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ARTICLE

Genotyping, Phage Typing, and Antimicrobial Resistance of *Salmonella* Typhimurium Isolated from Pigs, Cattle, and Humans

Min-Seok Ju[1], Zheng-Wu Kang, Ji Hun Jung[2], Seongbeom Cho[3], Sung Hun Kim[4], Young-Ju Lee[5], Chong Hae Hong, Son-Il Pak, and Tae-Wook Hahn*

College of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chuncheon 200-701, Korea

[1]National Veterinary Research and Quarantine Service, Incheon Regional Office, Incheon International Airport, Incheon 400-715, Korea

[2]Seoul Metropolitan Government Research Institute of Public Health and Environment, Seoul 137-734, Korea

[3]National Food Safety and Toxicology Center, Michigan State University, East Lansing, MI 48824, USA

[4]Division of Enteric Bacterial Infection, Center for Infectious Disease, National Institute of Health, Seoul 122-701, Korea

[5]College of Veterinary Medicine, Kyuongpook National University, Daegu 702-701, Korea

Abstract

Salmonella enterica serovar Typhimurium (ST) is one of the most common serovars isolated from humans and animals. It has been suggested that ST infections in Koreans are largely due to the consumption of contaminated pork and beef. To investigate the genotypes, phage types, and antimicrobial resistance patterns for ST isolates of different origins, a total of 70 ST strains, including 19 isolates from humans, 44 isolates from pigs, and 6 isolates from cattle, were analyzed using pulsed-field gel electrophoresis (PFGE), phage typing, and antimicrobial susceptibility tests. Forty-three distinct PFGE patterns were generated from 70 ST isolates, which were grouped into 14 PFGE groups (from A to N) at the level of 75% similarity. The most prevalent group was the A (A1-A17 subtypes) group, encompassing 54.5% (38/70) of ST isolates. ST isolates from pigs and cattle mostly belong to groups A and L, whereas ST isolates from humans mostly belong to groups F and C. Antimicrobial susceptibility tests using 11 antimicrobial agents showed that resistance to tetracycline (TE) (81.4%) was highly prevalent, followed by streptomycin (S) (64.3%) and nalidixic acid (NA) (31.4%) resistance. A total of seventeen antimicrobial resistance patterns were observed. Only 8.6% of isolates, including a reference strain, were susceptible to all antimicrobial agents tested. The most prevalent resistance pattern was TE-S (37.1%), which was seen in 66.6% of bovine, 40.8% of swine and 21.1% of human isolates. Three ST isolates from humans (15.9%) showed resistance to 7-8 antimicrobials. The most predominant phage type (PT) was U302 (64.3%), followed by DT170 (10.0%). PFGE types did not coincide with antimicrobial resistance patterns and phage types; therefore, the combination of those types allowed for further differentiation between tested ST isolates.

Key words: *Salmonella* Typhimurium, pulsed-field gel electrophoresis, antimicrobial susceptibility test, phage typing

Introduction

More than 2,500 *Salmonella* serotypes have been reported (Popoff *et al*., 2004). Among them, *Salmonella enterica* serovar Typhimurium (ST) is one of the most frequently isolated from humans and animals, primarily cattle, pigs, goats, sheep and poultry (Bender *et al*., 2001; Doran *et al*., 2005; Gross *et al*., 1998). The most common clinical manifestations of ST in those hosts are enteric disease and septicemia (Best *et al*., 2007). Most *Salmonella* serovars contaminate meats, eggs and their by-products via feces and intestinal contents in farms or slaughter houses. ST is mainly associated with meat contamination with being the most predominant serovar isolated from pork. ST is difficult to control in food animal environments because carrier animals may exhibit asymptomatic fecal shedding. These carrier animals likely play an important role in the spread of infection between herds or flocks, and consequently serve as source of food

*Corresponding author: Tae-Wook Hahn, College of Veterinary Medicine & Institute of Veterinary Science, Kangwon National University, Chuncheon 200-701, Korea. Tel: 82-33-250-8671, Fax: 82-33-250-8671, E-mail: twhahn@kangwon.ac.kr

contamination and transmission to humans (Yang *et al*., 2002). In Korea, ST has been one of the most frequently identified *Salmonella* serovars (Kim *et al*., 2004).

