The Journal of Microbiology, March 2004, p.14-19
Vol. 42, No. 1

Genomic Relationship of *Salmonella enterica* Serovar Typhimurium DT104 Isolates from Korea and the United States

Shukho Kim[1], Sung Guen Chun[1], Ok Young Lim[1], Mi Sun Park[1], Yeon Ho Kang[1], Yong Ho Park[2] and Bok Kwon Lee[1,*]

[1]Laboratory of Enteric Infections, Department of Microbiology, National Institute of Health, Nokbun-dong 5, Seoul 122-701, [2]Department of Veterinary Microbiology and Infectious Diseases, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul, Korea

(Received December 8, 2003 / Accepted February 12, 2004)

***Salmonella enterica* serovar Typhimurium DT104 (*Salmonella* Typhimurium DT104 or DT104) has been emerging as a common pathogen for human in Korea since 1997. In order to compare the genomic relationship and to search for the dominant strains in Korea, we conducted pulsed-field gel electrophoresis (PFGE) and IS*200* fingerprinting of 25 epidemiological unrelated isolates from human and animals from Korea and cattle from America. Two *Salmonella* Typhimurium DT104 isolates from human in Korea and all 8 isolates from American cattle had indistinguishable patterns from the PFGE and IS*200* fingerprinting but multidrug-resistant *Salmonella* Typhimurium isolates, including DT104, from Korean animals had diverse genetic patterns. The data suggest that a dominant DT104 strain might have circulated between Korean and American cattle and that it had a high level of clonality.**

Key words: DT104, IS*200*, fingerprinting, PFGE, resistance, clonality

Salmonella enterica serovar Typhimurium definitive type 104 (*S.* Typhimurium DT104) is a major virulent pathogen that has emerged as a world health problem (Threlfall *et al.*, 1996; Besser *et al.*, 1997; Brisabois *et al.*, 1997; Glynn *et al.*, 1998; Metzer *et al.*, 1998; Poppe *et al.*, 1998; Daly and Fanning, 2000). In Europe and in America, the multidrug-resistant *S.* Typhimurium DT104 has been isolated at an increased frequency from humans and animals (Poppe *et al.*, 1998; Paszti *et al.*, 2001). In Japan, multidrug-resistant *S.* Typhimurium that was similar to predominant isolates of western countries was reported recently (Izumiya *et al.*, 2001).

Salmonella enterica serovar Typhimurium has been one of the most frequent isolates of *Salmonella* serovars in Korea (Park *et al.*, 2002). The frequency of phage type DT104 in all isolated *S.* Typhimurium was 26.6%, 10.8%, and 2.5% in 1997, 1998, and 1999, respectively (our unpublished data). Furthermore, multidrug-resistant *S.* Typhimurium has been isolated from diarrheal patients with a gradual increase in recent years (Davis *et al.*, 2002). *S.* Typhimurium DT104 often has resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline; such a resistance to many antibiotics is referred to as a pentadrug-resistant pattern (ACSSuT) (Bolton *et al.*, 1999, Boyd *et al.*, 2000, Kahn *et al.*, 2000). The five genes that are responsible for triggering the ACSSuT resistance are located between two integrons and are clustered in a chromosomal region of about 13 kb in size. Briggs and Fratamico (1999) have sequenced the gene cluster region conferring ACSSuT antibiotics resistance in an American strain of DT104. Boyd *et al.* reported the complete nucleotide sequence of a genomic island that was associated with the multidrug resistance region and has also reported that the resistance region is harbored on a 43 kb-long genetic element, which has been inserted into the chromosomal *thdF* gene (Boyd *et al.*, 2001, Boyd *et al.*, 2002).

Food-producing animals are reservoirs for nontyphoidal *Salmonella*. In the USA, most human *Salmonella* infections result from eating foods of animal origin (Angulo *et al.*, 2000). Ribot *et al.* reported that ACSSuT-resistant *S.* Typhimurium DT104 isolates that were obtained in the years 1985, 1990, and 1995 shared an indistinguishable PFGE pattern. It was suggested that the emergence of *S.* Typhimurium DT104 during the mid-1990s in the US probably was a result of the dissemination of multidrug-resistant DT104 in food-producing animal reservoirs (Ribot *et al.*, 2002). In Singapore, dried anchovy was found to be the vehicle of transmission for multidrug-resistant *S.* Typhimurium DT104 (Ling *et al.*, 2002).

