ISSN 1021-366X

Analysis of Relationships between Several *Shigella sonnei* Outbreaks in the Taoyuan Area of Taiwan

Abstract

In the period between November 1995 and January 1996, there was a large-scale *Shigella sonnei* outbreak in the Taoyuan area. During the period between October 1998 and February 1999 in the same area, there were again five *S. sonnei* outbreaks. To understand the relationships between these outbreaks a study of the typing of the microbe was conducted using antimicrobial susceptibility testing, plasmid profile analysis, and pulsed-field gel electrophoresis (PFGE) methods. During the six outbreaks, a total of 55 *S. sonnei* isolates were collected. By antimicrobial susceptibility testing, these isolates were grouped into two types; by plasmid profile analysis, they were classified into three types; the PFGE method demonstrated only one type. The finding and the locality and timing of these outbreaks suggested that the *S. sonnei* isolates of the six outbreaks were closely related. It was therefore decided that the *S. sonnei* strains isolated in the period 1998-1999 and the strains isolated during the 1995 large-scale outbreak had, a molecular-

epidemiological and genetic clonal relationship.

Key Words: *Shigella sonnei* outbreak, pulsed-field gel electrophoresis (PFGE), plasmid profile analysis, antimicrobial susceptibility testing

Introduction

Shigella is a gram-negative bacillus of the Enterobacteriaceae family. It is one of the major pathogenic agents of diarrhea throughout the world(1). The infection is transmitted primarily by the fecal-oral route. Infections can be inapparent or with symptoms such as high fever, diarrhea, and bloody stools. According to their biochemical and serological characteristics, S. sonnei is classified into four groups, group A being S. dysenteriae; group B, S. flexneri; group C, S. boydii; and group D, S. sonnei. Using the O antigen, A, B and C groups can be further classified into 45 serotypes, and group D into only one serotype(1). Group D is mainly responsible for bacillary dysentery in developed countries. In the US, group D accounts for about 60-80% of all bacillary dysentery infections(2).

In recent years, bacillary dysentery has been the third most common infection among all notifiable diseases(3) in Taiwan. Groups B and D account for almost 95% of all infections. Group D is more important in major outbreaks. Infections occur more often between June and October, and except in the case of major outbreaks, more commonly in the eastern and northern parts of the island, particularly in the Taoyuan area(4) in the north. In the two months between October 1998 and February 1999 in the Taoyuan area, there were several large-scale outbreaks of *S. sonnei* infections, indicating the unusually high prevalence of bacillary dysentery in this area, and calling for further epidemiological studies.

Modern molecular biological technologies such as plasmid profile analysis,

pulsed-field gel electrophoresis (PFGE), ribotyping, and polymerase chain reaction (PCR) have been applied successfully to the differentiation studies of various pathogenic agents[5-11]. Of these methods, pulsed-field gel electrophoresis (PFGE) is the most effective method for typing bacterial strains[6,9]. The present study was designed, by using primarily the PFGE method together with the plasmid profile analysis for typing, to analyze the epidemiological relationships of the isolates in the 1998-1999 *S. sonnei* outbreaks in the Taoyuan area, and at the same time, to set up bacterial finger-print databases for disease prevention and control references in the future. The isolates of a major outbreak in a primary school in the Taoyuan area in November 1995 were also compared.

Materials and Methods

Sources of *Shigella sonnei* Isolates

Of the 55 *S. sonnei* isolates collected (53 human and two environmental specimens), five were from the major outbreak in a primary school in Taoyuan in November 1995; and the other 50, from the five outbreaks in the same area between November 1998 and February 1999 (Table 1). Isolation and assessment of the microbes followed the specifications in the "Standard Operational Procedures of Laboratory Testing for Disease Control" issued by the Department of Health.