Numerous phenotypic and genotypic methods have been described to subtype ST and to investigate any relatedness among strains in animals and humans. These methods include phage typing (Corbett-Feeney and Riain, 1998), plasmid profiling, ribotyping, and amplified fragment length polymorphism. Phage typing is widely used for subtyping ST but is useful only in a limited number of serotypes. Plasmid profiling is also useful but may be confounded by the instability of certain plasmids; it is also complicated by its requirement of special reagents as well as training and experience in its performance and in the interpretation of results (Doran *et al*., 2005).

In recent years, standardized pulsed-field gel electrophoresis (PFGE) has been applied as an additional typing method in *Salmonella* reference laboratories. PFGE has been known as "gold standard" of genotypic typing of *Salmonella* and other foodborne pathogens for outbreak identification and source identification (Bessa *et al*., 2007; Best *et al*., 2007; Corbett-Feeney and Riain, 1998; Doran *et al*., 2005). In the absence of timely phage typing, PFGE is a simple way to detect and monitor multi-drug-resistant strains (Bender *et al*., 2001).

Antimicrobial resistance in ST isolates has been increasing dramatically in recent years. Multi-drug resistant ST isolates have been isolated from animals and humans suffering from diarrhea. The detection and monitoring of multi-drug resistant ST isolates is important for the selection of antibiotics to treat clinical salmonellosis and to assess the risk of the dissemination of the multi-drug resistant strains (Yang *et al*., 2002). Antimicrobial susceptibility tests and genotyping are important epidemiological tools to determine potential sources of infection. Moreover, data on the distribution of ST in different animals is useful for understanding the epidemiology of antimicrobial resistance and the spread of particular clonal genotypes.

Analysis of ST isolates from different origins, including humans and animals, indicates the spread of certain genetically identical or similar clones of ST in humans and animals. It is interesting to ask whether the clonal identity or similarity of ST isolates between humans and animals may be due to the limited diversity of ST isolates or the spreading of certain types of isolates between humans and animals. Comparison of subtypes for unrelated ST strains isolated from different origins, such as humans, pigs, cattle and other animals may help to answer this question. Such comparisons may also identify the most prevalent subtypes in ST infection in both humans and animals in Korea.

Previously, we have used the PFGE method with *Xba*I to analyze 155 *S.* Enteritidis isolates from humans and chickens and found that A5 was the major subtype (Kang *et al*., 2009). In this study, 70 ST isolates from different origins were analyzed by PFGE, antimicrobial susceptibility tests and phage typing.

Materials and Methods

Bacterial strains

A total of 70 ST isolates (44 from swine, 6 from bovines, 19 from humans and one type strain, ATCC14028) were analyzed. All swine and bovine ST isolates were from Gangwon and Gyeonggi provinces from 2000 to 2007. All human ST isolates were from Seoul from 2003 to 2005. ST isolates were taken from the feces of pigs and cattle regardless of clinical symptoms, while ST isolates from humans were isolated from diarrheal patients. All isolates were identified by the API kit (bioMérieux, Montalieu Vercieu, France) and *Salmonella* specific PCR and were serotyped with the Kauffman method (Popoff *et al*., 2004).

Antimicrobial susceptibility test

All ST isolates were tested for antimircobial susceptibility on Muller-Hinton agar plates by the disk diffusion method (NCCLS, 2000). The media and the disks were purchased from BBL (Becton Dickinson Microbiology systems, Cockeyville, USA). A total of 11 antimicrobial agents were used: tetracycline (TE) 30 μg, streptomycin (S) 10 μg, kanamycin (K) 30 μg, gentamicin (GM) 12 μg, neomycin (N) 30 μg, ampicillin (AM) 10 μg, ticarcillin (TIC) 75 μg, sulfamethoxazole/trimethoprim (SXT) 23.75 μg, cephalothin (CF) 30 μg, nalidixic acid (NA) 30 μg, ciprofloxacin (CIP) 5 μg. The inhibition zones were interpreted as recommended by the supplier except that the intermediate and sensitive isolates were grouped together.

