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Subtyping of *Salmonella Enterica* Serovar Typhimurium from Clinical Samples by Multiple-Locus Variable-Number Tandem Repeat Analysis and Pulsed-Field Gel Electrophoresis

Sana A. Khan[1], Steven Foley[2], Rossina Stefanova[3], Mohamed S. Nawaz[2], Carl E. Cerniglia[2] and Ashraf A. Khan[2*]

[1]*University of Arkansas- Fayetteville, USA.*

[2]*National Center for Toxicological Research, USA.*

[3]*The Arkansas Department of Health, USA.*

[*]Correspondence:
Ashraf A. Khan, Ph.D., Research Scientist, Division of Microbiology, U.S. Food and Drug Administration, National Center for Toxicological Research, Jefferson, AR 72079, USA, Tel: 870-543-7601; Fax: 870-543-7307.

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ABSTRACT

A total of 47 Salmonella enterica ser. Typhimurium strains were isolated from patients and were analyzed for antibiotic susceptibility, virulence genes, plasmids, Multiple-locus Variable-number Tandem Repeat Analysis (MLVA) and Pulsed-field Gel Electrophoresis (PFGE). The composite analysis was based on equal weighting of PFGE restriction enzyme XbaI and MLVA data. Clustering for both data sets was performed using the unweighted pair group method with arithmetic mean (UPGMA). All the isolates were susceptible to gentamicin, kanamycin, nalidixic acid, and trimethoprim-sulfamethoxazole. Six isolates were resistant to ampicillin, one isolate to chloramphenicol, and another isolate to tetracycline. Sixteen virulence genes (sopB, pagC, pefA, spiA, msgA, tolC, spvB, spaN, invA, iroN, orgA, sifA, ipfC, prgH, sipB, and sitC), were screened by the polymerase chain reaction (PCR). All isolates were positive for ten genes: msgA, tolC, spaN, invA, iroN, sifA, ipfC, prgH, sipB, and sitC; and negative for the pefA gene. Seven strains carried one or more plasmids. Twenty-five MLVA patterns were detected among the 47 clinical isolates. The six most common patterns in the data set were all highly related with each other. Most of the patterns had been seen before in the PulseNet national MLVA database; however, patterns for seven of the isolates were new. Additionally, 14 isolates with 9 different MLVA patterns were deemed rare to the database (0.21%). Analysis of the PFGE and MLVA data as a composite dataset improved the discrimination between isolates compared to either data set alone, dividing the isolates into 29 different profiles. The highly discriminatory nature of MLVA, and its usefulness as a complementary technique for PFGE, were well illustrated in this study. There were 4 different XbaI PFGE restriction patterns. MLVA, PFGE, plasmid, antibiotic susceptibility, and virulence gene analysis were useful and important tools to discriminate S. Ser. Typhimurium strains from clinical samples.

Keywords

Salmonella enterica serovar Typhimurium, PFGE, MLVA.

Introduction

Salmonellosis, is one of the most common food-borne diseases that occur in humans. Every year in the United States, nontyphoidal *Salmonella* causes an estimated 1.2 million illness, 23,000 hospitalizations, and 450 deaths [1]. There are many milder cases of salmonellosis that are not reported. Children are prone to an infection caused by *Salmonella*, but infants, elderly, and immunocompromised people are more likely to attract severe infections [2]. *Salmonella* infections are usually caused by the consumption of fecal contaminated water and food, including beef, pork, poultry, vegetables, seafood, and dairy [3-7]. The Centers for Disease Control and Prevention (CDC) has estimated that non-typhoid *Salmonella* has caused about one million cases of food-borne illnesses (11% of total food-borne illnesses), 19,336 cases of hospitalization (35% of total food-borne illnesses resulting in hospitalization) in the United States [8,9].