Antimicrobial Susceptibility Testing

The dilution and disk diffusion method was used for the antimicrobial susceptibility testing of *S. sonnei*[13]. Antibiotics used for testing were Ampicillin 10 μg, Cefixime 5 μg, Nalidixic acid 30 μg, and Cotrimoxazole 25 μg.

Plasmid Profile Analysis

A single colony cultured overnight was picked up from the nutrient plate and inoculated on a 2 mL nutrient broth (Difco, USA), agitated overnight at 37^0C, and finally, underwent plasmid extraction by the rapid alkaline extraction procedure outlined by Brinboim and Doly[14]. This broth was then placed under 0.8% gel electrophoresis at 100 volts for two hours. The DNA marker-X (Boehringer Mannheim, New York, USA) was used as an index of molecular weight. It was then stained with g/mL ethidium bromide for 15 minutes, washed with water for one hour, developed under ultraviolet ray and filed.

Pulsed-Field Gel Electrophoresis

The procedure followed that of the US CDC[15] with some modifications. A single colony cultured overnight was picked up from the nutrient plate, inoculated on either 2 mL nutrient broth or tryptic soy broth (TSB), agitated overnight at 37^0C, and washed once with SE solution (75 mM NaCl and 25 mM EDTA, pH 8.0) at 610 nm wavelength. The solution density was adjusted to an OD value of 1.15 and 1.25. 1% agar gel of low melting point (Bio-Rad Laboratories, Richmond, CA, USA) of the same volume was taken and mixed evenly with the solution, and placed into a plug mold (Bio-Rad Laboratories, Richmond, CA, USA) for ten minutes to solidify. The plug was placed in a lysis buffer (50 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0, 1% sodium lauryl sarcosine, and 1 mg/mL protease K) overnight at 53^0C. It was then washed twice for 30 minutes each time with TE solution (10 mM Tris, pH 8.0 and 10 mM EDTA, pH 8.0) containing 1 mM phenyl methylsulfony fluoride (Sigma Chemical Co., St Louis, MI, USA) . A 2.0 to 2.5 mm slice of plug was placed in a reagent solution containing 20 units of Xbal (Boehringer Mannheim Biochemicals, New York, USA) restriction enzymes at 37^0C undergoing agitation for four hours. It was then placed in CHEF-DR III (Bio-Rad Lab) in 1.2% gel, at 14^0C, at 6 volts/cm electric field and angle 120^0. The changing time was 5 to 33.5 seconds, and the electrophoresis time was 22

hours. PFGE marker I (Boehringer Mannheim Bicochemicals) was used as an index of molecular weight. It was stained for 30 minutes with 0.5 μg/mL ethidium bromide, washed for two hours, and developed with ultraviolet ray.

The Dice coefficient formula[16] was used to compare the PFGE typing patterns. The D coefficient was the number of bands at the corresponding position in two isolates multiplied by 2 and divided by the sum of the bands. When D coefficients of these strains were >=0.8, they were considered to have come from related sources. Differences in positions of a few bands could be due to simple gene insertions or deletions, or the functioning or malfunctioning of position identification by the restriction enzyme[16-19].

Results

By antimicrobial susceptibility testing, the 55 isolates could be grouped into two antibiogram patterns. *S. sonnei* isolates collected from Pingchen city and Pateh city of Taoyuan county were of pattern I; those from the primary schools, kindergartens and factories of Lungtan township, Taoyuan county, were of pattern II. Pattern I isolates were resistant to Ampicillin and Cotrimoxazole, and sensitive to Cefixime and Nalidixic acid. Pattern II isolates were resistant to Nalidixic acid and Cotrimoxazole, and sensitive to Cefixime and Ampicillin (Table 1).