* To whom correspondence should be addressed.
(Tel) 82-2-380-1462; (Fax) 82-2-352-4767
(E-mail) bokrates@nih.go.kr ("OR" shokim@kebi.com)

In our previous studies, we characterized phenotypes and genotypes of multidrug-resistant *S.* Typhimurium that were isolated from Korean and American animals (Yang *et al.*, 2002), and the molecular epidemiological characteristics of DT104 isolates from the patients in Korea (Park *et al.*, 2002). A pulsed-field gel electrophoresis (PFGE) was performed to characterize clinical and food isolates of diseases caused by foodborne bacteria and to chase the origin of infection in outbreak investigations (Gautom, 1997; Swaminathan *et al.*, 2001). Through the analysis of the insertion sequence IS*200* distribution among *Salmonella* serovars, clonal relationships among the isolates were delineated (Burnens *et al.*, 1996). In this study, representative isolates of *S.* Typhimurium DT104 and other phage types from Korean patients, Korean animals and American cattle were analyzed by IS*200* fingerprinting and PFGE in order to compare the genomic relationship among these isolates.

Materials and Methods

Bacteria

A total of 25 isolates of *Salmonella* serovar Typhimurium were tested in this study. Representative 4 *S.* Typhimurium DT104 strains were isolated from the patients (Park *et al.*, 2002), and 8 *S.* Typhimurium DT104 strains were from Washington State University (Yang *et al.*, 2002). Two *S.* Typhimurium DT104 and 11 multidrug-resistant *S.* Typhimurium strains were isolated from animals in Korea (Yang *et al.*, 2002). All of the isolates were identified and confirmed by using the API 20E (bioMerieux, Marcy IEtoile, France) kit and serums (Difco, USA) in biochemical and serological tests, respectively.

Preparation of nonradioactively labeled IS200 probe

An IS*200* probe was prepared from genomic DNA of *Salmonella typhimurium* ATCC 14028 by PCR with IS*200*-L2 (5'-CCT AAC AGG CGC ATA CGA TC-3') and IS*200*-R2 primers (5'-ACA TCT TGC GGT CTG GCA AC-3') as described elsewhere (Burnens *et al.*, 1996). After the electrophoresis run in TAE buffer, the 556-bp PCR product was excised from the agarose gel and was purified with the QIAquick Gel Extraction Kit (Qiagen, USA). The probe was labeled using Enhanced Chemiluminescence (ECL™) direct nucleic acid labeling and detection systems (Amersham Pharmacia Biotech, USA).

Southern blotting and chemiluminescent detection.

Bacterial genomic DNA was extracted through the use of the G-Spin Genomic DNA Purification Kit (iNtRON Biotechnology, Korea). Two micrograms of extracted DNA was digested to completion with *Bam*HI and *Pst*I (IS*200* sequences do not have the recognition sites of these enzymes) as directed by the manufacturer (New England Biolabs, USA). Restriction fragments were separated through electrophoresis by using 1.0% w/v agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 0.9 V/cm, and these fragments were transferred by rapid downward transfer systems (Schleicher & Schuell, BioScience, Germany) to the Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, USA). DNA was fixed to the membrane by incubating it at 80°C for 2 h. Prehybridization, hybridization, and detection of hybrids that uses ECL detection reagents were carried out as recommended by the manufacturer (Amersham Pharmacia Biotech, USA).

PFGE

The preparation of genomic DNA blocks and the digestion of them with a restriction enzyme were carried out as described in an earlier publication (Gautom, 1997, Kim *et al.*, 2003). All *Salmonella* Typhimurium isolates were analyzed through the use of the restriction enzyme, *Xba*I (New England Biolabs, USA). Typing by PFGE of genomic DNA digested with *Xba*I was carried out in a CHEF Mapper system (Bio-Rad Laboratories, USA). The PFGE pulsing and running conditions were an initial 2.2 sec to a final 63.8 sec for 15 h and 6 Volts/cm at 14°C. Lambda ladder (New England Biolabs, USA) was used as the molecular size marker. After electrophoresis, the gels were stained with ethidium bromide for 20 min and were photographed using Gel Doc 2000 (Bio-Rad Laboratories, USA).