Typhimurium is one of the most common serovars of *Salmonella enterica*, which is predominantly found in the intestinal lumen

[9]. Fresh and processed poultry products account for one third of *Salmonella* infections in humans [10]. Chicken, ground beef, hedgehogs, peanut butter, cantaloupes, and tomato have been major vehicles of transmission of *S. ser.* Typhimurium in several outbreaks [11]. Serovar Typhimurium is not the most virulent type of *Salmonella*, but it is the leading cause of foodborne salmonellosis infection [12]. Infection caused by *S. ser.* Typhimurium are typically not fatal and often resolve, without the need for antimicrobial therapy. Fatal cases are typically associated with people who are immunocompromised [13].

Pulsed field gel electrophoresis (PFGE) is a gold standard subtyping method for foodborne bacterial pathogens used by PulseNet, the national molecular subtyping network for foodborne disease surveillance in the United States [14-17]. The PFGE method has been consistently used during foodborne outbreak investigations for discriminating strains [8]. Currently *Salmonella* surveillance efforts have focused on the serotype, location, PFGE typing and date of infection [8], but this approach sometime is not enough to distinguish the outbreak strains.

Among these typing methods PFGE has been proven to be important for establishing genetic relatedness of different bacterial strains and is commonly used during foodborne outbreaks investigations associated with specific pathogens as well as routine surveillance [10,16,18-20]. MLVA is another highly discriminatory subtyping method for bacterial pathogens that is based on the detection of short sequence repeats in the microbial genome [21]. Recently, MLVA has been used as an alternative to PFGE for subtyping of *Salmonella* ser. Enteritidis and several other pathogenic bacteria [15,22-25].

In this report we utilized antibiotic resistance, virulence genes, PFGE, and MLVA to fingerprint the forty-seven clinical *S. ser.* Typhimurium. Routine PFGE typing was unable to distinguish several isolates. To further type these clinical isolates, we used a MLVA and PFGE composite dataset to improve the discrimination between isolates compared to either dataset alone. We also report seven new MLVA patterns of *S. ser.* Typhimurium which have not been reported before in the PulseNet national MLVA database.

Materials and Methods

Bacterial strains and culture conditions

The forty-seven *Salmonella enterica* ser. Typhimurium clinical strains used in this study were obtained from Arkansas Department of Health investigations (Figure 1). *S. enterica* was grown overnight at 37°C in Luria-Bertani (LB, Becton Dickinson and Company, Franklin Lakes, NJ) broth or on a tryptic soy agar plate supplemented with 5% sheep blood (Thermo Fisher Scientific, Waltham, MA). All isolates were stored in LB broth containing 20% glycerol at -70°C.

Antimicrobial susceptibility testing by disk diffusion

All of the ser. Typhimurium isolates that were used to conduct this study were tested for resistance to eight antibiotics on Mueller-Hinton agar (Becton Dickinson and Company) by a agar disk diffusion method [26]. The antibiotics used were: kanamycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), trimethoprim-sulfamethoxazole (5 µg), ampicillin (10 µg), and gentamicin (10 µg) (Becton Dickinson and Company). Susceptibility and resistance were determined in accordance with the criteria of the Clinical and Laboratory Standards Institute [27]. *Escherichia coli* ATCC 25922 was used for quality control, because it is susceptible to all the antibiotics.

PCR detection of virulence genes

The ser. Typhimurium isolates were screened for sixteen virulence genes by the simplex PCR method using single set primers [28]. The PCR reaction mixtures, with a final volume of 10 µl, contained 2 µl template DNA, 5 µl GoTaq Green Master Mix (Promega Corporation, Madison, WI), 1 µl each of forward and reverse primer, and 1 µL distilled water (Table 1). The PCR cycling conditions were as follows: 5 min for the initial denaturation at 95°C; 30 cycles of 40s at 94°C for denaturation, 60s at 66.5°C for annealing, and 90 s at 72°C for elongation; and a final elongation step of 10 min at 72°C. The PCR products were analyzed by electrophoresis on 2% E-gel 96 (Fisher Scientific). For PCR products smaller than 500 bp the E-gel 96 ran for 8 min, and larger PCR products ran for 10 min. E-gel Low Range DNA Ladder (Thermo Fisher Scientific) was used as the molecular weight marker.

