

부산지역에서 분리된 *Salmonella enterica* serovar Typhi균에 대한 PFGE를 이용한 Molecular typing

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Molecular Typing of *Salmonella enterica* serovar Typhi Strains Isolated in Busan by Pulsed-Field Gel Electrophoresis

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Abstract

We obtained 424 *Salmonella enterica* serovar Typhi isolates from sporadic case of infection in Busan during 1996 to 2005. We investigated the trend of antimicrobial resistance and molecular typing by pulsed-field gel electrophoresis (PFGE). Of the total 424 isolates, 6 strains (1.4%) were multidrug resistant (MDR) *S. enterica* serovar Typhi isolates, 2 strains (0.5%) were resistant to only nalidixic acid, and the remaining 416 strains (98.1%) were fully susceptible to the 18 antimicrobial agent. PFGE of XbaI-digested chromosomal DNA was performed on 50 sporadic *S. enterica* serovar Typhi isolates with the objective of investigating the extent of genetic diversity of these isolates in our region.

We could find that these isolates were much more heterogeneous and at least 32 different PFGE patterns were generated according by dice coefficient, between 0.69 and 1.0. Restriction fragment patterns consisted of 13 to 18 fragments ranged in size from c. 20 to 630 kbp.

The results confirmed that PFGE would be an useful tool for investigating surveillance of sporadic or outbreak case and assessing clonality for *S. enterica* serovar Typhi in Busan area. Our finding will be valuable in developing rational strategies to control this pathogen and setting the basis of an effective PulseNet system in Korea.

Key Words: *Salmonella enterica* serovar Typhi, antimicrobial susceptibility, MDR, PFGE

INTRODUCTION

Typhoid caused by *Salmonella enterica* serovar Typhi belongs to the category of legislated communicable disease group I in Korea as it spreads rapidly and poses a high level of health risk to national health, requiring immediate control measures at the development or onset of an outbreak. It is a significant infectious disease in developing countries with an annual incidence of 16 million case and approximately 600,000 deaths worldwide¹. The incidence of typhoid fever results from mainly consuming foods or water contaminated by feces of patients or carriers

Thus, government adopted various measures to prevent public health from this disease including

purification of water supplies, sewage control, treatment of chronic carriers, and sanitary and hygiene education especially among food handlers in our nation.

It is known that human is exclusively the only infectious reservoir of *S. enterica* serovar Typhi in nature, so eradication of this etiological agent could be possibly achieved by effective antimicrobial chemotherapy or adequate vaccines against patient and chronic carrier. Notwithstanding, typhoid fever continues to be a public health problem in developed country and remains epidemic disease in most parts of Central America^{4, 5)}, Southeast Asia^{6, 7, 8)}, the Indian subcontinent^{9, 10)}, Africa^{11, 12)}, Turkey¹⁰⁾. Moreover, the emergence of multidrug resistant (MDR) *S. enterica* serovar Typhi strain have become a serious problem

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Table 1. No. of *Salmonella enterica* serovar Typhi isolates collected by year

Year	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	total
No. of isolates	67	50	77	53	42	35	40	34	13	13	424

globally in recent year⁶⁾.

In Korea, *S. enterica* serotype Typhi is endemic pathogen occurring by sporadic or rarely outbreak case in recent year. According to annual report of communicable disease published by Korea Center for Disease Control and Prevention (KCDC), its incidental case appears to be as follows: 265 case in 1997, 380 case in 1998, 308 case in 1999, 234 case in 2000, 401 case in 2001, 221 case in 2002, 199 case in 2003, and 174 case in 2004 by year²⁾. And after MDR *S. enterica* serovar Typhi strains was firstly confirmed in 1992, which was isolated from a patient who returned from Southeast Asia travel, National Institute of Health of Korea (NIHK) reported additionally 10 more MDR strains during 1996 to 1997 through the national laboratory communicable disease surveillance system, but the origins of the strains were not clear^{22, 29)}.