Preparation of DNA for PFGE

PFGE was performed according to the protocol used by the 'Pulse Net' system of the Center for Disease Control and Prevention. *Salmonella* embedded in agarose plugs was prepared by the previously described method (Seo *et al*., 2006). ST was cultured on LB agar plates and incubated overnight at 37ºC. Colonies on agar plates were harvested and suspended in TE cell suspension buffer (100 mM Tris and 100 mM EDTA, pH 7.5). The turbidity of the bacterial cell suspension was adjusted to 20%

transmittance using a colorimeter (bioMérieux). Proteinase K and 1.2% agarose (SeaKem Gold agarose, FMC Bioproducts, Rockland, ME, USA) were mixed with the cell suspension. This mixture was then dispensed into disposable plug molds (Bio-Rad, Richmond, CA, USA). ES buffer (0.5 M EDTA, pH 9.0; 1% sodium-lauroyl-sarcosine) and proteinase K were added to the plugs and they were incubated in a 55ºC water bath for 1 h. After incubation, the plugs were washed once for 15 min in sterile water and four times for 30 min in TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5) preheated to 50ºC. The washed plugs were stored in TE buffer at 4ºC until restriction enzyme digestion. The stored plugs were cut into two 1 mm-wide slices with a razor blade and then incubated at 37ºC for 3 h with the restriction enzyme *Xba*I (30U, Promega, Madison, WI, USA).

Pulsed-field gel electrophoresis

Electrophoresis of bacterial DNAs was performed in a Contour Clamped Homogenous Field (CHEF) DR II electrophoresis system (Bio-Rad). The DNA in the plugs was separated on 1% agarose gel in 0.5× TBE buffer. The electrophoresis conditions were as follows: initial switch time, 2.0; final switch time, 40.0s; run time, 16 h; gradient, 6.0 V/m; buffer temperature, 14ºC. *Salmonella* Serovar Braenderup H9812 (ATCC, USA) was included as a molecular weight standard. After electrophoresis, the gel was stained in 500 ml of sterile water containing 50 mg/ml ethidium bromide for 30 min and de-stained in water for 20 min. The stained gel was viewed on a UV transilluminator and photographed.

Data analysis

PFGE analysis was conducted according to the methods of Tenover *et al*. (1995) and Wonderling *et al*. (2003), with some modifications. Macro-restriction patterns were analyzed with BioNumerics software (Applied-Maths, Sin-Martens-Latem, Belgium) and banding patterns were compared using Dice coefficients with a 1% band position tolerance.

Bacteriophage typing

Phage typing was performed and results were determined in accordance with the method of the Public Health Laboratory Service (PHLS), London, United Kingdom (Anderson *et al*., 1977). The typing phages (n=29), standard type strain (n=10) and scheme were also provided by PHLS. Non-typable isolates were described as RDNC (reaction dose not confirmed).

Results

The most frequently observed antimicrobial resistance was to tetracycline (81.4%), followed by streptomycin

Table 1. Distribution of antimicrobial resistance by origin

Antimicrobial agents	No. (%) of resistant strains			
	Pigs (*n*=44)	Cattle (*n*=6)	Humans (*n*=19)	Total (*n*=70)
Tetracycline (TE)	42 (95.5)	6 (100)	9 (47.4)	57 (81.4)
Aminoglycosides				
Streptomycin (S)	31 (70.5)	6 (100)	8 (42.1)	45 (64.3)
Kanamycin (K)	1 (2.3)	-	4 (21.1)	5 (7.1)
Gentamicin (GM)	1 (2.3)	-	2 (10.5)	3 (4.3)
Neomycin (N)	1 (2.3)	-	4 (21.1)	5 (7.1)
Penicillins				
Ampicillin (AM)	4 (9.1)	-	-	4 (5.7)
Ticarcillin (TIC)	4 (9.1)	-	5 (26.3)	9 (12.9)
Sulfonamide				
Trimethoprim/sulfamethoxazole (SXT)	7 (15.9)	1 (16.7)	3 (15.8)	11 (15.7)
Cephalosporin				
Cephalothin (CF)	-	-	1 (5.3)	1 (1.4)
Quinolones and fluoroquinolones				
Nalidixic acid (NA)	12 (27.3)	1 (16.7)	9 (43.4)	22 (31.4)
Ciprofloxacin (CIP)	-	-	-	-

