CLINICAL AND MOLECULAR EPIDEMIOLOGY

Molecular analysis of *Shigella sonnei* isolated from three well-documented outbreaks in school children

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Fifty-eight isolates of *Shigella sonnei* from three outbreaks in school children and eight control isolates from epidemiologically unrelated sporadic clinical infections in Taiwan were compared by antibiotic susceptibility testing and molecular typing. Antibiotic susceptibility testing showed that all strains except one sporadic isolate were multi-resistant. Ribotyping after restriction endonuclease digestion with *Sal*I, *Pvu*II and *Hind*III generated the same ribosomal pattern in 65 of the 66 isolates. Plasmid profile analysis and pulsed-field gel electrophoresis (PFGE) produced eight and nine distinct patterns, respectively, and were in agreement with the epidemiological relationship of the outbreak strains. Nevertheless, some of the sporadic isolates could be discriminated only by a combination of these two methods. This study showed that plasmid profiling in combination with PFGE may be superior to ribotyping in molecular epidemiological investigations of *S. sonnei*.

Introduction

Infection caused by *Shigella* spp. remains a serious public health problem in both developing and developed countries and is the major cause of diarrhoeal disease in Taiwan [1, 2]. Outbreaks of infection due to *Shigella* spp. are difficult to control because of their low infective dose [3], the ease of spread by person-to-person transmission by the faecal–oral route [4] and their ability to spread indirectly by faecal contamination of food and water [5]. In recent years, *S. flexneri* and *S. sonnei* have been the most prevalent species causing shigellosis in Taiwan [1], but few epidemiological studies have been reported. In epidemiology, tracing the source of an infection is an important step in the control of transmission and the identification of the epidemic strain in the course of an outbreak investigation is the prerequisite for the discrimination of the causative clone and its reservoir.

In the past, several conventional typing methods including biotyping, colicin typing, bacteriophage typing and drug resistance pattern have been used in

epidemiological subtyping of *S. sonnei* [2, 6, 7], but the usefulness of these techniques is limited by problems common to typing systems based on phenotypic properties [8]. Recently, approaches at the DNA level have been used to discriminate between bacterial isolates. Plasmid profile analysis [9, 10], ribotyping [11, 12] and pulsed-field gel electrophoresis (PFGE) [13, 14] have proved to be useful in discriminating isolates of *Shigella* spp.

The present study was designed to determine the usefulness of these typing methods (plasmid profile analysis, ribotyping and PFGE) for differentiating strains of *S. sonnei* obtained from three well-documented outbreaks in three different counties of Taiwan during the period 1993–1997 and to investigate the clonal relationships between them. At the same time, eight epidemiologically unrelated isolates of *S. sonnei* obtained from sporadic clinical sources between 1985 and 1987 were studied for comparison.

Materials and methods

Diarrhoeal outbreaks

Three common-source epidemics (waterborne) of *S. sonnei* shigellosis occurred in three primary schools during the period Sept. 1993–Oct. 1997. The first

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outbreak (outbreak 1) occurred in Taichung City, Taiwan, in September 1993. One hundred stool specimens from 739 children with diarrhoea were cultured and isolates were confirmed as *S. sonnei*. The second outbreak (outbreak 2) occurred in Taoyuan County, Taiwan, in November 1995. In this outbreak, 404 isolates from stool specimens from 715 children with diarrhoea were confirmed as *S. sonnei*. The third outbreak (outbreak 3) of diarrhoea was in Hsinchu County, Taiwan, in October 1997. Stool specimens from 442 school children were cultured, with 123 isolates confirmed as *S. sonnei*; 76 of these children had diarrhoeal symptoms and 47 were symptom-free.

Bacterial strains

A total of 66 randomly selected isolates of *S. sonnei* were analysed in the study (Table 1). These included 25 isolates from outbreak 1, 20 isolates from outbreak 2, 13 from outbreak 3, and 8 isolates from epidemiologically unrelated sporadic clinical infections that occurred between 1985 and 1987. A type strain of *S. sonnei* (ATCC 9290) was included for comparison. All the isolates were characterised by biochemical and serological tests. Subsequently, all strains were stored at -70°C in Tryptic Soy Broth (Difco, Detroit, MI, USA) containing glycerol 15% until processing.