Genomic clustering of isolates

IS*200* profiles and PFGE patterns of *Salmonella* Typhimurium isolates were visually compared and numbered in sequence according to the molecular sizes of the bands. Coefficients of dice similarity were calculated, and cluster analysis was performed with the unweighted pair group mathematical average (UPGMA) algorithm in the Molecular Analyst Fingerprinting software version 1.12 (Bio-Rad, USA) (Sneath and Sokal, 1973).

Results and Discussion

Screening of the presence of insertion element IS*200* in *S.* Typhimurium DT104 and other phage types from animals were screened for the presence of the insertion element IS*200* using the restriction enzymes: *Bam*HI and *Pst*I. IS*200* fingerprinting with *Bam* HI showed five different patterns including the pattern for the strain ATCC 14028(Fig. 1A and C). Two *S.* Typhimurium DT104 human isolates from Korea and eight DT104 cattle isolates from USA had exactly the same IS*200* fingerprinting pattern, B1(Fig. 1E). IS*200* fingerprinting with *Pst*I showed more alternate patterns than fingerprinting with *Bam*HI, but two DT104 human isolates from Korea and eight DT104 cattle isolates from USA had an indistinguishable pattern between each other, namely P1(Fig. 1B, D, and F). Two DT104 swine isolates had different IS*200* fingerprinting patterns, B2 and B4 with *Bam* HI,

