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Molecular subtyping of *Shigella flexneri* 3a isolates by plasmid profile analysis and pulsed-field gel electrophoresis

Chein-Sheng Lin, Tien-Kwei Wang, Jin-Lai Tsai, Su-Ing Ho, Chih-Lung Lee, Cheng-Hsiung Lu

Bacteriology Division, Center for Disease Control, Department of Health, Taipei, Taiwan, ROC

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Twenty-two clinical isolates of *Shigella flexneri* 3a that were obtained from stool specimens from seven outbreaks of infection in northern Taiwan were subtyped and compared using plasmid profile analysis and pulsed-field gel electrophoresis (PFGE). Among 32 isolates, three distinct patterns were found by plasmid profile analysis and six by PFGE, with three different restriction enzymes, *Xba*I, *Sfi*I, or *Not*I. Seven PFGE patterns were observed in a comparative analysis using a combination of these three enzymes. Isolates collected from the same outbreaks had the same PFGE combination patterns and were distinguishable from those obtained during other outbreaks. These findings suggest that PFGE may be the most powerful method of genotyping *S. flexneri* 3a in epidemiological studies.

Key words: Plasmid profile, pulsed-field gel electrophoresis (PFGE), *Shigella flexneri* 3a

Shigellosis is a global gastrointestinal illness. There are four species or serogroups in the genus *Shigella*: *Shigella dysenteriae* (serogroup A), *Shigella flexneri* (serogroup B), *Shigella boydii* (serogroup C), and *Shigella sonnei* (serogroup D). On the basis of the presence of O-antigen, serogroups A, B, and C can be further divided into 45 serotypes. However, only a single serotype is found in serogroup D, *S. sonnei*. Shigellosis caused by serogroups A, B, and C is very common in developing countries, whereas serogroup D is an important pathogen of shigellosis in developed countries [1-2].

S. flexneri 2a and *S. sonnei* have been reported to account for 95% cases of shigellosis in Taiwan [3-6]. Before 1995, infections caused by *S. flexneri* 3a had only been sporadically reported in Taiwan. During the period from 1996 through 1997, however, seven outbreaks of infection that were associated with *S. flexneri* 3a were sequentially confirmed in northern Taiwan. Subtyping beyond the serotyping level is needed for epidemiological investigation of these isolates.

Many classic methods for typing microorganisms have been used, such as finding the drug resistance pattern, phage typing, biotyping, and colicin typing [6]. But these typing methods all have some disadvantages or limitations in subtyping [6]. In contrast, molecular typing methods such as plasmid profile analysis (PPA), pulsed-field gel electrophoresis (PFGE), ribotyping, and polymerase chain reaction, have been applied with success to the study of many microorganisms [6-13]. PFGE is considered to have the greatest discriminatory power in bacterial subtyping [6,10]. Although its discriminatory power of PPA is low, the technique is very simple and can be performed rapidly to screen specimens during outbreak in-vestigations. For this reason, PPA and PFGE were used in this study to characterize the clinical isolates of *S. flexneri* 3a .

Corresponding author: Dr. Chih-Lung Lee, Bacteriology Division, Center for Disease Control, Department of Health, Nan-Kang, Taipei 115, Taiwan, ROC.

Materials and Methods

Bacterial strains

A total of 22 isolates of *S. flexneri* 3a were collected by the Center for Disease Control, Taiwan, from seven outbreaks of *Shigella* infection with diarrheal syndrome in northern Taiwan during 1996 through 1997. Methods of cultivation, isolation, and identification of these isolates were described previously [14]. Briefly, *Salmonella-Shigella* agar (Difco Laboratories, Detroit, MI, USA) was used for primary isolation. Suspected colonies were tested for glucose utilization by triple sugar iron agar (Difco) and lysine decarboxylation by lysine iron agar (Difco). After these tests, biochemical identifications were carried out with the API 20E system (BioMerieux, Marcy-L'Etiole, France). Finally, serological grouping against O-antigens of *S. flexneri*

3a was performed according to the manufacturers' instructions.

Plasmid profile analysis

Lysates of *S. flexneri* 3a isolates were prepared according to the methods described by Birnboim and Doly [15]. Plasmid DNA was detected by gel electrophoresis using 0.8% agarose. The gels were stained with ethidium bromide (0.5 μg/mL) for 15 min and were destained with distilled water for 1 h. DNA bands were visualized and photographed under ultraviolet (UV) light. Plasmid size was determined by using a linear DNA ladder marker (Boehringer Mannheim, Mannheim, Germany).