Antibiotic susceptibility testing

Isolates were tested for susceptibility to ampicillin (AM, 10 μg), piperacillin (PIP, 100 μg), cefotaxime (CTX, 30 μg), ceftizoxime (ZOX, 30 μg), imipenem (IPM, 10 μg), amikacin (AN, 30 μg), gentamicin (GM, 10 μg), netilmicin (NET, 30 μg), tetracycline (TE, 30 μg), nalidixic acid (NA, 30 μg), chloramphenicol (C, 30 μg) and trimethoprim/sulphamethoxazole (SXT, 25 μg) by the Kirby-Bauer disk diffusion method [15] and the resistance breakpoints used were those recommended by the National Committee for Clinical Laboratory Standards [16].

Plasmid profile analysis

Plasmid DNA was extracted by the method of Kado and Liu [17]. Plasmid sizes were calculated by the method of Rochelle *et al.* [18], with plasmids obtained from *Escherichia coli* 39R861 (NCTC 50192) as a reference standard. Only small plasmids, which appeared as bright bands below the band of chromosomal DNA on the gel, were included in the analysis. Large plasmids above 20 kb were not investigated because of their instability [10].

Ribotyping

Whole-cell DNA was prepared as described by Nastasi *et al.* [19]. DNA was digested with restriction enzymes *Hind*III, *Sal*I and *Pvu*II (Boehringer Mannheim GmbH, Mannheim, Germany). DNA fragments were separated

by electrophoresis at 100 V for 4 h, transferred to a nylon membrane, and fixed by spectrolinker (Spectroline, XL-1000/Spectronic, Westbury, NY, USA). Hybridisation was performed with a random primed digoxigenin-11-dUTP-labelled cDNA probe obtained by reverse transcription of a commercially available preparation of *E. coli* 16S + 23S rRNA (Boehringer Mannheim). The digoxigenin-labelled cDNA probe was detected with anti-digoxigenin antibodies labelled with alkaline phosphatase and luminescent substrate Lumi-gen-PPD as recommended by the manufacturer (Boehringer Mannheim).

PFGE

PFGE was performed as described previously [20]. Briefly, genomic DNA in agarose plugs was digested with endonuclease *Xba*I (Boehringer Mannheim). DNA fragments were then separated by electrophoresis on Seakem GTG agarose (FMC, No. 50072; FMC BioProducts, USA) 1.2% gels in $0.5 \times$ Tris-borate-EDTA buffer with Pharmacia-LKB 2015 equipment. The running conditions were 200 V at 14°C for 24 h. The pulse time was increased from 5 to 35 s. Multimeric phage λ (48.5 kb) DNA (Boehringer Mannheim) was used as the molecular size marker. After electrophoresis, the gel was stained with ethidium bromide for 30 min and de-stained in distilled water for 2 h. DNA bands were then visualised under UV light and photographed.

Results

Antibiotic susceptibility testing

According to the antibiotic susceptibility tests (Table 1), all 25 isolates from outbreak 1 were resistant to tetracycline, co-trimoxazole, piperacillin, chloramphenicol and ampicillin, while the strains isolated from outbreaks 2 and 3 were resistant to tetracycline and co-trimoxazole only. Resistance to tetracycline, chloramphenicol and nalidixic acid was found most frequently among the sporadic isolates.

Plasmid profile analysis

Plasmid profiles of *S. sonnei* isolates are illustrated in Fig. 1 and summarised in Table 1. As expected, strains from the same outbreak possessed their own identical profiles (Table 1; Fig. 1, lanes 9–15). Among the eight sporadic isolates (S59–S66) six had distinct plasmid profiles (Table 1; Fig. 1, lanes 3–8). Isolates S59 and S60 shared the same profile (e) (Fig. 1, lanes 2 and 3). Likewise isolates S63 and S64 had an indistinguishable profile (g) (Fig. 1, lane 6). When profiles of outbreak strains were compared with the profiles of sporadic isolates, outbreak 1 strains shared the same profile (b) (Table 1) with one of the sporadic isolates (S62) isolated 7 years previously.