As entitled to the public referral laboratory in our region, we collected most of the *S. enterica* serovar Typhi isolated from local health center and hospital in Busan. We conducted antimicrobial agent susceptibility test over all the collected strains to survey the drug resistance change by year. Particularly we selected a panel of 50 isolates comprising the representative strains of each year and 6 MDR strains, which are all obtained from sporadic cases of typhoid fever and performed a molecular typing by pulse-field gel electrophoresis (PFGE).

The study of epidemiologic marker is important in an attempt to trace the source of infection. Although various typing methods, such as the antibiogram, plasmid profiles, phage typing, PFGE, ribotyping, PCR-based amplification fragment length polymorphism, single-strand conformation polymorphism, and nucleotide sequence analysis has been used for the subtyping of *S. enterica* serovar Typhi, PFGE has proved to be the most effective method because of its reliable, reproducible results and high discriminatory ability^{8, 21, 30)}. Also, PFGE is able to differentiate between clonally related strains and strains which represent independent clones²³⁾.

The objective of this study was to investigate the

extent of genetic diversity using PEGE and the trend of antimicrobial resistance among *S. enterica* serovar Typhi isolates during 1996 to 2005 in Busan, Korea

MATERIALS AND METHODS

Bacterial isolates

We collected 424 *S. enterica* serovar Typhi strains which were isolated from local health center and hospital during 1996 to 2005 in Busan metropolitan city, Korea (Table 1).

The identity of *S. enterica* serovar Typhi isolates was based on conventional methods, including the use of selenite broth, MacConkey agar, salmonella-shigella agar and kligler-iron agar and confirmed with the API 20E system (BioMerieux, France). O-group antigen was serotyped using agglutination antisera produced by the National Institute of Health of Korea (NIHK) or commercial antisera (Difco, Detroit, Mich.). Isolates were stored at -70°C deep freezer until analyzed.

Antimicrobial susceptibility test

Serovar Typhi isolates were tested for susceptibility to antimicrobials by the disc diffusion method (Bauer et al, 1966: National Committee for clinical Laboratory Standards 1998). The 18 antimicrobials were used: ampicillin (10 μg), amikacin (30 μg), ampicillin/sulbactam (10/10 μg), cephalothin (30 μg), cefazolin (30 μg), cefepime (30 μg), cefotetan (30 μg), cefotaxim (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), gentamicin (10 μg), imipenem (10 μg), nalidixic acid (30 μg), tetracycline (30 μg), ticarcillin (75 μg), sulfamethoxazol/trimethorim (23.75/1.25 μg), kannamycin (30 μg) and amoxicillin/clavulanic acid (20/10 μg). Test strains were cultured in TSB broth at 37°C for 2–8 h. The culture was adjusted to 0.5 McFarland turbidity. The bacterial cells were then spread evenly on a Mueller-Hinton agar plate. The test antimicrobial discs (BBL Sensi-Disc Becton Dickenson, Cockeysville, MD, USA) were placed on the agar. After 18 h of incubation at 35°C , the diameter of antibiotic zone was measured. *Escheichia coli* ATCC

25922 and *Staphylococcus aureus* ATCC 29213 were used as controls for the potency of antibiotics. Interpretation of inhibitory zone was made in accordance with the interpretative standards provided by the manufacturer.

Restriction endonuclease digestion and PFGE

Chromosomal DNA for PFGE analysis was prepared by the method of Gautom with modifications²⁹.

Bacterial cells were suspended in cell suspension TE buffer (100 mM Tris, 100 mM EDTA, pH 7.5), and the cell density was adjusted to a turbidity of transmittance rate 10% to 15% (Vitek colorimeter, Hach company, USA). Ten microliter of Proteinase K (20 mg/mL) and 200 μ L of cell suspension was added to 200 μ L of 1.2% SeaKem Gold agarose (Cambrex, Rockland, ME, USA). The agarose mixture was pipetted into disposable plug molds (Bio-Rad Laboratories, Hercules, CA). Solidified agarose plugs were transferred to a tube containing 1.5 mL of ES lysis buffer (0.5 M EDTA, pH 9.0, 1% sodium-lauroylsarcosine) and 40 μ L of Proteinase K (20 mg/mL), and plugs were incubated in a shaking water bath at 55 for 1 h. Plugs were washed one time with D.D.W for 15 min and two times with TE buffer (10 mM Tris, 10 mM EDTA, pH 7.5) for 15 min, each time in a shaking water bath at 55.