Pulsed-field gel electrophoresis

Methods for PFGE were as described by Maslow *et al* [16] with slight modifications. Isolated colonies of *S. flexneri* 3a were inoculated into 3 mL tryptic soy broth (Difco) with shaking for 5 h at 37°C. Bacterial cells were harvested by centrifugation and were adjusted to an optical density of 1.2 at 610 nm. A portion of the bacterial suspension was then mixed with an equal volume of 1% low–melting-point agarose (Bio-Rad Laboratories, Hercules, CA, USA). The mixture was dispensed into a plug mold (Bio-Rad) and allowed to solidify. For bacterial lysis, the resulting plugs were then placed in a mixture solution with 50 mM Tris-buffer (pH 8), 50 mM EDTA (pH 8), 1% sodium lauryl sarcosine, and 1 mg per mL of proteinase K. They were incubated overnight at 53°C under gentle shaking. The plugs were then washed twice with TE buffer (10 mM Tris-buffer, pH 8, 10 mM EDTA) containing 1 mM phenylethyl-sulfonyl fluoride (Sigma, St. Louis, MO, USA) and were washed twice with TE buffer for 1 h at 4°C. A slice of each plug (2.5 mm) was cut and incubated for 4 h with 20 units of the following restriction enzymes: *Xba*I, *Not*I, and *Sfi*I. The manufacturer's recommendations (Boehringer Mannheim) for the specific buffers and reaction conditions were followed. The slices were then loaded into the wells of a 1.2% pulsed-field certified agarose (Bio-Rad) plate in 0.5x TBE buffer. Electrophoresis was performed with a contour-clamped homogeneous electric field apparatus (CHEF-DRIII, Bio-Rad) at 14°C with 200 V. The pulse times for *Xba*I digestions were ramped from 7 to 12 sec during the first 10 h and from 20 to 65 sec for the following 12 h. For *Sfi*I digestion, the pulse times were ramped from 5 to 33.5 sec for 22.6 h. For *Not*I digestions, the pulse times were ramped from 5 to 50 sec for 21.5 h. A lambda DNA ladder (Boehringer Mannheim) was used as the

Table 1. Epidemiological and microbiological characteristics of 22 isolates of *S. flexneri* 3a from seven outbreaks of *Shigella* infection

Isolate no.	PFGE pattern				Plasmid profile	Event no.	Source	Date of isolation (mo.day.yr)
	*Xba*I	*Sfi*I	*Not*I	Combination				
S1	A	A	A	1	I	1	Hsin-chu	01.03.97
S2	A	A	A	1	I	1	Hsin-chu	01.03.97
S3	A	A	A	1	I	1	Hsin-chu	01.03.97
S4	A	A	A	1	I	1	Hsin-chu	01.03.97
S5	A	A	A	1	I	1	Hsin-chu	01.03.97
S6	A	A	A	1	I	1	Hsin-chu	01.03.97
S7	A	A	A	1	I	1	Hsin-chu	12.31.96
S8	B	B	A	2	I	2	Taoyuan	04.25.96
S9	B	B	A	2	I	2	Taoyuan	04.25.96
S10	B	B	A	2	I	2	Taoyuan	04.25.96
S11	B	B	A	2	I	2	Taoyuan	04.25.96
S12	B	B	A	2	I	2	Taoyuan	04.25.96
S13	B	B	A	2	I	2	Taoyuan	04.25.96
S14	B	B	A	2	I	2	Taoyuan	04.25.96
S15	B	B	A	2	I	2	Taoyuan	04.25.96
S16	C	C	B	3	I	3	Hsin-chu	07.19.96
S17	C	C	B	3	I	3	Hsin-chu	06.29.96
S18	D	D	C	4	II	4	I-lan	01.06.97
S19	D	D	C	4	II	4	I-lan	01.21.97
S20	E	E	D	5	III	5	Taoyuan	01.23.97
S21	F	F	E	6	I	6	Hsin-chu	07.09.96
S22	A	A	F	7	I	7	Hsin-chu	01.03.97

Note: All *S. flexneri* 3a clinical isolates were obtained from stool samples.