Table 1: Primers used in PCR for detection of virulence genes in *S. ser.* Typhimurium.

Gene	Sequence of Nucleotides	size (bp)	Function of gene
spvB	F- CTATCAGCCCCGCACGGAGAGCAGTTTTTA	717	Growth within host
	R- GGAGGAGGCGGTGGCGGTGGCATCATA		
spiA	F- CCAGGGGTCGTTAGTGTATTGCGTGAGATG	550	survival within macrophage
	R- CGCGTAACAAAGAACCCGTAGTGATGGATT		
pagC	F- CGCCTTTTCCGTGGGGTATGC	454	survival within macrophage
	R- GAAGCCGTTTATTTTTGTAGAGGAGATGTT		
cdtB	F- ACAACTGTCGCATCTCGCCCCGTCATT	268	Host recognition / invasion
	R- CAATTTGCGTGGGTTCTGTAGGTGCGAGT		
msgA	F- GCCAGGCGCACGCGAAATCATCC	189	survival within macrophage
	R- GCGACCAGCCACATATCAGCCTCTTCAAAC		
invA	F- CTGGCGGTGGGTTTTGTTGTCTTCTCTATT	1070	Host recognition / invasion
	R- AGTTTCTCCCCCTCTTCATGCGTTACCC		
sipB	F- GGACGCCGCCCGGGAAAAACTCTC	875	Entry into nonphagocytic cells
	R- ACACTCCCGTCGCCGCCTTCACAA		
prgH	F- GCCCGAGCAGCCTGAGAAGTTAGAAA	756	Host recognition / invasion
	R- TGAAATGAGCGCCCCTTGAGCCAGTC		
spaN	F- AAAAGCCGTGGAATCCGTTAGTGAAGT	504	Entry into nonphagocytic cells
	R- CAGCGCTGGGGATTACCGTTTTG		
orgA	F- TTTTTGGCAATGCATCAGGGAACA	255	Host recognition / invasion
	R- GGCGAAAGCGGGGACGGTATT		
tolC	F- TACCCAGGCGCAAAAAGAGGCTATC	161	Host recognition / invasion
	RCCGCGTTATCCAGGTTGTTGC		
iroN	F- ACTGGCACGGCTCGCTGTCGCTCTAT	1205	Iron acquisition
	R- CGCTTTACCGCCGTTCTGCCACTGC		

sitC	F- CAGTATATGCTCAACGCGATGTGGGTCTCC R- CGGGGCGAAAATAAAGGCTGTGATGAAC	768	Iron acquisition
lpfC	F- GCCCCGCCTGAAGCCTGTGTTGC R- AGGTCGCCGCTGTTTGAGGTTGGATA	641	Host recognition / invasion
sifA	F- TTTGCCGAACGCGCCCCCACACG R- GTTGCCTTTTCTTGCGCTTTCCACCCATCT	449	Filamentous structure formation
sopB	F- CGGACCGGCCAGCAACAAAACAAGAAGAAG R- TAGTGATGCCCGTTATGCGTGAGTGTATT	220	Host recognition / invasion
pefA	F- GCGCCGCTCAGCCGAACCAG R- GCAGCAGAAGCCCAGGAAACAGTG	157	Host recognition / invasion

Plasmid profiles

Isolation and profiling of plasmids were performed following the alkaline lysis method described by Ponce et al. [16]. The strains were inoculated overnight at 32°C in 2 ml prepared LB broth (BD). From the overnight cultures of bacterial growth, 1.5 ml was used for the plasmid isolation as described previously [16]. The plasmids were separated on 0.8 % agarose gels in 1 X Tris- acetate-EDTA (TAE) buffer at 64 V for 110 min. The agarose gel was stained with ethidium bromide and visualized by using Bio-Rad Gel DOC XR imaging system (Bio-Rad Laboratories, Richmond, CA). The supercoiled DNA ladder (Thermo Fisher Scientific) was used as the molecular weight marker.