By plasmid profile analysis, the 55 *S. sonnei* isolates could be grouped into three types. Different plasmids showed a difference of 5 to 8 bands, and a common band could only be found at position 2.0 kb. The molecular weight of the plasmids outside the chromosomes ranged from 1.6 to 12 kb (Figure 1). Isolates collected from different incidents at different localities such as Pingchen, Pateh or Lungtan, showed one to three types of different plasmid

profiles (Figure 1). On the other hand, *S. sonnei* strains collected from the same locality had similar plasmid profiles; isolates of the same incident had the same plasmid profile (Table 1). The isolates, when sliced by Xbal restriction enzyme, showed 23 to 24 bands, their sizes ranging from 32.4 to 500 kb.

The 55 *S. sonnei* isolates showed only one major PFGE pattern, though there were slight differences between patterns, resulting in four subtypes, types A, A-1, A-2 and A-3 (Figure 2). *S. sonnei* isolates of the 1995 outbreak showed a Pattern A configuration, and was three bands different from Pattern A-1 at 200-300 kb. Pattern A-2 and Pattern A-1 were isolates of the late 1998 outbreaks; they differed by two bands at 100-150 kb. Pattern A-3 was an isolate of the late 1998 and early 1999 outbreaks. It was different from pattern A-1 by the upward position of one band at 75 kb. The stain density was also higher. This was verified when electrophoresis time was stretched to 50-100 kb (unpublished data). The D coefficients of similarity were between 0.82 and 0.95, higher than the 0.8 suggesting the same microbial sources. In the same incident, the outbreak of the primary school in Lungtan township for instance, the isolates were of the same type or subtype, such as A-1. Isolates from incidents of different localities such as Pingchen, Pateh and Lungtan although of the same type, were of different subtypes such as A, A-1, A-2 or A-3. Generally speaking, isolates from the same locality were of the same type and subtype. Incidents No. 3 and No. 4 for instance, were from Pateh and were of subtype A-2; incidents No. 5 and No. 6 were from Lungtan, and were of subtype A-3. There were exceptions. Incidents No. 2 and No. 5, though from Lungtan, were of different subtypes A-1 and A-3.

Discussion

The Taoyuan area in the north of Taiwan has been an area of high prevalence of bacillary dysentery for some years. There was a large-scale *S. sonnei* outbreak in November 1995. Between October 1998 and February 1999, there were five large-scale *S. sonnei* outbreaks. To understand whether their sources of infection were related, the present study was designed, using conventional antimicrobial susceptibility testing and primarily, plasmid profile analysis and pulsed-field gel electrophoresis, in order to conduct a molecular epidemiological investigation of the bacillary dysentery outbreaks in this area, and at the same time establish bacterial finger-print databases.

Antimicrobial susceptibility testing is a routine test performed by clinical laboratories. The genes and mechanism that control the resistance to antibiotics can be modified by external factors. Subsequent generations of the same strain may differ in their susceptibility to antibiotics. Though antibiotic susceptibility can be of reference value to disease control and medical affairs, it is not adequate for either typing or comparing incidents from different time periods. The antimicrobial susceptibility of the isolates from Lungtan though different from those of Pingchen and Pateh, differed in phenotype only. The reason for the difference was not detected by plasmid profile analysis or pulsed-field gel electrophoresis. The findings of antimicrobial susceptibility testing in the present study were used for reference in the comparison.

In plasmid profile analysis, small plasmids (1.6 to 12 kb) were used. Larger plasmids (>15 kb) could be lost in the course of culturing or transferring, and were unstable in extraction under high pH value. They were generally not used for differentiation[6,19]. The 55 *S. sonnei* had three different plasmid profiles by 5 to 8 bands. They could be easily differentiated. When isolates

of the same locality showed few differences using the pulsed-field gel electrophoresis, plasmid profile analysis could differentiate and verify the differences[20]. Isolates in smaller areas, townships for instance, could be differentiated by the differences in plasmid profiles (1, 2 and 3 types). Isolates of the same townships had the same plasmid profile pattern.