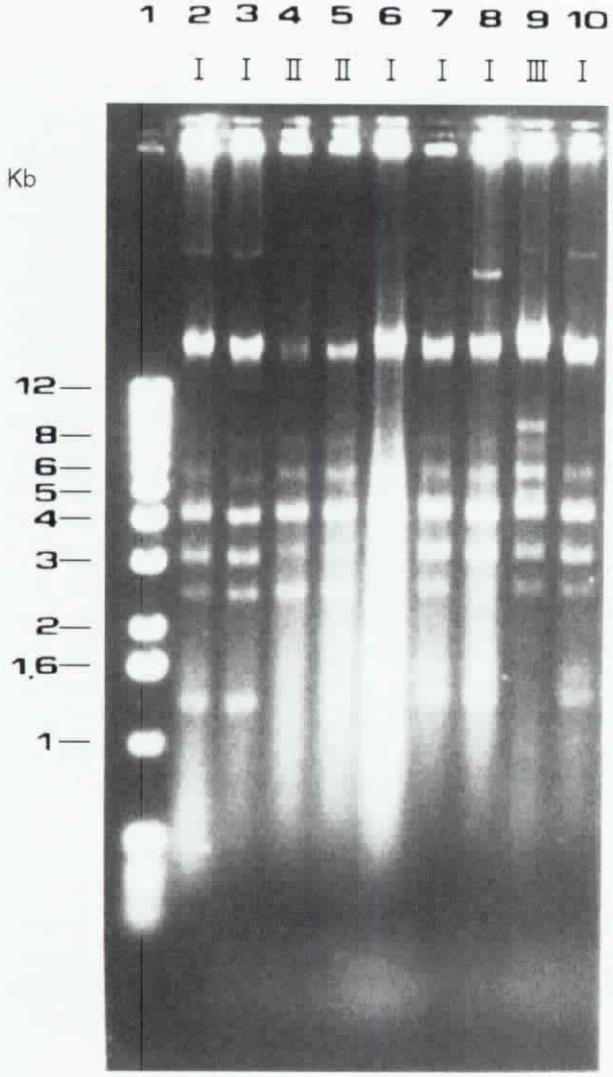

Fig. 1. Plasmid profiles of *Shigella flexneri* 3a isolates from northern Taiwan. Lanes: 2, isolate S1; 3, isolate S8; 4, isolate S18; 5, isolate S19; 6, isolate S16; 7, isolate S17; 8, isolate S21; 9, isolate S20; 10, isolate S22. Lane 1: linear DNA marker, starting from bottom at 1 kb. Track Roman letters correspond to the different plasmid profiles listed in Table 1.

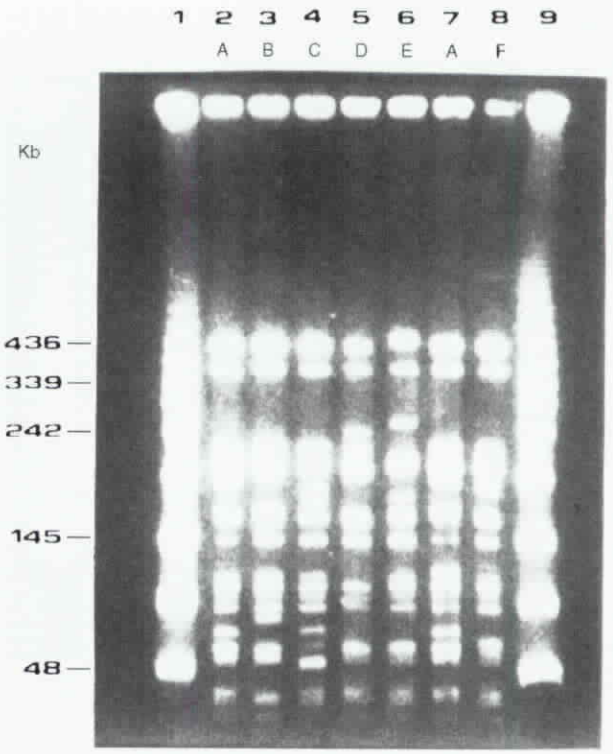

Fig. 2. PFGE patterns of *Shigella flexneri* 3a isolates with *Xba*I digestions. Lanes: 2, isolate S1; 3, isolate S8; 4, isolate S16; 5, isolate S18; 6, isolate S20; 7, isolate S22; 8, isolate S21. Lanes 1 and 9: lamda DNA marker, starting from bottom at 48 kb. Track letters correspond to the different PFGE patterns listed in Table 1.

molecular size marker. The gels were stained with ethidium bromide (0.5 μg/mL) for 30 min and were destained with distilled water for 3 h. DNA bands were visualized and photographed under UV light.