Table 2. Distribution of antimicrobial resistance patterns[1)] in *Salmonella* Typhimurium isolated from pigs, cattle and humans

Resistance patterns	No. (%) of isolates			
	Pigs	Cattle	Humans	Total
TE	8 (18.1)	-	-	8 (11.4)
NA	-	-	7 (36.7)	7 (10.0)
TE-S	18 (40.8)	4 (66.6)	4 (21.1)	26 (37.1)
TE-NA	3 (6.9)	-	-	3 (4.3)
TE-S-NA	5 (11.2)	1 (16.7)	-	6 (8.3)
TE-S-TIC	-	-	1 (5.3)	1 (1.4)
TE-S-SXT	1 (2.3)	1 (16.7)	-	2 (3.0)
TE-S-AM-TIC	1 (2.3)	-	-	1 (1.4)
TE-S-GM-SXT	1 (2.3)	-	-	1 (1.4)
TE-S-SXT-NA	1 (2.3)	-	-	1 (1.4)
TE-S-K-N-TIC	-	-	1 (5.3)	1 (1.4)
TE-S-AM-TIC-SXT	1 (2.3)	-	-	1 (1.4)
TE-S-K-N-SXT-NA	1 (2.3)	-	-	1 (1.4)
TE-S-AM-TIC-SXT-NA	2 (4.6)	-	-	2 (3.0)
TE-S-K-N-TIC-SXT-NA	-	-	1 (5.3)	1 (1.4)
TE-K-GM-N-TIC-SXT-NA	-	-	1 (5.3)	1 (1.4)
TE-S-K-GM-N-TIC-SXT-CF	-	-	1 (5.3)	1 (1.4)
pan susceptible	2 (4.6)	-	3 (15.7)	6[2)] (8.6)
Total	44 (100)	6 (100)	19 (100)	70 (100)

[1)]TE, tetracycline; S, streptomycin; K, kanamycin; GM, gentamicin; N, neomycin; AM, ampicillin; TIC, ticarcillin; SXT, trimethoprim/sulfamethoxazole; CF, cephalothin; NA, nalidixic acid; CIP, ciprofloxacin

[2)]One ATCC 14028 showed pan-susceptibility.

(64.3%), nalidixic acid (31.4%), trimethoprim/sulfamethoxazole (15.7%), ticarcillin (12.9%), kanamycin (7.1%), neomycin (7.1%), ampicillin (5.7%), gentamicin (4.3%), and cephalothin (1.4%) (Table 1). Cephalothin resistance was only observed in one human isolate (1.4%), whereas ampicillin resistance was only seen among swine isolates (5.7%). Resistance to tetracycline, streptomycin, trimethoprim/sulfamethoxazole, and nalidixic acid was observed among ST isolates of all origins. However, all ST isolates were susceptible to ciprofloxacin. When analyzed by origin, bovine isolates showed the highest rate of resistance to tetracycline and streptomycin (100%), followed closely by swine isolates (70.5-95.5%). In human isolates, resistance to tetracycline, streptomycin and nalidixic acid were similar (42.1-47.4%). Resistance to trimethoprim/sulfamethoxazole was approximately similar in all isolates (15.8-16.7%).

Seventeen distinct antimicrobial resistance patterns were observed among all 70 ST isolates (Table 2). Sixty-four (91.4%) ST isolates were resistant to at least one antimicrobial agent and forty-nine (70%) were resistant to two or more antimicrobials. Six isolates (8.6%), including the reference strain, were shown to be susceptible to all tested antimicrobial agents. The most prevalent pattern was TE-S (37.1%), which was observed in 66.6% of bovine, 40.8% of swine and 21.1% of human isolates (Table 2). Three ST isolates from humans (15.9%) showed resistance to 7-8 antimicrobials, while swine isolates showed resistance to a maximum of 6 antimicrobials.

