of the kit-supplied rep-PCR master mix (MM1). Thermal cycling parameters were as follows: initial denaturation at 94 °C for 2 min; followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, extension at 70 °C for 90 seconds; and a final extension at 70 °C for 3 minutes. The rep-PCR products were separated and detected by a micro-fluidics chip and Agilent model 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA). DNA fingerprint patterns were generated as electropherogram, which were automatically exported to the DiversiLab software for analysis. Both band position and intensity of all samples were analyzed and compared with web-based DiversiLab software (version 3.3) using the

extended Jaccard coefficient. Distance matrices and the unweighted pair-group method with arithmetic mean were used to create a dendrogram [21]. The isolates were categorized as follows: indistinguishable isolates had >97% similarity with no banding differences and no variation in intensities of individual bands; similar isolates had 95–97% similarity and one or two different bands difference; different isolates had <95% similarity and two or more different bands [21]. Isolates were assigned unique rep-PCR types unless classed as indistinguishable or similar using the above criteria.

2.6. MLST

MLST was performed according to the method described in previous studies [12,13,22–30] (<http://mlst.ucc.ie/mlst/dbs/Senterica>). DNA was extracted using an UltraClean Microbial DNA Isolation Kit. PCR reactions were performed in 20 µL reaction mixtures in a thermocycler (Biometra, Göttingen, Germany). For PCR reactions, 0.5 µM DNA template was added to 50 µL PCR mixture consisting of Pyrobest DNA polymerase (5 U/µL, 0.5 µL), 10× Pyrobest buffer II (Takara Bio Inc.), 1 µM of each primer (<http://mlst.ucc.ie/mlst/dbs/Senterica>), and dNTP mixture (2.5 mM each). The mixture was initially denatured at 98 °C for 5 minutes followed by 40 cycles of 98 °C for 10 seconds, 55 °C for 30 seconds, 72 °C for 1 minute, and finally elongated at 72 °C for 5 minutes. The PCR products were separated on 2% agarose gels. TIFF images of agarose gels were generated using the GelDoc XR+ gel documentation system (Bio-Rad) and Image Lab 3.0 Software (Bio-Rad). After PCR product purification, the DNA sequences of clones were analyzed by the Custom Oligonucleotide Synthesis Manufacture Office (Seoul, South Korea). Allele numbers were assigned an MLST type after the distinct allele sequences were submitted via the Internet to the dedicated database (<http://mlst.ucc.ie/mlst/dbs/Senterica>).

2.7. Data analysis

The diversity of PFGE, rep-PCR, and MLST was assessed using Simpson's index (D). Confidence intervals were calculated as described in a previous study [31]. We calculated Simpson's index as:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s n_j(n_j - 1)$$

where N is the total number of isolates in the sample population, s is the total number of types, and n_j is the number of isolates belonging to the j th type.

3. Results

3.1. Antibiotic susceptibility profiles

Six antibiotic susceptibility patterns of *S. Enteritidis* isolates are shown in Figure 1, and the Simpson's index of this method was 0.68 (Table 1). Twenty percent of food