Pulsed-field gel electrophoresis (PFGE)

S. ser. Typhimurium cells were grown overnight on blood agar plates (Thermo Fisher Scientific) at 37°C. PFGE was performed following a procedure described by Ribot et al. (2006) [17] with some modifications. The plugs were digested with 12 Units of restriction enzyme *XbaI* (Promega Crop.) for 5 h at 37°C. Digested fragments were resolved in 1% SeaKem Gold Agarose (Cambrex Bio Science Rockland Inc., Rockland, ME) gel in 0.5 x Tris-Borate-EDTA (TBE) buffer using a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF-Mapper, Bio-Rad).

Electrophoresis was performed at 6 V/cm with 2.16- 63.8 s linear ramp times of 19 h. Gels were cooled at 14°C throughout the run and then stained with ethidium bromide and de-stained with distilled water. Banding patterns were visualized by UV and photographed using the Gel Doc XR Imaging System (Bio-Rad). The *S. ser.* Braenderup strain H9812 PulseNet standard was used as a molecular weight marker after digestion with *XbaI*. Fingerprinting profiles were examined manually using the BioNumerics software (Applied Maths, Inc., Austin, TX). The extent of variability was determined by the Dice coefficient and clustering was based on the un-weighted pair group average (UPGA) method.

Multi-Locus Variable-Number Tandem Repeat Analysis (MLVA)

Multiple-Locus Variable-Number Tandem Repeat Analysis (MLVA) was performed according to the procedures described in the Laboratory Standard Operating Procedure for PulseNet MLVA of *Salmonella enterica* serotype Typhimurium – Applied Biosystems Genetic Analyzer 3500 and PulseNet Standard Operating Procedure for Analysis of MLVA data of *Salmonella enterica* serotype Typhimurium in BioNumerics – Applied Biosystems Genetic Analyzer 3500 data (http://www.pulsenetinternational.org/protocols/Pages/mlva.aspx).

The composite analysis for the combined PFGE and MLVA data was based on equal weighting of *XbaI* and MLVA data. Composite analysis for MLVA allele type data alone was performed using the categorical similarity coefficient. Clustering for both datasets was performed using the unweighted pair group method with arithmetic mean (UPGMA).

Results

Antibiotic resistance profiles and PCR amplification of virulence genes

Of the 273 *S. ser.* Typhimurium strains which were isolated during 2002-2011 from clinical samples in thirty-two counties of Arkansas, forty-seven strains were selected representing at least five isolates from each year (Figure 2). All forty-seven ser. Typhimurium isolates were susceptible to gentamicin, kanamycin, nalidixic acid, and trimethoprim-sulfamethoxazole. However, six isolates TM6, TM27, TM 35, TM40, TM46 and TM48 were resistant to ampicillin, and one isolate TM20 was resistant to both chloramphenicol and tetracycline (Figure 3). Thirty-six isolates showed an intermediate resistance to streptomycin (Figure 3).

The virulence gene amplification of sixteen genes showed that the forty-seven isolates were positive for at least nine virulence genes: *msgA, tolC, spaN, invA, prgH, sifA, ipfC, sipB*, and *sitC* (Figure 3). All isolates were negative for pefA gene. Nine isolates were positive for eleven virulence genes. Three isolates TM13, TM40 and TM48 were positive for fifteen virulence genes although they were isolated in 2009, 2008 and 2006 (Figure 3).

Plasmid profile

Among the forty-seven *S. ser.* Typhimurium isolates, seven isolates carried plasmids. Isolates T8, T12, T20, T36, T37, and T46 carried large plasmids sized greater than 50 kb. Isolate T20 carried three plasmids, one was 50 kb and the other two were sized less than 6 kb. Isolate T47 carried one small plasmid that was sized less than 6 kb.

Pulsed-field gel electrophoresis (PFGE)

The PFGE typing of forty-seven *S. ser.* Typhimurium isolates by *XbaI* resulted in 17-18 bands and gave four different profiles. The dendrogram of the PFGE profiles is shown in Figure 4. Out of 47 isolates, 44 isolates grouped in one cluster which could not be distinguished among each other. However, these isolates were isolated from different counties over a ten-year timespan.