Chromosomal DNA from the 70 ST isolates was digested with *Xba*I and analyzed with PFGE and BioNumerics, and a total of 43 distinct patterns were generated

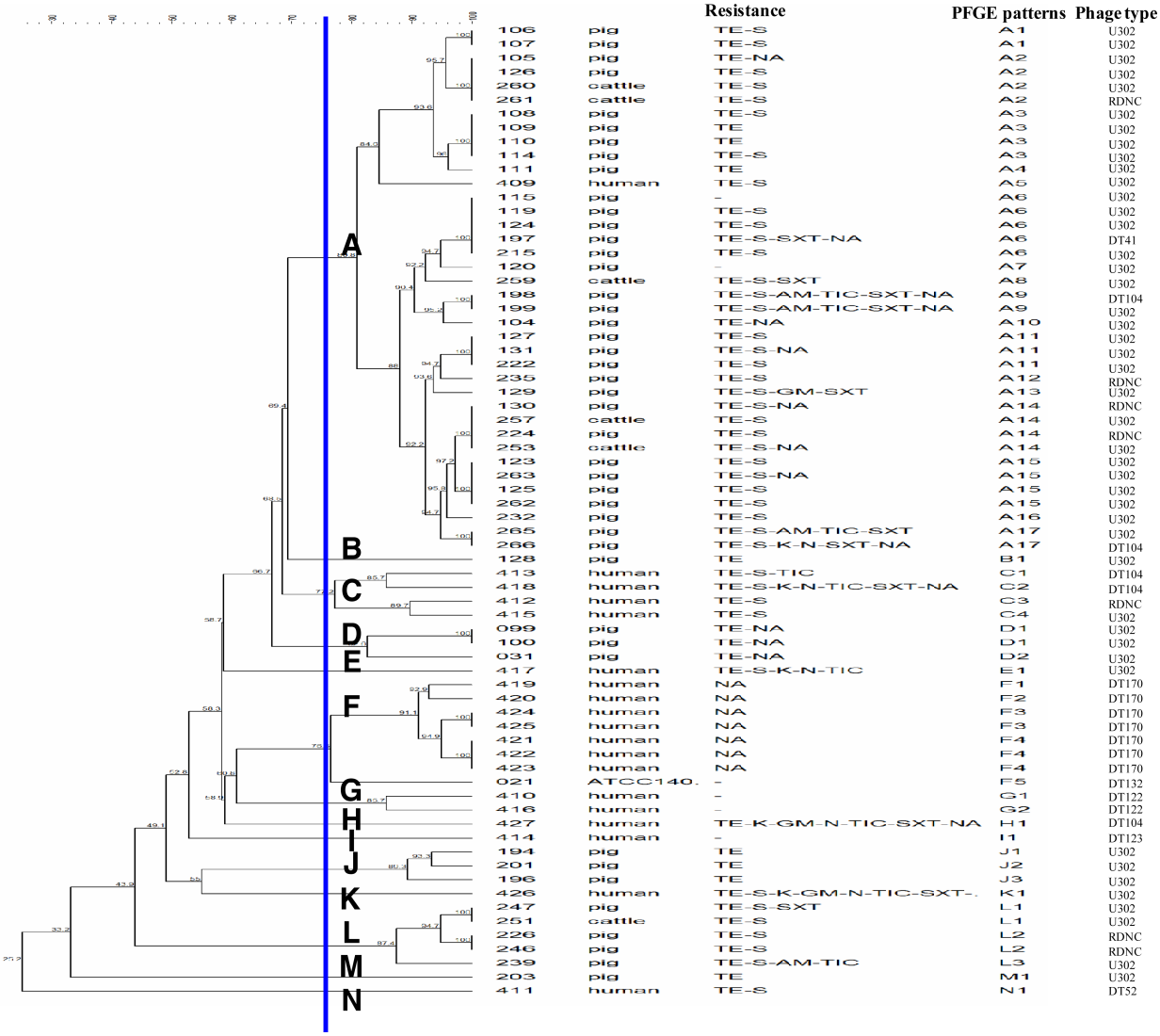

Fig. 1. Dendrogram representing the 70 isolates typed by PFGE using *Xba*I. The strain identification, source, PFGE subtype, antibiotic resistance pattern, and phage type are also shown for each isolate.