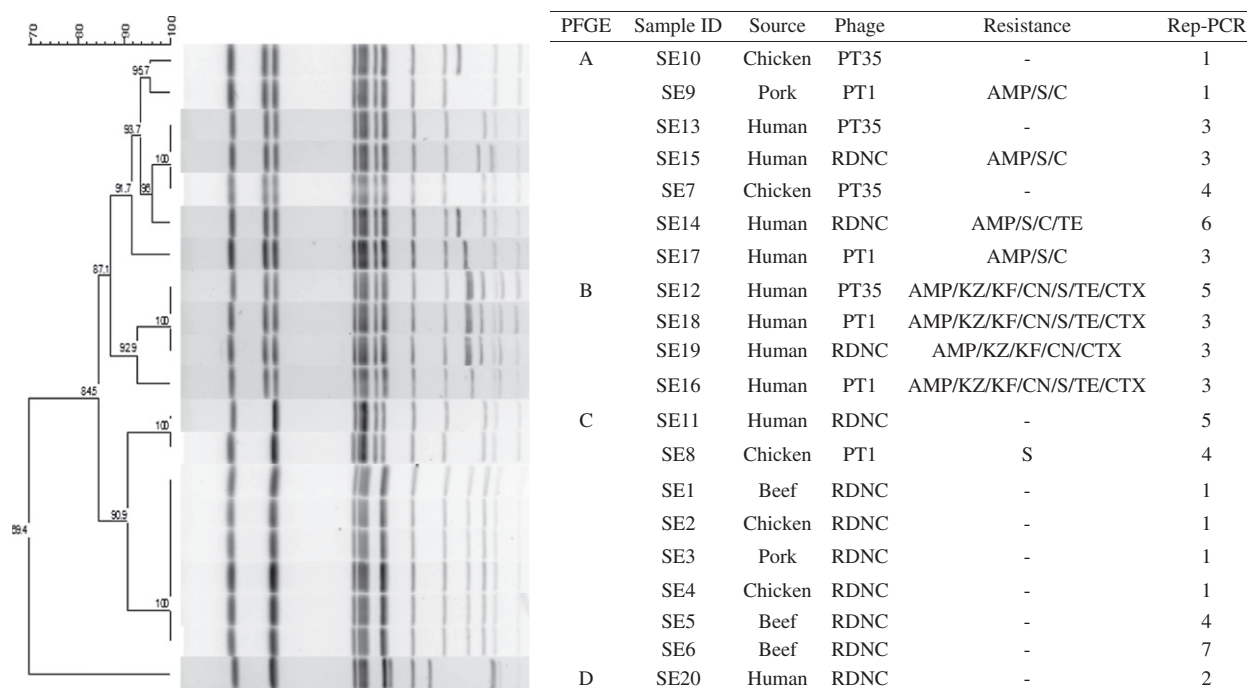


Figure 1. Pulsed-field gel electrophoresis (PFGE) patterns of the digested genomic DNA from food and human *Salmonella enterica* serovar Enteritidis isolates ($n = 20$). Six antimicrobial susceptibility patterns, three phage types, four PFGE types (A through D), and seven rep-PCR types (1 through 7) were identified among the 20 isolates. AMP = ampicillin, C = chloramphenicol, CN = gentamicin, CTX = cefotaxim, KF = cephalothin, KZ = cefazolin, S = streptomycin, RDNC = reaction does not confirm, TE = tetracycline.

isolates and 70% of human isolates were resistant to at least one of the 16 antibiotics tested. The highest resistance rate was observed for ampicillin and streptomycin (40%), followed by chloramphenicol, cephalothin, gentamicin, tetracycline, cefazolin, and cefotaxim (20%). Eight of the 20 isolates (40%) were resistant to two or more antibiotics, and seven of these were human isolates (Figure 1). Among the eight isolates resistant to two or more antibiotics, SE12, SE16, SE18, and SE 19 were resistant to ampicillin–cefazolin–cephalothin–gentamicin–cefotaxim–(streptomycin–tetracycline), and the other four were resistant to ampicillin–streptomycin–chloramphenicol (Figure 1).

3.2. Phage type

The *S. Enteritidis* isolate phage types are shown in Figure 1. The Simpson's index from the results of this method was the lowest, at 0.62 (Table 1). We found a total of three phage types, PT1, PT35, and reaction does not confirm (RDNC), among the 20 isolates tested. These phage types were as follows: RDNC (55%, six food and five human isolates), PT35 (25%, two food and three human isolates), and PT1 (20%, two food and two human isolates).

3.3. PFGE and rep-PCR patterns

A total of four types (A through D) of PFGE pattern were generated from the 20 *S. Enteritidis* isolates

Table 1. Comparison of different subtyping methods for *Salmonella enterica* serovar Enteritidis isolates

Method	No. of types	Frequent type	Simpson's index (CI) ^a
Antibiotic susceptibility	6	Non antibiotic resistance	<i>D</i> 0.68 (0.48–0.87)
Phage typing	3	RDNC	<i>D</i> 0.62 (0.48–0.77)
PFGE	4	Type C	<i>D</i> 0.71 (0.62–0.79)
Rep-PCR	7	Types 1 and 3	<i>D</i> 0.82 (0.73–0.91)
MLST	1	11	<i>D</i> 0

^aSimpson's index (*D*) represents the probability that two randomly selected strains will not belong to the same group. CI = confidence interval; RDNC = reaction does not confirm.

(Figure 1), and the Simpson's index was 0.71 (Table 1). In rep-PCR pattern, a total of seven types (1 through 7) were generated (Figure 1), and the Simpson's index was 0.82 (Table 1). Among the PFGE patterns, PFGE type C was the largest group with seven food isolates and one human isolate (Figure 1). Seven of eight isolates of PFGE type C with 100% similarity were phage type RDNC and not resistant to antibiotics, and they were included in rep-PCR types 1, 4, and 7. All four human isolates of PFGE type B had a similar antimicrobial resistance pattern, ampicillin–cefazolin–cephalothin–gentamicin–cefotaxim, and three of them showed 100% similarity in PFGE and separated to rep-PCR types 3 and 5 (Figure 1). PFGE type A included four human and three food isolates that were either not resistant to antibiotics or resistant to ampicillin–streptomycin–chloramphenicol (Figure 1). PFGE type A isolates were differentiated into rep-PCR types 1, 3, 4, and 6 (Figure 1).

Among rep-PCR patterns, rep-PCR types 1 and 3 were large groups, and four isolates of rep-PCR type 1 were phage type RDNC and not resistant to antibiotics and were included in PFGE type C. In addition, three isolates of rep-PCR type 3 were resistant to ampicillin–cefazolin–cephalothin–gentamicin–cefotaxim and were included in PFGE type B. However, rep-PCR types 4 and 5 did not show a similar phenotypic pattern, and rep-PCR types 2, 6, and 7 had one isolate.