Agarose plugs containing DNA were cut to slice of 1 mm thickness and restricted with 30 U of XbaI (New England Biolabs, Beverly, MA, USA) at 37°C for 90 min. The digested DNA plugs were loaded on the comb, and a 1% SeaKem gold agarose gel was prepared using 0.5 TBE (Tris-Borate-EDTA) buffer (Bioneer, Daejeon, Korea) and electrophoresed using CHEF Mapper (Bio-Rad) with switch times: an initial 2.16 sec and a final 63.8 sec, field strength: 6 V cm^{-1} , and run time: 18 hours. Gels were stained with ethidium bromide (0.5 μ g/mL: Bioneer) for 30 min, destained in distilled water for 30 min, and photographed under UV illumination (Vilber Lourmat, France).

Salmonella serovar Branderup H9812 (ATCC BAA-664) was used as a reference standard to normalize gels.

Data analysis

Patterns generated by PFGE were assessed visually

and the band differences were interpreted by the method of Tenover et al.²⁹. By this criteria, it has been proposed that isolates with similar PFGE band patterns were described as genetically indistinguishable, isolates with fewer than four band shift were assumed to be closely related (which caused by a single genetic event, e.g. a point mutation resulting in loss or gain of a restriction site, insertion, deletion, or chromosomal inversion), isolates with differences in four to six bands might be possibly related, and isolates showing a differences in more than seven bands were considered to be epidemiologically unrelated.

The similarity of fragment length patterns between two strains is scored by the Dice similarity coefficient. This coefficient, F, was calculated by the formular $F = 2n_{xy}/(n_x + n_y)$, where n_x is the total number of DNA fragments from isolate X, n_y is the total number from isolate Y, and n_{xy} is the number of fragments identical in the two isolates. An F value of 1.0 indicates that the two isolates have identical PFGE patterns. The original F value multiplied by 100 gives similarity percentages¹⁹.

Dendrogram was created by setting dice similarity coefficient and clustered by the method of unweighted pair group average (UPGMA), performed with BioNumerics (Applied Maths, Belgium)

Each unique PFGE pattern initial name was assigned as X based on the restriction endonuclease XbaI

RESULTS AND DISCUSSIONS

Antibiotic susceptibility testing

Of the total 424 *S. enterica* serovar Typhi isolates, only 8(1.9%) strains showed three resistance types (Table 1). The first type (A) was including two isolates that showed resistant to 5 antimicrobial agents: ampicillin, chloramphenicol, trimethoprim-sulfamethoxazol (co-trimoxazole), tetracycline and nalidixic acid

The second type (B) was including four isolates that showed the same resistant pattern with A type except tetracycline, and the last type (C) was including two isolates that were showing resistance to only nalidixic acid (Table 2). Besides 8 isolates, the remaining 416(98.1%) were fully susceptible to the 18

Table 2. Types of antimicrobial susceptibility for *S. enterica* serovar Typhi isolates

Type	No. of strains	resistant drug pattern ^a	isolates (yr)	PFGE pattern
A	2	AM, C, SXT, Te, NA	1998/1, 1999/2	X30
B	4	AM, C, SXT, NA	1998/2, 1999/1, 2001/1, 2003/3	X26, X29, X28
C	2	NA	2002/1, 2005/2	X27, X6
Others	416	susceptible to all drugs tested		

a) AM; ampicillin, C; chloramphenicol, SXT; trimethoprim-sulfamethoxazol, Te; tetracycline, NA; nalidixic acid

antimicrobial agents,

Although antimicrobial susceptibility test was also used traditionally epidemiologic marker, in this study, only three types of antibiogram were found. Therefore, results of antimicrobial susceptibility could not be useful to determine the transmission route of the *S. enterica* serovar Typhi strains in this area.