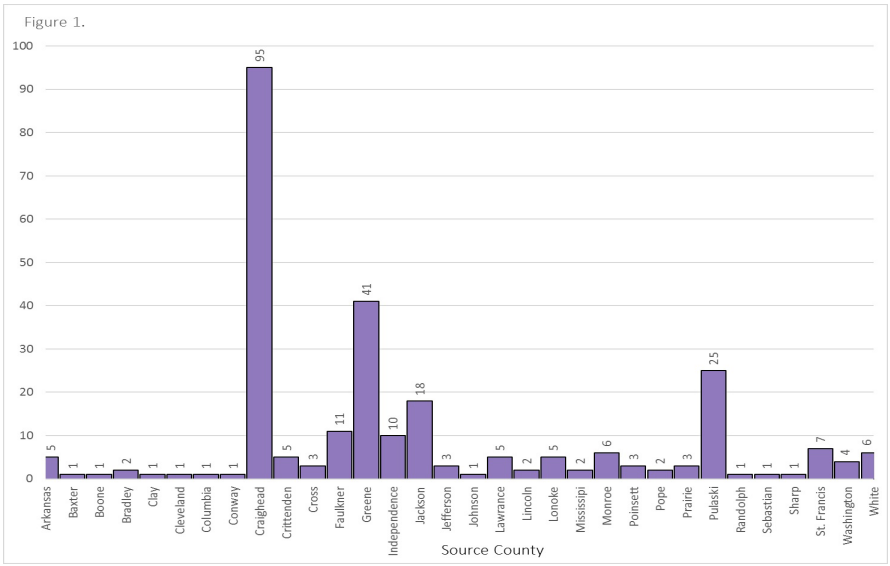
Multi-Locus Variable-Number Tandem Repeat Analysis (MLVA)

Twenty-five MLVA patterns were detected among the 47 clinical isolates, with the largest cluster of isolates displaying pattern A (Table 2). MLVA pattern names were assigned only to groups consisting of 3 or more isolates, and the largest cluster of isolates

consisted of 10 isolates (Figure 5). The six most common patterns in the dataset were all highly related with each other. Most of the patterns had been seen before in the PulseNet national MLVA database; however, patterns for seven of the isolates (TM 1, TM 4, TM 5, TM 10, TM 39, TM 40, TM 44, TM 46, TM47) were new. Additionally, 14 isolates with 9 different MLVA patterns were deemed rare to the database (0.21%). Analysis of the PFGE and MLVA data as a composite dataset improved the discrimination between isolates compared to either dataset alone, dividing the isolates into 29 different genotypes (Figure 3).

MLVA Type	Allele profile	No. of isolates	Prevalence of MLVA patterns in PN database (%)
A	4-10-2-(-2)-25-15-36	10	0.13%
B	4-10-2-(-2)-25-14-36	3	0.82%
C	4-10-2-(-2)-25-16-36	3	0.015%
D	4-9-2-(-2)-25-14-36	3	New pattern
E	4-15-2-(-2)-25-14-36	3	0.030%
F	4-16-2-(-2)-25-14-36	3	0.21%

Table 2: Six most common MLVA patterns detected among the 47 isolates belonging to the study set.



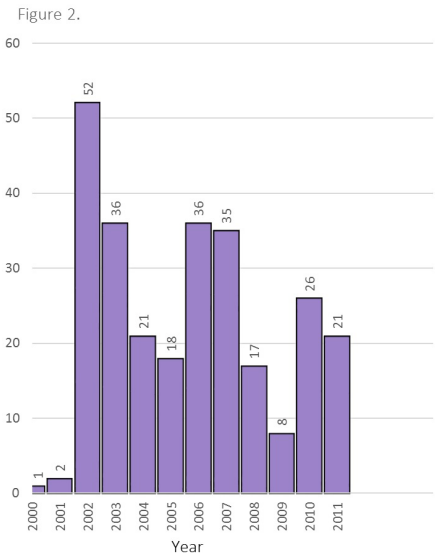


Figure 1: *Salmonella enterica ser.* Typhimurium isolated from each counties of Arkansas during 2000-2011. **Figure 2:** Number of *Salmonella enterica ser.* Typhimurium isolated each year from clinical samples during 2000-2011.