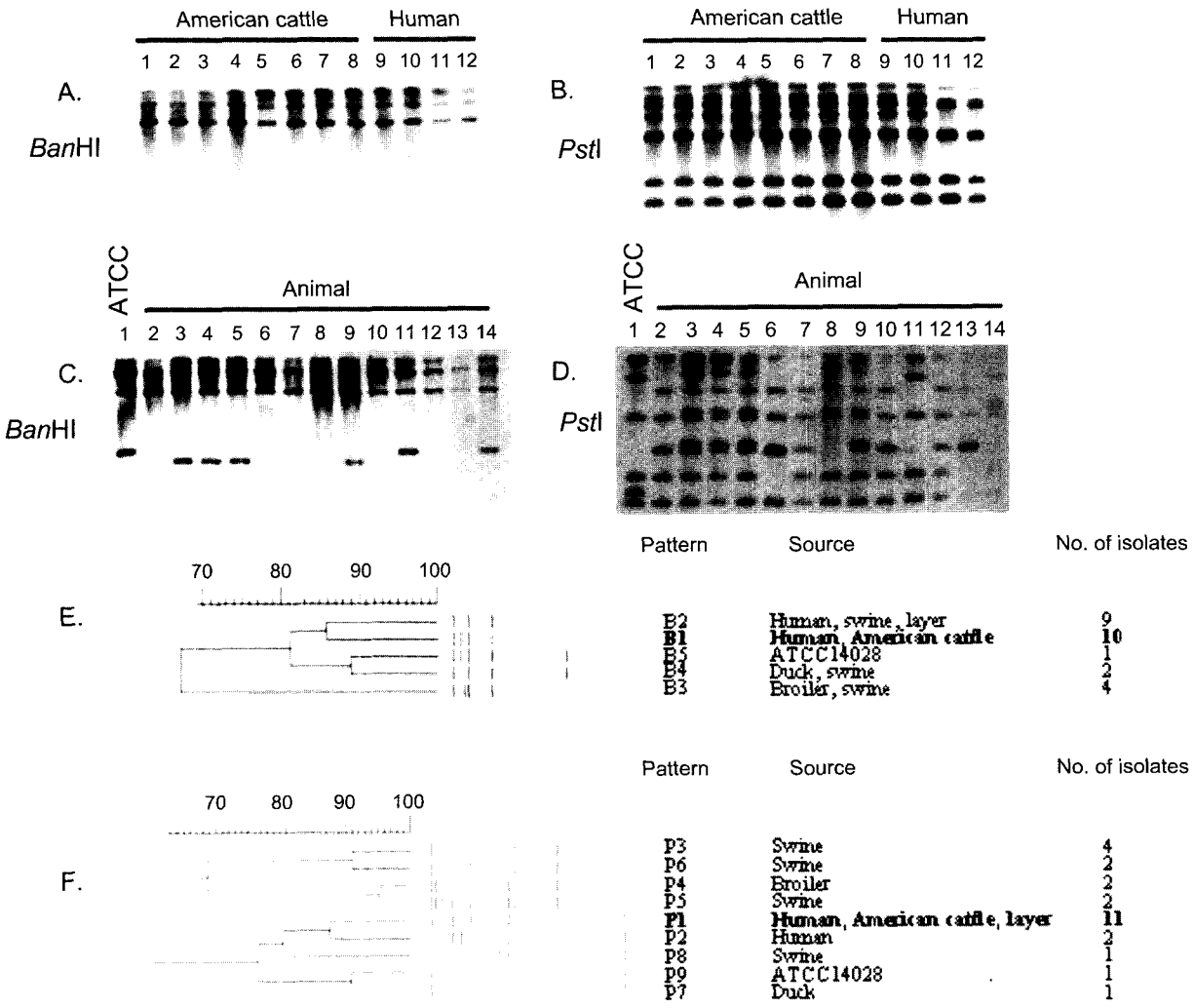

Fig. 1. IS*200* fingerprinting patterns and the dendrograms of multidrug-resistant *Salmonella* Typhimurium isolates from humans of Korean origin, American cattle, and Korean animals digested with the restriction enzymes, *Bam* HI (A, C, E) and *Pst* I (B, D, F). Lane 1-8 in panel A and B show the IS*200* fingerprinting patterns of *S.* Typhimurium DT104 isolates from American cattle, DT2380, DT2486, DT2490, DT2498, DT2501, DT2502, DT2505 and DT2581, respectively. Lane 9-12 in panel A and B show the patterns of DT104 isolates from humans of Korean origin, DT14, DT24, DT35 and DT42, respectively. Lane 2-14 in panel C and D show the patterns of multidrug-resistant *S.* Typhimurium isolates from Korean animals, ST1-ST6, ST10-ST12, and ST19-22, respectively. Lane 1 in panel C and D show the patterns of *S. typhimurium* ATCC 14028.

and P6 and P8 with *Pst*I, respectively, when compared with Korea human and American cattle isolates. Other multidrug-resistant *S.* Typhimurium isolates from Korean animals had a variety of IS*200* fingerprinting patterns that were distinguishable from ten DT104 human and American cattle isolates. *Xba*I PFGE patterns of all these *S.* Typhimurium isolates illustrated the most variable genomic relationship and the highest discriminative power among the molecular epidemiological results in this study (Fig. 2A). However, from the IS*200* fingerprinting results with *Bam*HI and *Pst*I, two DT104 human isolates and eight DT104 American cattle isolates had an indistinguishable pattern, called X1. This X1 PFGE pattern was highly similar to the pattern of human isolates from America (Ribot *et al.*, 2002). Other isolates had variable PFGE band patterns and were discriminated into each pattern in a similarity range greater than 75% with the exception of two broiler isolates (X 4, < 45% similarity) and the strain ATCC 14028 (X 14, < 40% similarity) (Fig. 2B).

A combination of IS*200* fingerprinting and PFGE identifying of genomic relationships between human DT104, animal DT104, other phage types, and American cattle DT104 isolates allowed a clear differentiation of the ACSSuT-resistant DT104 clone from the other *S.* Typhimurium, which originated from various sources. Among the total 14 clones, X1/B1/P1 was the most frequent and was found in all ACSSuT-resistant DT104 isolates from human and American cattle. Two DT104 from swine had two different clones, X12/B2/P6 and X13/B4/P8, compared to the most prominent type, X1/B1/P1. DT24 and DT35 isolates (X2/B2/P2) were also human DT104, but these isolates were resistant to ASSuT drugs but not ACS-