The corresponding positions of bands were used in typing by the pulsed-field gel electrophoresis method. When isolates differed by more than 3 bands, they were considered of different patterns. When the difference was between 1 and 3, they were considered subtypes[17]. Isolates of the six incidents differed only by 1 to 3 bands; they were classified further into subtypes. As their D coefficients were somewhere between 0.82 and 0.95, higher than the 0.8 suggestive of the same microbial source, they were considered to have significant relatedness. When the finding of the present study was compared with the bacterial finger-print databases of Taipei City, Taichung City or Indonesia[21], and the D coefficients of isolates of the six incidents being between 0.37 and 0.45, it was evident, though indirectly, that the isolates of the six incidents were locality-related. Typing, however, should be verified by further epidemiological studies.

Dysentery bacilli are highly infectious. Ingestion of 10 to 100 microbes is sufficient to induce infection. Carriers are capable of shedding bacilli for as long as one and a half years[22]. Incidents No. 2 and No. 6 overlapped somewhat in their time of occurrence, and they occurred in neighboring townships. Some patients of incidents No. 2 and No. 5 were relatives. It was likely that the source of infection was the same, as suggested by the findings of typing. Although incident No. 1 occurred three years before the other incidents, its D coefficient was as high as 0.8; therefore it was speculated that there were some carriers in the area during this period, and under certain weather and sanitary conditions, initiated outbreaks. In incidents No. 1 and

No. 3, since the pulsed-field gel electrophoresis and plasmid profiles of the environmental and human isolates were of the same typing, they likely had the same source of infection.; it is possible that the drinking water of the school could have been contaminated. Though no pathogenic agents were isolated from environmental specimens in other incidents, their drinking water came from sources other than tap water. Improvement of environmental sanitation and health education of the public is most vital in the prevention and control of further outbreaks in the Taoyuan area.

Acknowledgement

The authors wish to thank Dr Wang KJ, former Director of the National Institute of Preventive Medicine, DOH, for his guidance and encouragement. Our thanks are also due to the staff of the Taoyuan County Health Bureau, the Epidemiology Division of the former National Quarantine Service, DOH, and the FETP of the former National Institute of Preventive Medicine, DOH, for their assistance in the present study.

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References

1.Farmer III JJ. *Enterobacteriaceae*. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolk RH (ed.), Manual of Clinical Microbiology, 7th ed., Washington, DC, American Society for Microbiology, 1999; 465-67.

2.Dupont HL. *Shigella* species (bacillary dysentery). In: Mandell GL, Douglas RG, Bennett JE (ed.), Principles and Practice of Infectious Diseases, 3rd ed., Churchill Livingstone Inc., New York, 1990; 1716-22.

3.Center for Disease Control, Department of Health. Annual Report on Communicable Diseases, Taiwan Area, 1998. February 2000; 1-6.

4.Pan TM. Trend of dysentery and its prevention. Epidemiology Bulletin 1996; 12: 212-19.

5.Faruque SM, Haider K, Rahman MM, Alim AA, Ahmad QS, Albert MJ, Sack RB. Differentiation of *Shigella flexneri* strains by rRNA gene restriction patterns. J Clin Microbiol 1992; 30: 2996-9.

6.Liu PYF, Lau YJ, Hu BS, Shyr JM, Shi ZY, Tsai WS, Lin YH, Tseng CY. Analysis of clonal relationships among isolated of *Shigella sonnei* by different molecular typing methods. J Clin Microbiol 1995; 33: 1779-8.

7.Litwin CM, Strom AL, Chipowsky S, Ryan KJ. Molecular epidemiology of *Shigella* infections: plasmid profiles, serotype correlation, and restriction endonuclease analysis. J Clin Microbiol 1991; 29: 104-8.

8.Wong HC, Lu KT, Pan TM, Lee CL, Shih DY. Subspecies typing of *Vibrio parahaemolyticus* by pulsed-field gel electrophoresis. J Clin Microbiol 1996; 34: 1535-9.

9.Liebisch B, Schwarz S. Molecular typing of *Salmonella enterica* subsp. *enterica* serovar *enteritidis* isolates. J Med Microbiol 1996; 44: 52-9.