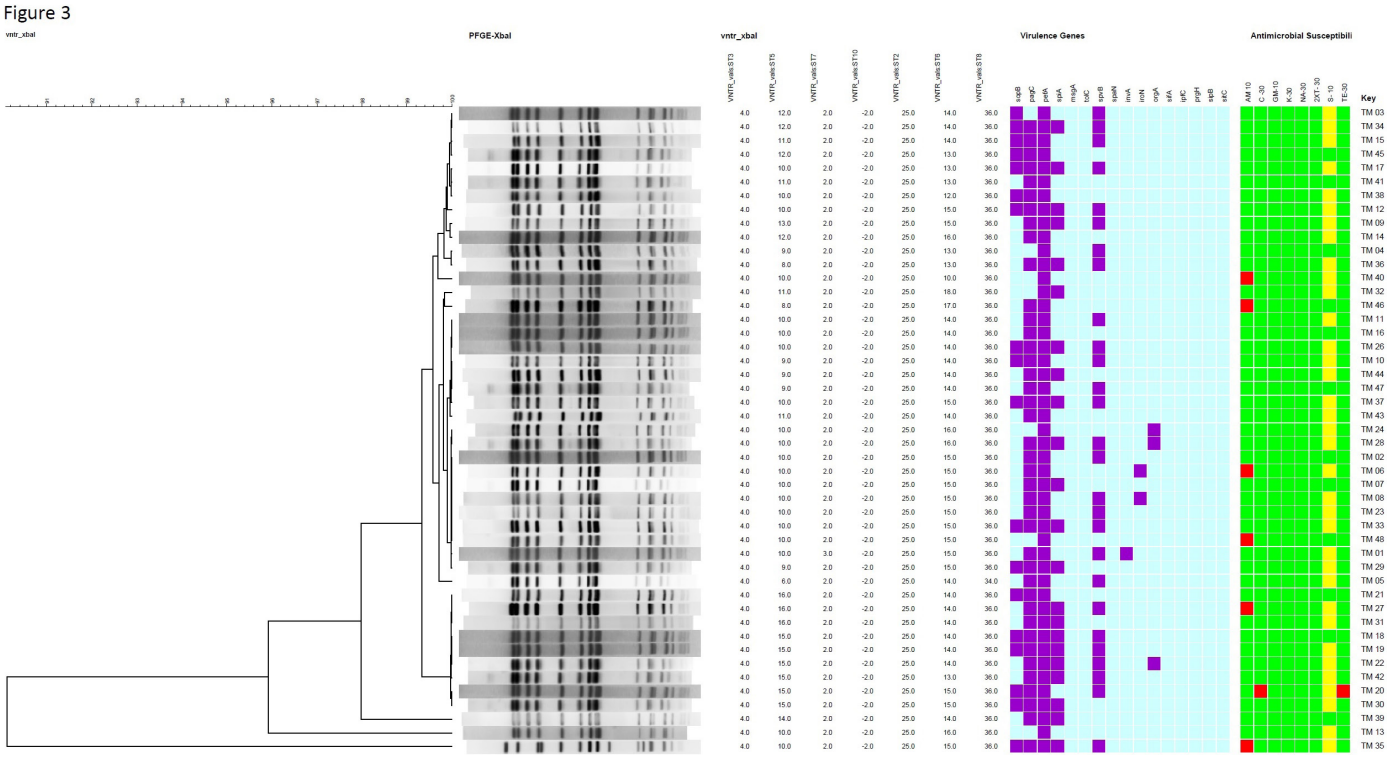


Figure 3: Dendrograms of MLVA and XbaI-PFGE analysis of clinical isolates of *S. enterica ser*. Typhimurium generated by BioNumerics software version-6. For the antibiotic susceptibility test (AST), the red color indicates resistance to the corresponding antimicrobials, the yellow color indicates intermediate susceptibility and green color indicates susceptibility. For the detection of virulence genes, the dark blue indicates the presence of the gene while light blue indicates the absence of the gene.