3.4. MLST analysis

Seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) were compared using MLST analysis (Table 1). All isolates were assigned to MLST 11 in the dedicated database (<http://mlst.ucc.ie/mlst/dbs/Senterica>), resulting in the Simpson's index (*D*) of zero (Table 1). All isolates possessed identical alleles at all seven loci; *aroC* allele type 5, *dnaN* allele type 2, *hemD* allele type 3, *hisD* allele type 7, *purE* allele type 6, *sucA* allele type 6, and *thrA* allele type 11.

4. Discussion

Given the public health hazard posed by *S. Enteritidis*, it is important to be able to quickly describe outbreaks, trace transmission routes, and define relationships between human and food isolates [32,33]. We characterized *S. Enteritidis* isolates ($n = 20$) from food and human sources by antibiotic susceptibility, phage typing, PFGE, rep-PCR, and MLST. We then evaluated the correlations between the types formed using the results of each subtyping method. Six antimicrobial susceptibility patterns, three phage types, four PFGE types, and seven rep-PCR types were identified among the 20 isolates. MLST typing had no discriminatory power, as only one MLST type was observed.

We also investigated the typing methods as tools for determining the source, either human or food, of *S.*

Enteritidis isolates. Antibiotic resistance to two or more antibiotics was more common in human isolates than in food isolates. PFGE and rep-PCR tended to generate human or animal-specific clustering. Many of the human isolates possessed similar PFGE patterns (type A and B), and food and human isolates were not included in same type in rep-PCR pattern.

The discriminatory power of five subtyping methods was measured by calculation of Simpson's index. Simpson's index is commonly used as an estimate of the discriminatory ability of subtyping methods, and it is a measure of the probability that two epidemiologically unrelated isolates will be characterized as being "different" by the typing method under evaluation [32,34]. The highest Simpson's index was obtained from rep-PCR followed by PFGE, antibiotic susceptibility, phage typing, and MLST. In a previous study, the semiautomated rep-PCR (DiversiLab) patterns have a limited ability to discriminate some serotypes of *Salmonella* from different sources [35,36]. In addition, PFGE and rep-PCR exhibited a similar discriminatory ability in patient and food samples from a large food-borne outbreak of *S. Enteritidis* [33]. However, in the current study using epidemiologically unrelated isolates, rep-PCR showed the best discriminatory power with the highest Simpson's index.

PFGE pattern appeared to be more related to antimicrobial resistance profiles and phage types of *S. Enteritidis* isolates than rep-PCR, and Simpson's index was higher in rep-PCR than in PFGE (Figure 1 and Table 1). *S. Enteritidis* isolates with similar antibiotic resistance pattern were indistinguishable in PFGE types A, B, and C. However, these isolates were separated into rep-PCR types 1, 3, and 6 (PFGE type A), types 3 and 5 (PFGE type B), and types 1, 4, 5, and 7 (PFGE type C). These results of PFGE pattern in this study are in agreement with those of previous studies by Foley et al [8] and Harbottle et al [13]. *S. Typhimurium* isolates with indistinguishable PFGE patterns exhibited resistance to at least five antimicrobials [8], and *S. Newport* isolates with indistinguishable PFGE patterns exhibited similar antibiotic resistance patterns [13]. These results indicate that *S. Enteritidis* isolates with similar antimicrobial susceptibility patterns might be indistinguishable by PFGE. This limitation can be overcome by the use of a second enzyme for PFGE analysis or a combination of rep-PCR, further increasing the ability of researchers to differentiate between *S. Enteritidis* isolates that have similar phenotypes [8,37].

In this study, all isolates of *S. Enteritidis* tested were assigned to MLST 11, according to the MLST database (<http://mlst.ucc.ie/mlst/dbs/Senterica>). In previous studies using the same database [12,26,30], MLST was highly correlated to *Salmonella* serotype. Noda et al [30] found that 30 *S. Enteritidis* isolates collected in Japan between 1973 and 2004 had homologous MLST type 11 sequences and no nucleotide differences in seven

housekeeping genes. Another study targeting seven different housekeeping and virulence genes found that MLST was not able to discriminate clinically relevant serotypes of *Salmonella* well [38]. The limited discriminatory ability of MLST may be a result of the moderate to slow rate of mutation accumulation within the targeted housekeeping genes [13]. Therefore, the discriminatory performance of MLST needs to be increased if more variable gene targets are examined.

To the best of our knowledge, this study is the first in which the utility of the five subtyping methods were compared to differentiate *S. Enteritidis* isolates from both humans and food sources. The present report describes the preliminary results of the use of a small number of *S. Enteritidis* isolates from sporadic cases. Further research is required on the use of a number of *S. Enteritidis* isolates that have various epidemical characteristics. A combination of various typing methods may increase the ability to discriminate among similar serotypes of *S. Enteritidis* and to determine the origin of pathogens. This, in turn, may lead to improved source tracking of foodborne pathogens during outbreaks.

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