Similarity among isolates

DNA fragment patterns were visually assessed and were referred to as restriction endonuclease analysis (REA) patterns. Isolates were considered to be genetically identical if there was complete concordance of DNA fragment profiles, and were considered different if there was a difference of one or more DNA bands. REA patterns generated by PFGE for various isolates were compared, and the similarity of fragment length patterns between two isolates was scored using the Dice coefficient or coefficient of similarity [17-20]. The Dice coefficient, F, expresses the proportion of shared DNA fragments in two isolates and is calculated using the formula $F = 2nxy/(nx + ny)$, where nx is the total number of DNA fragments from isolate X, ny is the total number of fragments from isolate Y, and nxy is the number of fragments identical in the two isolates. An F value of 1 indicates that the two isolates have identical REA patterns, and a value of 0 suggests complete dissimilarity.

Results

Plasmid profile analysis

The 22 clinical isolates, which contained five to six small plasmids, that ranged from 1.2 to 10 kb in molecular weight, can be classified into three distinct plasmid profiles, type I, II, and III (Table 1). Isolates obtained from the same outbreak had identical plasmid

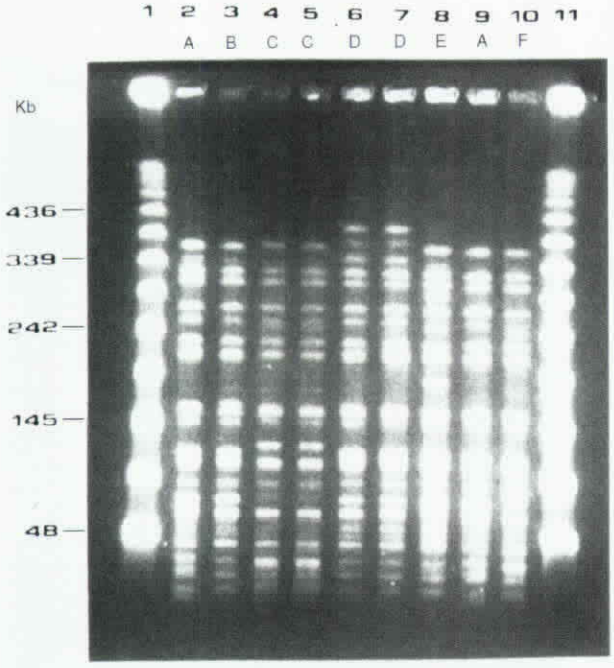

Fig. 3. PFGE patterns of *Shigella flexneri* 3a isolates with *Sfi*I digestions. Lanes: 2, isolate S1; 3, isolate S8; 4, isolate S16; 5, isolate S17; 6, isolate S18; 7, isolate S19; 8, isolate S20; 9, isolate S22; 10, isolate S21. Lanes 1 and 11: lamda DNA marker, starting from bottom at 48 kb. Track letters correspond to the different PFGE patterns listed in Table 1.

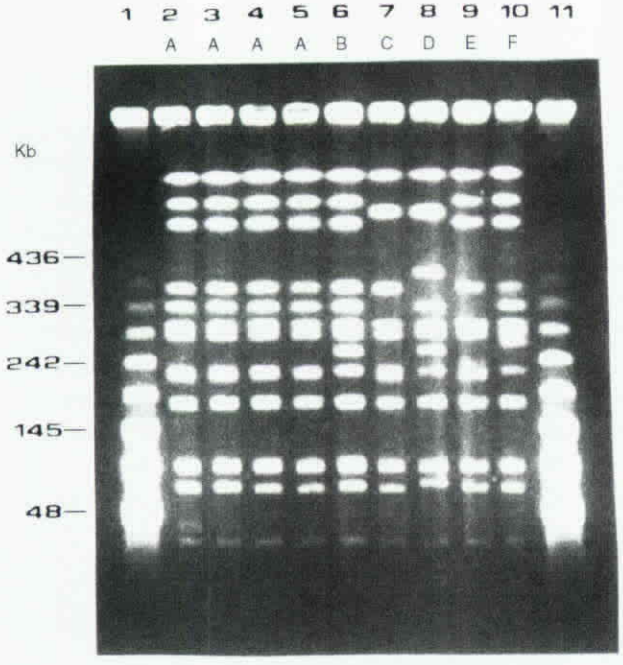

Fig. 4. PFGE patterns of *Shigella flexneri* 3a isolates with *Not*I digestions. Lanes: 2, isolate S1; 3, isolate S8; 4, isolate S16; 5, isolate S17; 6, isolate S18; 7, isolate S19; 8, isolate S20; 9, isolate S22; 10, isolate S21. Lanes 1 and 11: lamda DNA marker, starting from bottom at 48 kb. Track letters correspond to the different PFGE patterns listed in Table 1.

profiles, but identical plasmid profiles were also found in isolates from unrelated events. Specifically, isolates obtained from events No. 1, 2, 3, 6, and 7 all had the type I plasmid profiles. Isolates obtained from events No. 4 and 5 were classified as type II and type III, respectively (Fig. 1).