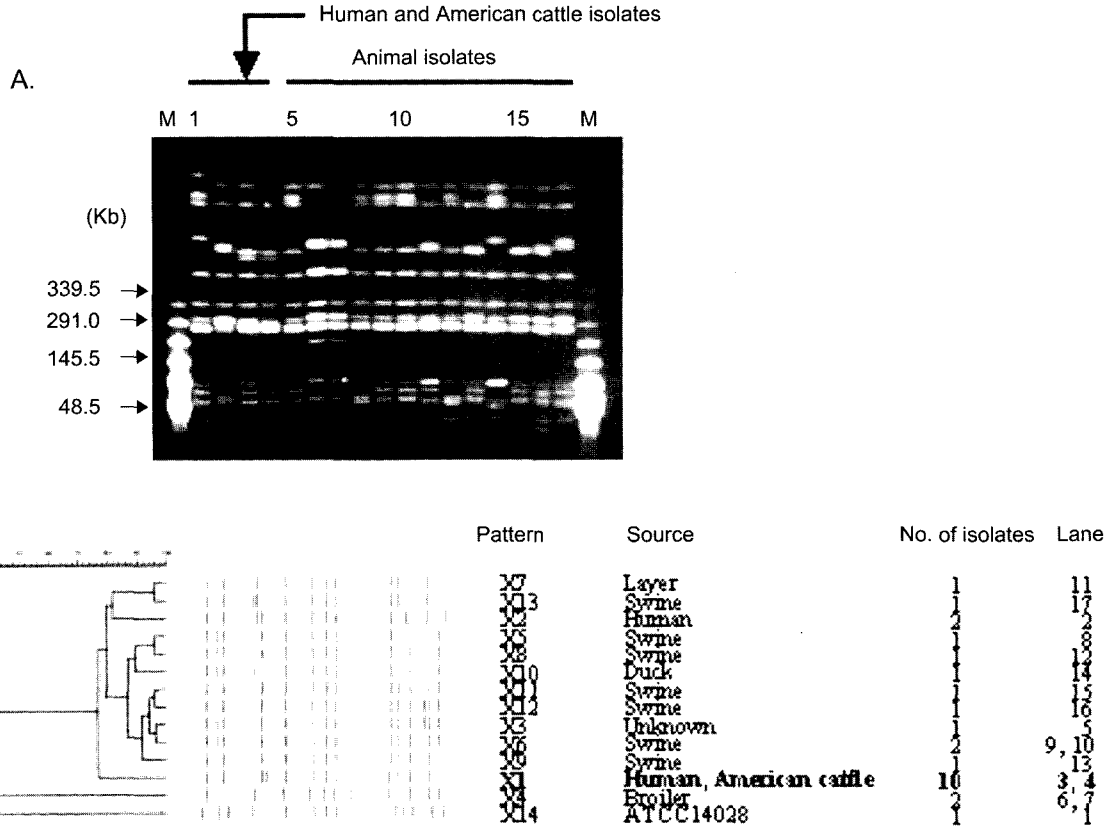

Fig. 2. *Xba*I PFGE patterns of multidrug-resistnat *Salmonella* Typhimurium isolates from humans of Korean origin, American cattle, and Korean animals. M is a Lambda ladder as the molecular size marker.

Table 1. Origins, phage types, PFGE, and IS*200* patterns of *Salmonella* Typhimurium isolates

Strain designation	Date and source	Phage type	*Xba* I PFGE pattern	*Bam* HI IS*200* pattern	*Pst* I IS*200* pattern	Reference
DT14	1997, human	DT104	X 1	B 1	P 1	19
DT24	1997, human	DT104	X 2	B 2	P 2	19
DT35	1997, human	DT104	X 2	B 2	P 2	19
DT42	1997, human	DT104	X 1	B 1	P 1	19
DT2380	WSU, bovine	DT104	X 1	B 1	P 1	25
DT2486	WSU, bovine	DT104	X 1	B 1	P 1	25
DT2490	WSU, bovine	DT104	X 1	B 1	P 1	25
DT2498	WSU, bovine	DT104	X 1	B 1	P 1	25
DT2501	WSU, bovine	DT104	X 1	B 1	P 1	25
DT2502	WSU, bovine	DT104	X 1	B 1	P 1	25
DT2505	WSU, bovine	DT104	X 1	B 1	P 1	25
DT2581	WSU, bovine	DT104	X 1	B 1	P 1	25
ST1	1983, unknown	RDNC[1]	X 3	B 2	P 3	25
ST2	1985, unknown	RDNC	X 4	B 3	P 4	25
ST3	1989, broiler	RDNC	X 4	B 3	P 4	25
ST4	1992, swine	RDNC	X 5	B 3	P 5	25
ST5	1992, swine	4	X 6	B 2	P 6	25
ST6	1992, swine	17	X 6	B 2	P 3	25
ST10	1993, layer	RDNC	X 7	B 2	P 1	25
ST11	1993, swine	RDNC	X 8	B 3	P 5	25
ST12	1994, swine	RDNC	X 9	B 2	P 3	25
ST19	1997, duck	RDNC	X 10	B 4	P 7	25
ST20	1997, swine	RDNC	X 11	B 2	P 3	25
ST21	1997, swine	DT104	X 12	B 2	P 6	25
ST22	1997, swine	DT104	X 13	B 4	P 8	25

[1]RDNC: Reaction Does Not Conform to any recognized phage types.