(Fig. 1). The genetic relatedness of these isolates ranged from 25.2 to 100%. However, some patterns were very similar, differing by three or fewer bands, indicating that such isolates were likely to be highly related according to Tenover's criteria (Tenover *et al*., 1995).

With a 75% similarity index, 14 large clusters were referred to 14 PFGE groups (A to N), each containing its own subtypes (Fig. 1). The A group was the most prevalent PFGE group observed, encompassing 17 subtypes (A1-A17) and 54.3% (38/70) of the isolates. It was followed by F (F1-F5, 11.4%), L (L1-L3, 7.1%), C (C1-C4, 5.7%), D (D1-D2, 4.3%), and J (J1-J2, 4.3%). Six groups (B, E, H, K, M, and N) contained only a single subtype and one isolate (1.4%).

Swine isolates belonged mostly to the A group, followed by D, J, L, B, and M. Bovine isolates belonged to A and L. Human isolates belonged mostly to the F group, followed by C, E, G, I, K, and N. The reference strain ATCC 14028 belonged to the F group.

The phage type (PT) distribution of the 70 ST isolates is shown in Table 3. Six phage types from animals and 8 phage types from humans were identified (Table 3). In phage types from animals (n=50), U302 was the most common, found in 36 (81.8%) isolates from pigs and 5 (83.3%) isolates from cattle. The most common PT in humans was DT170, found in 7 (35.0%) ST isolates. PT DT104 was found in 2 ST isolates from pigs and 3 isolates from humans.

The combinations of PFGE patterns, PT, and antimicrobial resistance patterns were also analyzed (data not shown). However, PFGE types did not coincide with PT or antimicrobial resistance patterns. Therefore, the combination of antimicrobial susceptibility patterns, PT, and PFGE types allowed further differentiation of tested ST isolates.

Table 3. Distribution of phage types of *S*. Typhimurium isolated from animals and humans

Phage type	No. (%) of isolates			Total
	Pig	Cattle	Human	
U302	36 (81.8)	5 (83.3)	4 (20.0)	45 (64.3)
DT41	1 (2.3)	0	0	1 (1.4)
DT52	0	0	1 (5.0)	1 (1.4)
DT104	2 (4.5)	0	3 (15.0)	5 (7.1)
DT122	0	0	2 (10.0)	2 (2.9)
DT123	0	0	1 (5.0)	1 (1.4)
DT132	0	0	1[2)] (5.0)	1 (1.4)
DT170	0	0	7 (35.0)	7 (10.0)
RDNC[1)]	5 (11.4)	1 (16.7)	1 (5.0)	7 (10.0)
Total	44 (100)	6 (100)	20 (100)	70 (100)

[1)]RDNC: Reacts with phages but does not confirm to a recognized pattern

[2)]ATCC 14028 (isolated from human)

Discussion

A total of 70 ST isolates (44 from pigs, 6 from cattle, 19 from humans and 1 reference strain) in Korea were analyzed using PFGE and antimicrobial resistance patterns to investigate the distribution of genotypes and antimicrobial resistance.

Many molecular biological tools can be used for genotyping. However, many epidemiological typing studies have used PFGE to identify *Salmonella* clones because of its high discriminatory power and reliability. Moreover, PFGE using the restriction endonuclease *Xba*I has been widely recognized as a sensitive method for molecular fingerprinting in several *Salmonella* serotypes (Liebana *et al*., 2001).