Table 1. Characteristics of *S. sonnei* isolates examined in the present study

Source and isolate	Date of isolation (day/month/yr)	Antibiogram pattern	Plasmid profile	rDNA RFLP pattern with			PFGE pattern
				<i>Sal</i> I	<i>Pvu</i> II	<i>Hind</i> II	
Type strain ATCC 9290	...	*	a	s1	p1	h1	i
Outbreak 1 (Taichung city [†])							
S1–S7	13/9/93	PIP ^r TE ^r C ^r SXT ^r AM ^r	b	s1	p1	h1	ii
S8–S13	15/9/93	PIP ^r TE ^r C ^r SXT ^r AM ^r	b	s1	p1	h1	ii
S14–S17	24/9/93	PIP ^r TE ^r C ^r SXT ^r AM ^r	b	s1	p1	h1	ii
S18–S22	2/10/93	PIP ^r TE ^r C ^r SXT ^r AM ^r	b	s1	p1	h1	ii
S23–S25	6/10/93	PIP ^r TE ^r C ^r SXT ^r AM ^r	b	s1	p1	h1	ii
Outbreak 2 (Taoyuan County [‡])							
S26–S38	20/11/95	TE ^r SXT ^r	c	s1	p1	h1	iii
S39	5/11/95	TE ^r SXT ^r	c	s1	p1	h1	iii
S40–S45	27/11/95	TE ^r SXT ^r	c	s1	p1	h1	iii
Outbreak 3 (Hsinchu County [§])							
S46	13/10/97	TE ^r SXT ^r	d	s1	p1	h1	iv
S47–S58	25/10/97	TE ^r SXT ^r	d	s1	p1	h1	iv
Sporadic clinical isolates [§]							
S59	14/11/87	TE ^r NA ^r C ^r PIP ^r SXT ^r AM ^r	e	s1	p1	h1	v
S60	16/11/87	TE ^r NA ^r C ^r PIP ^r SXT ^r AM ^r	e	s1	p1	h1	v
S61	19/6/86	TE ^r NA ^r C ^r	f	s1	p1	h1	vi
S62	11/8//86	TE ^r NA ^r C ^r AM ^r	b	s1	p1	h1	vii
S63	21/10/86	TE ^r NA ^r C ^r PIP ^r AM ^r	g	s1	p1	h1	viii
S64	4/11/86	TE ^r C ^r PIP ^r	g	s1	p1	h1	ix
S65	2/8/85	TE ^r NA ^r C ^r	h	s1	p1	h1	ix
S66	5/9/85	TE ^r	i	s2	p2	h2	x

*Sensitive to all tested drugs.

[†]Middle Taiwan.[‡]Northern Taiwan.[§]Southern Taiwan. **Fig. 1.** Agarose gel electrophoresis profiles of plasmid DNA from *S. sonnei* isolates. Lane 1, type strain ATCC 9290; 2–6, sporadic clinical isolates S59–S63, respectively; 7, isolate S65; 8, isolate S66; 9 and 10, isolates S11 and S15 from outbreak 1; 11–13, isolates S26, S39 and S45 from outbreak 2; 14 and 15, isolates S46 and S47 from outbreak 3; 16, *E. coli* 39R861 as mol. wt marker (kb); linear DNA, fragmented plasmids and chromosomal DNA.

Ribotyping

To determine the optimal enzymes for achieving good resolution of DNA, preliminary tests were performed with restriction endonucleases *Hind*II, *Pvu*II, *Sal*I, *Eco*RI, *Bam*HI, *Sca*I, *Hind*III and *Hin*FI. Of the enzymes tested, *Hind*II, *Pvu*II and *Sal*I provided the best distribution of bands and patterns were easily distinguishable. After restriction with these enzymes, all 58 outbreak strains, seven of eight sporadic isolates and type strain ATCC 9290 exhibited identical ribotypes as illustrated in Fig. 2a. Only isolate S66 possessed a distinct pattern regardless of the enzyme

used. As shown in Fig. 2b (lanes 2, 4 and 6), the patterns of isolate S66 were different from those of isolate S1 (as representative of the other 65 isolates).

PFGE

PFGE resolution of DNA restricted with *Xba*I showed 10 restriction patterns consisting of 17–20 DNA fragments ranging in size from *c.* 20–500 kb in all 66 clinical isolates of *S. sonnei* and the type strain of the species (Table 1, Figs. 3 and 4). The strains from individual outbreaks were indistinguishable, whereas patterns from the three different outbreaks were distinct from each

Fig. 2. (a) Ribosomal patterns of *Hind*III restriction fragments of *S. sonnei* chromosomal DNA hybridised with dig-labelled *E. coli* 16S+23S rRNA. Lane 1, type strain ATCC 9290; 2 and 3, outbreak 1 isolates (S1, S10); 4 and 5, outbreak 2 isolates (S26, S39); 6 and 7, outbreak 3 isolates (S46, S47); 8 and 9, sporadic isolates (S59, S65); 10, digoxigenin-labelled phage λ DNA cut with *Hind*III as mol. wt marker (kb). (b) Ribosomal patterns of *Sal*I (lanes 1 and 2), *Pvu*II (3 and 4); *Hind*II (5 and 6) restriction fragments of *S. sonnei* isolates S1 and S66 chromosomal DNA hybridised with dig-labelled *E. coli* 16S+23S rRNA. Lanes 1, 3 and 5, isolate S1; 2, 4 and 6, isolate S66; 7, digoxigenin-labelled phage λ DNA cut with *Hind*III as mol. wt marker (kb).