SuT (Park *et al.*, 2002). Table 1 summarizes the molecular epidemiological results for the combined types.

The purpose of this study was to investigate the genomic relationships among human and animal *S.* Typhimurium DT104 isolates from Korea and America. IS*200* fingerprinting and PFGE are already established as molecular epidemiological tools which have been performed to provide the genomic relationship and proper discriminatory power for *Salmonella* and other gram-negative bacteria (Burnens *et al.*, 1996, Izumiya *et al.*, 2001, Kim *et al.*, 2002, Park *et al.*, 2002, Ribot *et al.*, 2002). In recent studies that pertain to *Salmonella* fingerprinting, there has been a higher preference and confidence for PFGE than any other subtyping methods. For fine structure subtyping, many researchers have used a combination of methods. For example, combinations of PFGE and IS*200* fingerprinting or PFGE and plasmid profiling were used. Recently, a group of Norwegian scientists developed a method of DNA fingerprinting based on a variable number of tandem repeat loci, which showed greatly improved resolution when compared to the *Xba*I PFGE and other subtyping methods (Lindstedt *et al.*, 2003). However, the method has not been used well in other countries and needs more verification by other scientists in order to be recognized for use.

Although we performed PFGE and IS*200* fingerprinting as a combinational subtyping, pentadrug-resistant DT104 isolates from Korean and American cattles were indistinguishable. This result indicated that a dominant and highly clonal DT104 strain might have circulated among Korean and American cattles. We did not elucidate substantial evidence of any link between isolates from humans and animals. More DT104 isolates are required for substantial tracking of epidemiological linking between human and animal isolates.

References

Angulo, F.J., K.R. Johnson, R.V. Tauxe, and M.L. Cohen. 2000. Origins and consequences of antimicrobial-resistant nontyphoidal *salmonella*: implications for the use of fluoroquinolones in food animals. *Microb. Drug. Resist.* 6, 77-83.

Besser, T.E., C.C. Gay, J.M. Gay, D.D. Hancock, D. Rice, L.C. Pritchett, and E.D. Erickson. 1997. Salmonellosis associated with *S typhimurium* DT104 in the USA. *Vet. Rec.* 140, 75.

Bolton, L.F., L.C. Kelley, M.D. Lee, P.J. Fedorka-Cray, and J.J. Maurer. 1999. Detection of multidrug-resistant *Salmonella enterica* serotype *typhimurium* DT104 based on a gene, which confers cross-resistance to florfenicol and chloramphenicol. *J. Clin. Microbiol.* 37, 1348-1351.

Boyd, D., A. Cloeckaert, E. Chaslus-Dancla, and M.R. Mulvey. 2002. Characterization of Variant *Salmonella* Genomic Island 1 Multidrug Resistance Regions from Serovars Typhimurium DT104 and Agona. *Antimicrob. Agents Chemother.* 46, 1714-22.

Boyd, D., G.A. Peters, A. Cloeckaert, K.S. Boumedine, E. Chaslus-Dancla, H. Imberechts, and M.R. Mulvey. 2001. Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *J. Bacteriol.* 183, 5725-32.

Boyd, D.A., G.A. Peters, L. Ng, and M.R. Mulvey. 2000. Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica* Typhymurium DT104. *FEMS Microbiol. Lett.* 189, 285-291.

Briggs, C.E. and P.M. Fratamico. 1999. Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. *Antimicrob. Agents Chemother.* 43, 846-849.

Brisabois, A., I. Cazin, J. Breuil, and E. Collatz. 1997. Surveillance of antibiotic resistance in *Salmonella. Euro. Surveill.* 2, 19-20.

Burnens, A.P., J. Stanley, I. Sechter, and J. Nicolet. 1996. Evolutionary origin of a monophasic *Salmonella* serovar, 9, 12: l, revealed by IS*200* profiles and restriction fragment polymorphisms of the *fljB* gene. *J. Clin. Microbiol.* 34, 1641-1645.

Daly, M. and S. Fanning. 2000. Characterization and chromosomal mapping of antimicrobial resistance genes in *Salmonella enterica* serotype Typhimurium. *Appl. Environ. Microbiol.* 66, 4842-4848.

Davis M.A., D.D. Hancock, and T.E. Besser. 2002. Multiresistnat clones of *Salmonella enterica*: The importance of dissemination. *J. Lab. Clin. Med.* 140, 135-141.