Since emergence of chloramphenicol resistance *S. enterica* serovar Typhi was reported firstly in the early 1970s³⁹, antimicrobial resistant *S. enterica* serovar Typhi had not been found before 1997 in Korea. Recently, chloramphenicol resistant *S. enterica* serovar Typhi isolates were appeared in this country as following resistant rates according to the reports of KCDC : 4.7% in 1999, 6.8% in 2000, 11.7% in 2001, 3.3% in 2002, 6.6% in 2003, 3.0% in 2004²⁷.

Since chloramphenicol has traditionally been the drugs most frequently used to treat typhoid fever, serovar Typhi isolates resistant to this drug and two more first-line drugs (ampicillin and co-trimoxazole) were defined as MDR *S. enterica* serovar Typhi¹¹.

Acquisition of resistance to chloramphenicol, ampicillin, trimethoprim, sulfonamides, and tetracyclines is usually associated with an incompatibility group HI plasmid^{32, 10}. These plasmids are large (~180 kb) and conjugative and originated from Southeast Asia³⁶. Recently, it was reported that MDR serovar Typhi also resistant to nalidixic acid and the fluoroquinolones revealed in Bangladesh²⁶, India²⁷, Thailand, Vietnam^{28, 31}, and Tajikistan²⁹.

Although MDR serovar Typhi has been raised a global problem, the incidence rate has been decreased recently in our nation because the condition of environmental and personal sanitation have been improved substantially. Also we found that the spread and emergence rate of MDR serovar Typhi wasn't increased in our region. Although the frequency of drug resistant strains are rarer comparing to other

Salmonella serovars, it is needed to continue evaluation of antimicrobial resistance patterns because resistance may be easily spread by genetic exchange between *S. enterica* serovar Typhi and other members of the family Enterobacteriaceae in normal gut flora.

PFGE patterns for *S. enterica* serovar Typhi isolates

When chromosomal DNA of all 50 *S. enterica* serovar Typhi isolates were restricted with XbaI and analysed by PFGE, a total of 32 different PFGE subtype pattern were generated according by dice coefficient, between 0.69 and 1.0.

Patterns produced by PFGE consisted of 13 to 18 fragments ranging in size from c. 20 to 630 kbp.

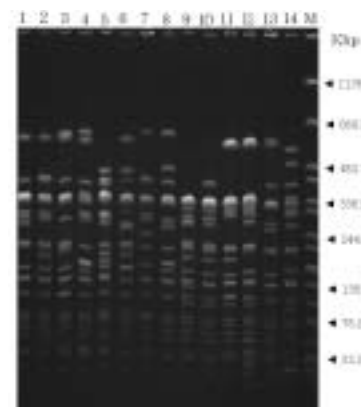


Fig. 1. PFGE (XbaI digested) patterns of representative *S. enterica* serovar Typhi isolates from sporadic case in Busan from 1996 to 2005.

Lane 1, 2005/13 (X3); Lane 2, 2005/6 (X6); Lane 3, 1999/4 (X11); Lane 4, 2000/1 (X12); Lane 5, 2004/6 (X17); Lane 6, 2005/12 (X18); Lane 7, 1997/4 (X20); Lane 8, 2005/1 (X21); Lane 9, 2004/12 (X22); Lane 10, 1998/3 (X24); Lane 11, 1999/1 (X26); Lane 12, 1998/1 (X30); Lane 13, 1997/6 (X31); Lane 14, 2005/9 (X32); Lane 15, molecular size marker, *Salmonella* serovar Branderup H9812 reference standards restricted with XbaI.