10.Pan TM, Lin CS, Wang TK, Tsai JL, Ho SI, Lee CL. Molecular subtyping of *Salmonella enterica* serover *paratyphi* A from Southeast Asia. J Food Drug Anal 1998; 6: 573-8.

11.Pan TM, Lin CS, Wang TK, Tsai JL, Ho SI, Lee CL, Chiou CS, Hsu SY, Huang HC, Wang GR. Preliminary report on genotyping of *Vibrio cholerae* non-O1 isolates in Taiwan by pulsed-field gel electrophoresis. J Microbiol Immunol Infect 1998; 31: 257-60.

12.National Institute of Preventive Medicine, Department of Health. Isolation and assessment of bacillary dysentery bacilli. In Standard Operational Procedures of Laboratory Testing for Disease Control. Taipei, 1995; 1-5.

13.Wood GL, Washington JA. Antibacterial susceptibility tests: Dilution and disk diffusion methods. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (ed.), Manual of Clinical Microbiology, 7th ed., Washington, DC, American Society for Microbiology, 1999; 1526-43.

14.Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 1979; 7: 1513-23.

15.Maslow JN, Slutsky AM, Arbeit RD. Application of pulsed-field gel electrophoresis to molecular epidemiology. In: Persing DH, Smith TF, Tenover FC, White TJ eds. Diagnostic Molecular Microbiology—Principles and Applications. Washington, DC, American Society of Microbiology, 1993; 563-72.

16.Dice LR. Measures of the amount of ecological association between species. Ecology 1945; 26: 297-302.

17.Goering RV. Molecular epidemiology of nosocomial infection: analysis of chromosomal restriction fragment patterns by pulsed-field gel electrophoresis. Infect Control Hosp Epidemiol 1993; 14: 595-600.

18.Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing

DH, Swaminathan B. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. J Clin Microbiol 1995; 33: 2233-9.

19.Casalino M, Micoleetti M, Salvia A, Colonna B, Baimone F. Characterization of endemic *Shigella flexneri* in Somalia: antimicrobial resistance, plasmid profile, and serotype correlation. J Clin Microbiol 1994; 32: 1179-83.

20.Brain MJ, Van R, Townsend I, Murray BE, Cleary TG, Pickering LK. Evaluation of the molecular epidemiology of an outbreak of multiply resistant *Shigella sonnei* in a daycare center by using pulsed-field gel electrophoresis and plasmid DNA analysis. J Clin Microbil 1993; 31: 2152-6.

21.Lin CS, Wang TK, Tsai JL, Ho SI, Lee CL, Pan TM. Subspecies typing of *Shigella* isolated from outbreaks in Taiwan, 1995-1996. Formosan J Med 1997; 1: 152-8.

22.Levine MM, Dupont HL, Khodabandelou M, Hornick RB. Long-term *Shigella*-carrier state. New Eng J Med 1973; 288: 1169-71.

Table 1 Shigella sonnei outbreaks in Taoyuan and their Typing

No.	Time	Locality	No. of Strains Collected@	PFGE Patterns	Plasmid Typing	Susceptibility Typing**
1	95/11—96/01	Primary school A in Pingchen	5*	A	1	I
2	98/10—98/12	Primary school B in Lungtan	23	A-1	2	Π
3	98/11	Primary school C in Pateh	7*	A-2	3	I
4	98/11	Primary school D in Pateh	7	A-2	3	I
5	98/12—99/01	Kindergarten in Lungtan	10	A-3	2	Π
6	99/02	Factory in Lungtan	3	A-3	2	Π

@: strains, 53 human specimens and 2 environmental specimens

*: one strain of them was environmental strain

**：pattern I：Resistant to ampicillin, cotrimoxazole；susceptible to cefixime, nalidixic acid

pattern II：Resistant to nalidixic acid, cotrimoxazole；susceptible to cefixime, ampicillin