Pulsed-field gel electrophoresis

Four restriction endonucleases, *Xba*I, *Sfi*I, *Not*I, and *Spe*I, were used to determine which of them would give a suitable number of well-separated restricted fragments. Digestion with *Xba*I, *Sfi*I, and *Not*I all provided an optimum number of fragments greater than 50 kb with a clear PFGE profile. Digestion with *Spe*I produced many small fragments of less then 50 kb, which were difficult to separate by PFGE (data not shown). PFGE analysis of the digested DNA yielded 12 to 23 DNA fragments, ranging from 32 to 727 kb in size. The three enzymes, *Xba*I, *Sfi*I, and *Not*I, all produced six different PFGE patterns. With *Xba*I digestion, six distinct PFGE patterns were found with 17 to 19 fragments, ranging from 32 to 436 kb (Fig. 2). With *Sfi*I digestion, six PFGE patterns were found with 20 to 23 fragments, ranging from 32 to 436 kb (Fig. 3). With *Not*I digestion, six REA patterns were found with 12 to 14 fragments, ranging from 32 to 727 kb (Fig. 4). However, a total of seven distinct PFGE combination patterns were produced among all 22 isolates using comparative analyses using three different enzymes [21]. Isolates collected from the same event had the same PFGE combination patterns and were distinguishable from those obtained during other events.

Similarity analysis

It was found that the coefficient of similarity ranged from 0.74 to 0.95 with *Xba*I digestion, from 0.75 to 0.88 with *Sfi*I digestion, and from 0.72 to 0.96 with *Not*I. The coefficient of similarity ranged from 0.72 to 0.96 among these isolates. The REA patterns of these isolates were very similar.

Discussion

Several molecular typing methods have been applied to sporadic and epidemic isolates of *S. flexneri* [7,13, 20]. We found encouraging results in our previous molecular epidemiologic studies of *Shigella* species (B or D species) with PPA and PFGE [13,22]. *Shigella* species usually harbor a heterogenous population of plasmids [6,8,13]. Large plasmids (> 15 kb) are usually unstable and cannot be used for PPA. Only small plasmids, which are below the band of chromosomal DNA on the gel, are suitable for PPA. In this study,

PPA produced three distinct patterns from seven different events. However, this technique could not completely distinguish the epidemiologically related strains from unrelated strains isolated from different events. Thus, the use of the PPA method for studies involving *S. flexneri* 3a can provide only limited information about molecular epidemiology. In recent years, PFGE has been used successfully for the differentiation of *Shigella* isolates within the same serovar [8,13]. Previous studies found that using a specific restriction enzyme provided only limited value for strain differentiation. However, by comparative use of several different enzymes, the discriminatory value of PFGE could be enhanced [11,21]. In this study, although event No. 1 and 7 (Table 1) exhibited the same PFGE pattern on digestion with *Xba*I or *Sfi*I, a difference between their PFGE patterns was achieved using the enzyme *Not*I. In contrast, event No. 1 and 2 could be differentiated with *Xba*I or *Sfi*I, but not with *Not*I. By using a combination of three enzymes, seven comparative patterns were produced from seven different events. Similarity analysis among these *S. flexneri* 3a isolates found that the coefficient of similarity ranged from 0.72 to 0.96. A previous report suggested that isolates with an *F* value over 0.8 could be considered to be derived from a common source [21]. Therefore, some of the strains included in this study, which were isolated within a close geographic areas and during the same period of time, may have originated from a common ancestor.

Outbreaks caused by *Shigella* infection are difficult to prevent due to the low infectious dosage (10-100 microorganisms). Person-to-person transmission readily occurs in day-care centers from chronic carriers [23, 24]. Accumulation of PFGE profiles will be useful in the follow-up of the sources of *Shigella* within a population. Further studies analyzing *S. flexneri* 3a isolates are needed to build up a database of PFGE patterns in Taiwan.

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