Figure 4

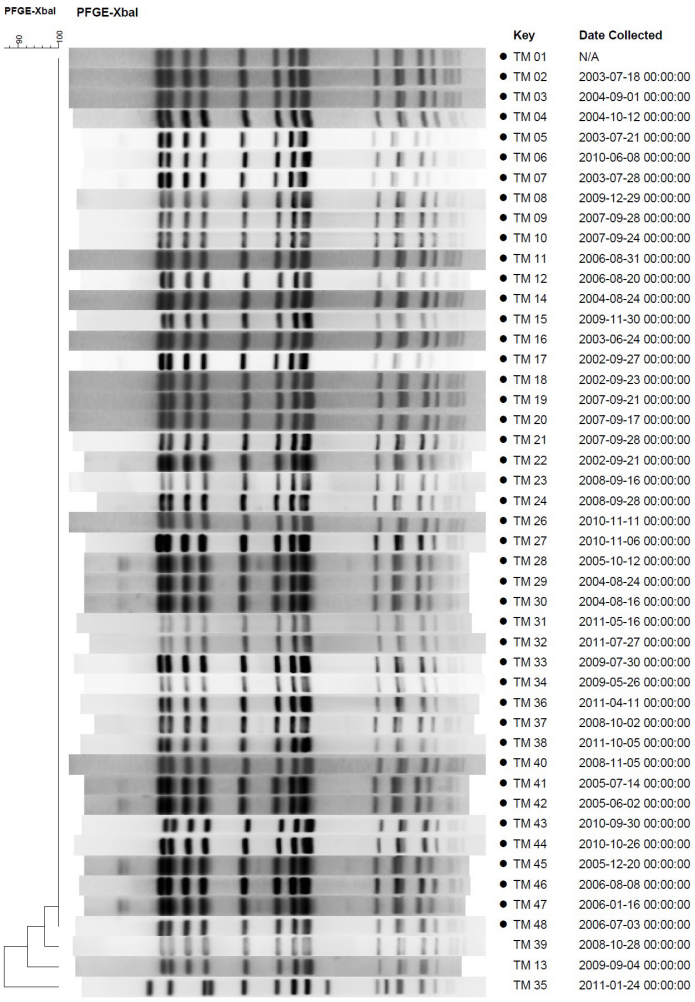


Figure 4: Dendrograms of XbaI-PFGE analysis of clinical isolates of *S. enterica ser.* Typhimurium generated by BioNumerics software version-6. Similarity was determined using the Dice coefficient with 1.3 % optimization and 1.1 % tolerance, and the clustering was performed by UPGMA.