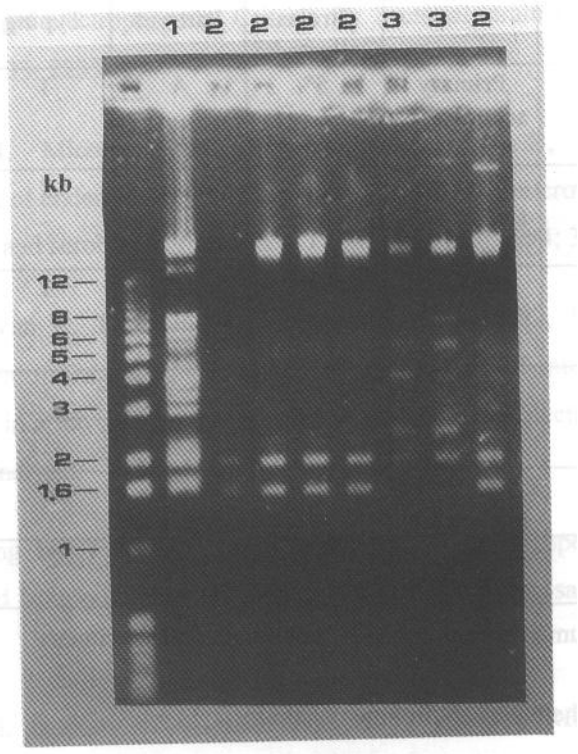

Figure 1. Plasmid Patterns of *Shigella sonnei* Isolated from Outbreaks in Taoyuan

Strains in each row: Row 2, isolates of incident No. 1; Rows 3 and 4, isolates of incident No. 2; Rows 5 and 6, isolates of incident No. 5; Row 7, isolates of incident No. 3; Row 8, isolates of incident No. 4; Row 9, isolates of incident No. 6. Row 1, index of molecular weight. Figures on top stand for the plasmid typing of the isolates (Table 1).

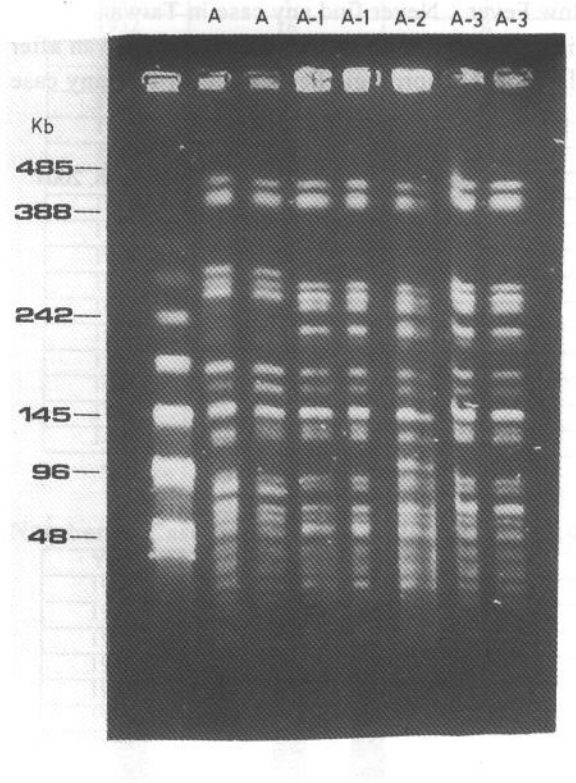

Figure 2. PFGE Typing of *Shigella sonnei* Isolates in Outbreaks of Taoyuan

Strains in each row: Rows 2 and 3, isolates of incident No. 1; Rows 4 and 5, isolates of incident No. 2; Row 6, isolates of incident No. 3; Row 7, isolates of incident No. 5; Row 8, isolates of incident No. 6. Row 1, index of molecular weight. Letters and figures on top stand for the PFGE typing of the isolates (Table 1).