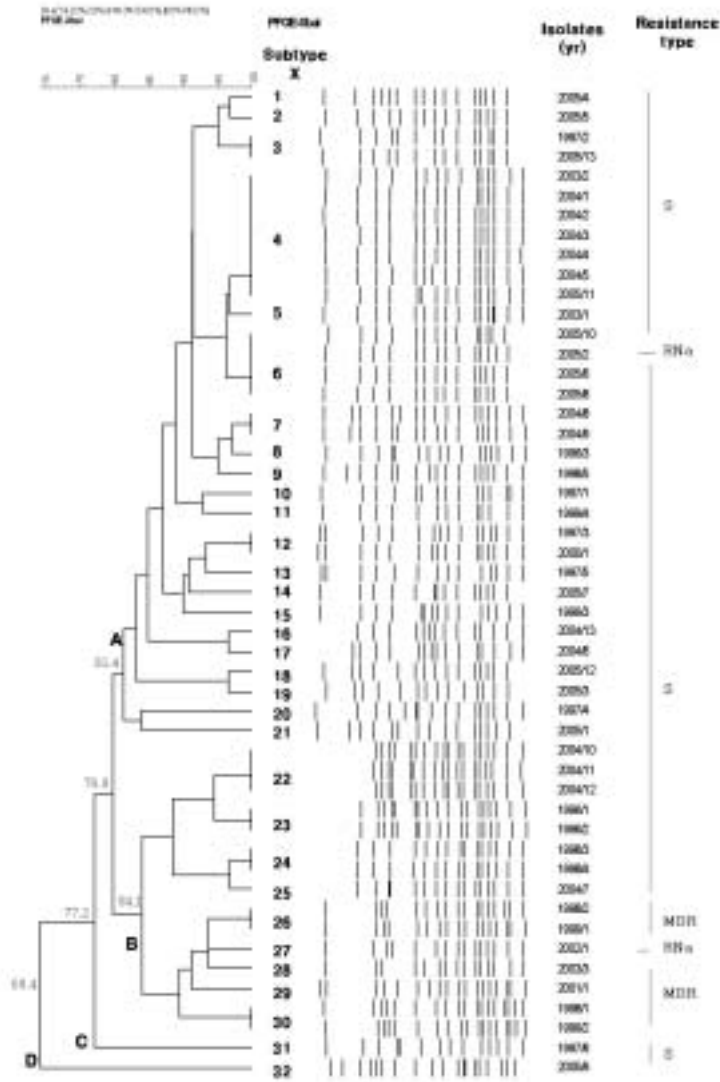


Fig. 2. Dendrogram showing similarities among the XbaI PFGE pattern of the 50 sporadic case *S. enterica* serovar Typhi strains.

Electrophoresis on an agarose gel of representative PFGE pattern are shown in Fig. 1. The number of fragments were shown 13 in two type patterns, 14 in 12 type patterns, 15 in 4 type patterns, 17 in 6 type patterns and 18 in 8 type patterns respectively. Thong et al. reported that PFGE produced restriction endonuclease analysis (REA) pattern consisted of 11 to 24 DNA fragments¹⁷. Also they reported that PFGE following digestion with restriction endonuclease XbaI (5' -TCTAGA-3'), SpeI (5' -ACTAGT-3'), and AvrII (5' -CCTAGG-3') was able to differentiate isolates of *S. enterica* serovar Typhi chromosomal DNA. And in conclusion, XbaI is the most useful restriction enzyme for PFGE of serovar Typhi because it's more discriminatory and cheaper than AvrII or SpeI.

Ten subtypes (F = 1,0) were shared by two or more strains: X3 (n = 2), X4 (n = 7), X6 (n = 4), X7 (n = 2), X12 (n = 2), X22 (n = 3), X23 (n = 2), X24 (n = 2), X26 (n = 2), X30 (n = 2). Of these, X4 type was the major subtype and shared by seven strains, of which five strains were isolated sporadically in 2004. These strain may be the same or at least, clonally highly related strains. Dendrogram of the 50 PFGE pattern is shown in Fig. 2.

At a similarity level of 81.4%, there are two major cluster designated A and B arbitrarily. The A cluster contained significant number of 33 strains and that was major cluster. Among 26 strains isolated in 2004 and 2005, 21 (80.7%) isolates were amongst this predominant pattern.