Fig. 3. *Xba*I restriction patterns of outbreak strains of *S. sonnei* isolates obtained by PFGE. Lanes 1-3, outbreak 1 strains (S1, S8, S13); 4-6, outbreak 2 strains (S26, S39, S40); 7-9, outbreak 3 strains (S46, S47, S58); 10, λ ladder as mol. wt marker (kb).

Fig. 4. *Xba*I restriction patterns of *S. sonnei* isolates obtained by the PFGE. Lane 1, type strain ATCC 9290; 2, isolate S1 (outbreak 1); 3, isolate S26 (outbreak 2); 4, isolate S46 (outbreak 3); 5-9, isolates S60-S64, respectively; 10, isolate S66; 11, λ ladder as mol. wt marker (kb).

other. In contrast, eight sporadic isolates possessed six patterns (Table 1, v–x; Fig. 4, lanes 5–10). Among them, four isolates had individual patterns (vi, vii, viii, x). The other four isolates shared two different patterns. Isolates S59 and S60 possessed pattern v, and isolates S64 and S65 had pattern ix (Table 1).

Discussion

In recent years, outbreaks of infection with *S. sonnei* have become an important public health problem in Taiwan. The concept of clonal expansion of micro-organisms during common-source epidemics in contrast to multiple-source outbreaks is important in the epidemiology of infectious diseases. This study investigated the clonal relationship of *S. sonnei* among three well-documented outbreaks and compared them to those isolated from sporadic clinical infections by molecular typing methods.

Within a single outbreak, strains had identical antibiogram patterns. This result showed that the identification of a new or unusual pattern of antibiotic resistance among isolates cultured from multiple patients is often the first indication of epidemic spread. A previous study [2] and the report by Yagupsky *et al.* [21] showed that the genes of multiple drug resistance of *S. sonnei* were on the plasmids of 68–114 kb (45–75 MDa). Although the outbreak strains in the present study shared three different plasmid profiles, they all possessed a plasmid of *c.* 68–114 kb (*c.* 45–75 MDa), which may carry the genes of multiple drug resistance.

Plasmid profile analysis is useful and extensively applied in the differentiation of isolates. Although this technique has limitations because it relies on the detection of extrachromosomal genetic elements which can readily be lost [22], an earlier study found that they had high specificities in different strains with different plasmid profiles in studies on *Shigella* spp. isolated from Southern Taiwan [2].

The present study showed the poor discriminatory power of ribotyping for *S. sonnei* isolates. This finding is similar to an earlier report on strains from Taiwan [14]. This limits the usefulness of ribotyping in epidemiological investigations of *S. sonnei*. Nastasi *et al.* [19] observed that 52 isolates from four Italian outbreaks at four different geographically dispersed sites during the years 1975–1991 exhibited the same ribotype, although three other outbreaks could be differentiated into three different ribotypes. However, ribotyping has been used successfully for *S. sonnei* in Spain [23] and other countries [24, 25].

PFGE has been shown to be a valuable typing method for epidemiological investigation of several bacterial pathogens [13, 26, 27] and clonality studies [28]. In interpreting chromosomal DNA restriction patterns

produced by PFGE, Tenover *et al.* [29] have suggested that macro-restriction profiles of an isolate differing by more than six fragment positions can be considered to represent different strains. Thus, it is obvious that these three different well-documented outbreak strains should be considered as genetically distinct.

As plasmid profiling and PFGE are two different typing systems, and one cannot be replaced by another, they can complement each other in the subtyping of *S. sonnei*. Thus, strains that cannot be differentiated by either plasmid profiling or PFGE should be subtyped by a combination of these two means. In conclusion, PFGE and plasmid profiling were shown to be typing methods with a relatively high degree of discriminatory power that was superior to ribotyping. The diversity of PFGE patterns and plasmid profiles in this study suggests that these two techniques in combination are useful tools for epidemiological analysis of *S. sonnei* isolates.

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