At a level of 75% similarity, 14 PFGE groups (A-N) were identified. Most isolates belonged to group A, especially the isolates from pigs (32/44) and cattle (5/6). However, only one human isolate belonged to group A and most human isolates belonged to groups F, C, and G. This result indicated that ST isolates from pigs and cattle were not quite similar to the isolates from humans. The differences in the genotypes of the ST isolates tested may be due to their different isolation times, areas and origins. They may also be due to the acquisition of mobile genes, either temperate phages or plasmids, and new sets of genes acquired by transduction, transposition, or transformation (Pang *et al*., 2007). However, previous reports have suggested that ST infections in humans are largely due to the consumption of contaminated pork and beef (Gudmundsdottir *et al*., 2003). If this were true, the genotypes of ST isolates between animals and humans would be expected to be quite similar. Baggesen *et al*. (2000) reported a high degree of homogeneity among ST isolates from Europe and the United States. It has also been reported that the genotypes of ST isolates from humans and animals were closely related in some cases (Tsen *et al*., 2002). Pang *et al*. (2007) reported that PFGE subtypes of *S.* Enteritidis showed about 75% homogeneity regardless of the geographic area and host source of the isolates. It was reported that PFGE patterns generated from the *Xba*I enzyme were very similar among *S.* Enteritidis strains (Kang *et al*., 2009; Laconcha *et al*., 2000; Woo, 2005). In this study, PFGE patterns with a single band difference were considered distinct, and 43 PFGE

patterns were grouped into 14 main clusters at 75% similarity by BioNumerics software. Therefore, there is little genetic similarity between ST isolates collected from pigs, cattle, and humans. Further investigation using more ST isolates from diverse sources isolated within a limited time period may be needed to conclusively determine the relationship between animal and human origins.

ST isolates from cattle were susceptible to kanamycin, gentamicin, neomycin, ampicillin, ticarcillin, cephalothin and ciprofloxacin. Most ST isolates from pigs were not resistant to cephalothin or ciprofloxacin, but the ST isolates from humans were all susceptible to ampicillin and ciprofloxacin. Antunes *et al*. (2003) reported that resistance to streptomycin and tetracycline was often found in *Salmonellae* from poultry as well as pigs and cattle. A high frequency of resistance to tetracycline and streptomycin has been reported in many bacterial species, including ST. Tetracycline has been most commonly used in livestock, and these results might indicate that this drug is being used without proper controls in the environment or in meat producing animals (Bessa *et al*., 2007). It is interesting to note that all of the isolates in this study that were resistant to streptomycin were also resistant to tetracycline. This result is consistent with a previous study (Antunes *et al*., 2003). A low frequency (2.7%) of ST isolates resistant to ciprofloxacin was reported in Taiwan from 1996 to 2001 (Hsueh *et al*., 2004), whereas our study and other previous studies identified no ST isolates that were resistant to ciprofloxacin (Gorman and Adley, 2004; Gross *et al*., 1998). *Salmonella* isolates from cattle and turkey are more often multi-drug resistant (MDR) than isolates from other species (Wonderling *et al*., 2003). The rate of MDR against >2 antimicrobial agents was 100% in cattle, 77.3% in pigs and 47.6% in humans. However, the MDR patterns of ST isolates from humans were more diverse than those in other species (Table 2).

Phage typing is a useful technique for phenotyping and epidemiologically tracing the infection source of *S.* Typhimurium. This study showed that U302 is the most common type in pigs and cattle while DT170 is the most common type in humans. Interestingly, DT104 was found in two isolates from pigs and three isolates from humans.

It is not easy to differentiate between strains using a single method, but a combination of methods can be highly reliable and effective. According to other studies, correlation among plasmid profile, PFGE type, and ribotype was not clear, but PFGE types were highly related to antimicrobial resistance patterns (Liebana *et al*., 2001; Navarro *et al*., 1996). However, this study produced very different results from our study because we found no apparent correlation between PFGE types and antimicrobial resistance patterns. Some strains with the same genotype showed different antimicrobial resistance patterns and the same antimicrobial resistance pattern was observed for strains with different genotypes. However, genetic information used in conjunction with antimicrobial resistance patterns may help to detect the emergence of potential new strains and the spread of antimicrobial resistance among existing strains.

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