Interestingly, all of 6 MDR serovar Typhi isolates had similar PFGE pattern (F=0.88 to 1.0) grouped to B cluster. By the criteria of Tenover et al.²⁹, isolates differing in one to four bands (F=0.88 to 0.97) assumed to be closely related. Also Maslow et al. pointed out that strains with one or two bands' shift in PFGE analysis have been considered to be clonally related²⁹. From this suggestion, these MDR serovar Typhi strains can be designated closely related strains.

Because MDR serovar Typhi include the plasmid DNA ranging from 40 to 220 kbp⁶⁾, it seemed that the restriction fragment pattern of these isolates were somewhat different from susceptible serovar Typhi isolates in band position and number especially below 100 kbp. (Fig. 1)

Kariuki et al.¹¹⁾ found that there was no correlation between the antimicrobial susceptibility phenotype and the PFGE restriction fragment pattern of serovar Typhi isolated in Kenya. However, our observations seemed to be in contrast to these findings since the MDR serovar Typhi strains (ampicillin, chloramphenicol, tetracycline, nalidixic acid, co-trimoxazole) shared similar PFGE patterns.

Some patterns such as X3 and X12, although belonging to the same PFGE pattern, were isolated at different time(1997 vs 2005 and 1997 vs 2000). Thus, recirculation of certain infectious strains from sporadic case may be possible, suggesting that certain genotype of *S. enterica* serovar Typhi are stably maintained and persist over this considerable time period.

In Asia including Malaysia, Indonesia, Thailand, Taiwan, and Papua New Guinea, there have been reports describing many PFGE types in circulation^{16, 17, 18, 33, 19)}.

In Malaysia, PFGE suggested that individual outbreaks were associated with closely related strains, whereas isolates of serovar Typhi from sporadic case were very diverse. Forty-six XbaI digestion patterns were found for the 60 strains of sporadic serovar Typhi from Malaysia, and nine for 10 serovar Typhi strains obtained in Thailand. Kubota et al. showed that XbaI digested genomic DNA produced 79 unique patterns associated with international travel to 31 different country¹⁹⁾.

Our findings are similar to those of Thong et. al.¹⁷⁾ who found a significant genetic diversity as

demonstrated PFGE fingerprint patterns within the same region

We could find that multiple genetic variants of *S. enterica* serovar Typhi are simultaneously present in our region.

These results confirmed the utility of PFGE in assessing clonality for *S. enterica* serovar Typhi and proved to be an useful tool for investigating local epidemics and for surveillance of sporadic case in Busan area.

In Korea, PulseNet (The national molecular subtyping network) has recently been set to develop a standardized PFGE protocol including *S. enterica* serovar Typhi isolates.

Despite the limited geographic region and sample size, our findings would be valuable in developing rational strategies to control this pathogen and setting the basis of an effective PulseNet system in our nation.

국문초록

1996년부터 2005년까지 부산지역에서 분리된 *Salmonella enterica* serovar Typhi 주에 대한 항균제내성 변화양상 및 Pulsed-field gel electrophoresis (PFGE)를 이용한 분리주의 분자역학적 형별을 분석하였다. 전체 424주에 대한 항균제 감수성 시험결과 multidrug resistant (MDR) 6주(1.4%), nalidixic acid에만 내성을 보이는 균 2주를 제외한 나머지 416(98.1%)가 시험 항균제 18종 모두에 감수성을 보였다. 부산지역 분리 장티푸스균의 유전적 이질성을 확인하고자 실시한 산발 분리주 50주 PFGE/XbaI 시험결과, 최소 32종의 다양한 패턴이 나타났다. 각 패턴별로 제한효소 절편 수는 13개에서 18개 까지였고, 절편크기는 약 20 kbp에서 63 kbp 범위였다. 본 시험결과 부산지역의 장티푸스의 산발 또는 집단발생시 PFGE는 유용한 역학적 지표로 사용가능함을 알 수 있었으며, 또한 전국적 PulseNet구축의 기초 자료로서 활용도가 높을 것으로 사료된다.

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