Figure 5

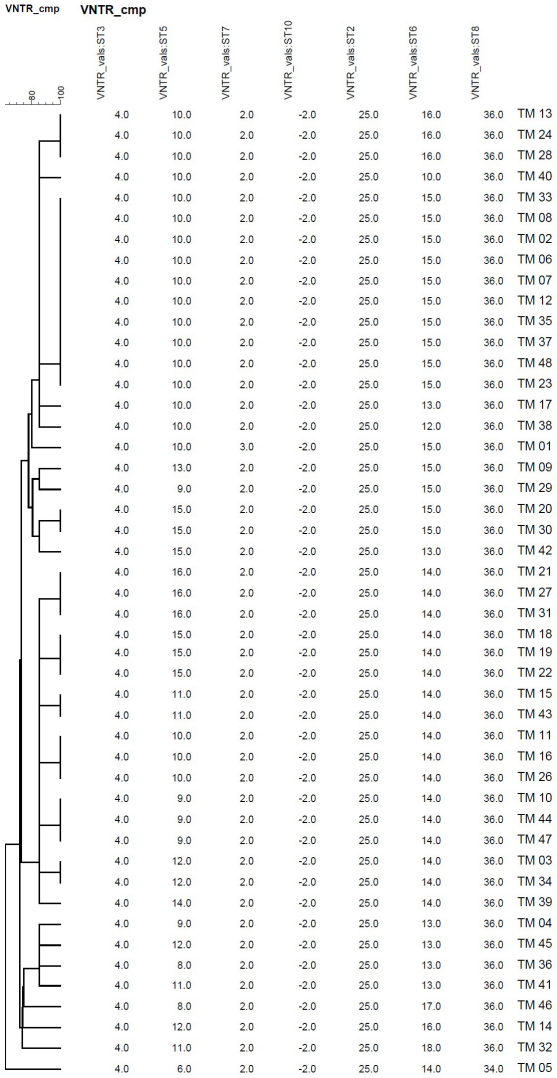


Figure 5: Dendrograms of MLVA analysis of clinical isolates of *S. enterica ser.* Typhimurium generated by BioNumerics software version-6.

Discussion

Salmonella ser. Typhimurium is one of the most common serovars of *Salmonella* that cause foodborne outbreaks in the United States. Accurate surveillance of *S. ser.* Typhimurium is important for public health. It is important to subtype pathogens as soon as possible to detect suspected infection outbreaks and to find the source.

Most of the isolates used in this study were susceptible to antibiotics, except a few which were resistant to ampicillin, chloramphenicol and tetracycline. Lower rates of resistance in this study are in agreement with other studies that have reported a low prevalence of antimicrobial resistance among S. Typhimurium isolates from different sources [29]. Previously Skyberg et al., 2006 [28] used virulence genotyping by amplifying seventeen virulence genes of *Salmonella* spp. PCR detection of virulence genes, which are mostly targeted at the pathogenicity islands of *S. ser.* Typhimurium, showed the presence of several important virulence genes known to contribute to severe salmonellosis. We found nine isolates that encoded eleven virulence genes and three isolates (TM13, TM40 and TM48) that amplified fifteen virulence genes (Figure 3). Plasmid profiling, historically helpful in differentiating and characterizing *Salmonella* serotypes, was not a useful tool in this study due to the minimal number of isolates (n=7) carrying plasmids, which made it impossible to differentiate the isolates based on these data alone.

The PFGE bacterial fingerprinting is a gold standard method used by regulatory agencies and clinical laboratories to assess relatedness among *Salmonella* isolates from different food, clinical or environmental sources [14-16,30]. The PFGE analysis of ser. Typhimurium isolates in this study showed a somewhat low level of diversity, with the strains only yielding four *XbaI* profiles. PFGE data alone was not able to discriminate the majority of *Salmonella* isolates used in this study.

The high discriminatory nature of MLVA and its usefulness as a complementary technique to PFGE was well illustrated in this study, such that 29 different profiles were detected in the dataset when the methods were combined. This is in great contrast to the 4 different *XbaI* PFGE restriction patterns observed (Figure 4). The 44 isolates that comprised the most common PFGE genotype were distributed among 24 different MLVA patterns. However, the 10 isolates that comprised the most common MLVA pattern exhibited two different PFGE types. When assessing the interrelationships of the MLVA patterns it should be noted that the assay is comprised of both conserved and highly variable loci. This provides both the ability to assign isolates to the same cluster or outbreak with high confidence, and resolution to differentiate highly related isolates that may not be associated. Many of the patterns seen in this study, such as MLVA patterns A and B, are considered close variants of each other, differing at a single highly variable locus (ST5 or ST6) by fewer than four repeats. Such differences have been seen in multiple past outbreaks [31-33].

The analyzed results based on both PFGE and MLVA experiments produced 29 different profiles. Some of the more common PFGE-MLVA composite genotypes were further discriminated by the antimicrobial resistance and virulence gene profiles. It can, therefore, be concluded that the best resolution of *S. enterica* serotype Typhimurium isolates are achieved using a combination of different typing methods and whole genomic sequencing. However, subtyping data must be concordant with epidemiological data when used for public health surveillance.

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