Gautom, R.K. 1997. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157 : H7 and other gram-negative organisms in 1 day. *J. Clin. Microbiol.* 35, 2977-80.

Glynn, M.K., C. Bopp, W. Dewitt, P. Dabney, M. Mokhtar, and F.J. Angulo. 1998. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. *N. Engl. J. Med.* 338, 1333-1338.

Izumiya, H.,J. Terajima, S. Matsushita, K. Tamura and H. Watanabe. 2001. Characterization of multidurg-resistant *Salmonella enterica* serovar Typhimurium isolated in Japan. *J. Clin. Microbiol.* 39, 2700-2703.

Khan, A.A., M.S. Nawaz, S.A. Khan, and C.E. Cerniglia. 2000. Detection of multidrug-resistant *Salmonella typhimurium* DT104 by multiplex polymerase chain reaction. *FEMS Microbiol. Lett.* 182, 355-360.

Kim, S., B.K. Lee, Y.H. Kang, H.J. Nam, O.Y. Lim, W.S. Seok, and J.K. Park. 2003. A virulent strain of *Salmonella enterica* serovar London isolated in infants with enteritis traced by active surveillance and molecular epidemiological study. *J. Kor. Med. Sci.* 18, 325-330.

Lindstedt, B.A., E. Heir, E. Gjernes, and G. Kapperud. 2003. DNA fingerprinting of *Salmonella enterica* subsp. *enterica* serovar *typhimurium* with emphasis on phage type DT104 based on variable number of tandem repeat loci. Lindstedt BA, Heir E, Gjernes E, Kapperud G. *J. Clin. Microbiol.* 41, 1469-79.

Ling, M.L., K.T. Goh, G.C. Wang, K.S. Neo, and T. Chua. 2002. An outbreak of multidrug-resistant *Salmonella enterica* subsp. enterica serotype Typhimurium, DT104L linked to dried anchovy in Singapore. *Epidemiol. Infect.* 128, 1-5.

Metzer, E.,V. Agmon, N. Andoren, and D. Cohen. 1998. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium phage-type DT104 among salmonellae causing enteritidis in Israel. *Epidemiol. Infect.* 121, 555-559.

Park, M.S., Y.H. Kang, S.J. Lee, C.Y. Song, and B.K. Lee. 2002. Characteristics of epidemic multidrug resistant *Salmonella*

enterica serovar Typhimurium DT104 strains first isolated in Korea. *Korean Journal of Infectious Diseases* 34, 1-8. (Korean).

Paszti, J., V.G. Laszlo, I. Gado, H. Milch, K. Krisztalovics, M. Kiraly, J. Orban, and V. Koppany. 2001. The spread and antibiotic resistance of *Salmonella enterica* serotype typhimurium DT104 in Hungary. *Acta. Microbiol. Immunol. Hung.* 48, 95-105.

Poppe, C., N. Smart, R. Khakhria, W. Johnson, J. Spika, and J. Prescott. 1998. *Salmonella typhimurium* DT104: A virulent and drug-resistant pathogen. *Can. Vet. J.* 39, 559-565.

Ribot, E.M., R.K. Wierzba, F.J. Angulo, and T.J. Barrett. 2002. *Salmonella enterica* serotype Typhimurium DT104 isolated from humans, United States, 1985, 1990, and 1995. *Emerg. Infect. Dis.* 8, 387-391.

Sneath, P.H.A. and R.R. Sokal. 1973. Numerical Taxonomy. San Francisco: Freeman.

Swaminathan, B. and T.J. Barrett, S.B. Hunter, R.V. Tauxe and the CDC PulseNet Task Force. 2001. PulseNet: The molecular subtyping network for foodborne bacterial disease surveillance, United States. *Emerg. Infect. Dis.* 7, 382-389.

Threlfall, E.J., J.A. Frost, L.R. Ward, and B. Rowe. 1996. Increasing spectrum of resistance in multiresistant *Salmonella typhimurium*. *Lancet.* 347,1053-1054.

Yang, S.J., K.Y. Park, S.H. Kim, K.M. No, T.E. Besser, H.S. Yoo, S.H. Kim, B.K. Lee, and Y.H. Park. 2002. Antimicrobial resistance in *Salmonella enterica* serovars Enteritidis and Typhimurium isolated from animals in Korea: comparison of phenotypic and genotypic resistance characterization. *Vet. Microbiol.* 86, 295-301.