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Analysis of Clonal Relationships among Isolates of *Shigella sonnei* by Different Molecular Typing Methods

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***Shigella sonnei* is a major cause of diarrheal disease in developed as well as in developing countries. Epidemiologic studies of this organism have been limited by the lack of a simple and effective method for comparing strains. In this study, we have compared different molecular typing methods, i.e., plasmid profile analysis, restriction endonuclease analysis of plasmids, rRNA gene restriction analysis (ribotyping), pulsed-field gel electrophoresis (PFGE), and enterobacterial repetitive intergenic consensus (ERIC) sequence-based PCR (ERIC-PCR) for typing 20 clinical isolates of *S. sonnei* collected from six incidents of infection. PFGE and ERIC-PCR fingerprintings had the highest discriminatory power for discrimination of epidemiologically related isolates from epidemiologically unrelated strains of *S. sonnei*, and both gave seven distinct strain types among these isolates and the type strain of the species. Plasmid study and ribotyping produced only six and two distinct patterns, respectively, among these strains. All of these molecular typing techniques demonstrated an identical fingerprint for eight temporally related sporadic isolates. It is possible that these temporally related isolates belonged to a single bacterial clone and circulated obscurely through the community. Our results indicate that the ERIC-PCR technique represents a rapid and simple means for typing *S. sonnei* with a level of discrimination equivalent to that of PFGE but greater than those of plasmid profile analysis, restriction endonuclease analysis of plasmids, and ribotyping.**

Shigella sonnei has emerged as a major cause of diarrheal disease in developed as well as in developing countries. It accounts for about 60 to 80% of all cases of shigellosis currently reported in the United States (2). The study of epidemiologic markers is important in an attempt to trace the source of infection. Unlike the other *Shigella* spp., *S. sonnei* contains only one serovar, and this has hindered the development of a serologic typing schema. As a result, other typing procedures, such as colicin typing, phage typing, biotyping, and drug resistance pattern, have been used (15, 18, 24, 26). However, all of these typing systems, which are based on the phenotypic properties of the microorganism, have some disadvantages or limitations.

Recently, approaches at the molecular level have been used to assess the relatedness of bacterial isolates. Plasmid pattern analysis was found to be useful for the characterization of epidemic strains harboring plasmids (8, 9, 19, 21). Its discriminatory power is further increased by restriction endonuclease digestion (20, 26). More recently, sensitive and reproducible molecular markers, including those used in ribotyping (5, 11) and pulsed-field gel electrophoresis (PFGE) (1, 16), have been applied with success to *S. sonnei* and other microorganisms. Despite the broad applicability of these techniques, their use in clinical microbiology laboratories has been limited because they are time-consuming and labor intensive. To circumvent these problems, a novel DNA fingerprinting strategy based on the PCR amplification of variable-length chromosomal sequences with a variety of primers was developed. One of these approaches, known as the random-amplified polymorphic DNA assay, is based on the use of simple arbitrary primers in a PCR of low stringency to amplify segments of the genome and has been used successfully for the typing of several bacterial species (25). This method has the advantage that no prior sequence information is required, but the fingerprint patterns have a critical dependence on reaction conditions and substrate concentration (3). On the other hand, Versalovic et al. (23) used consensus primers in the PCR to amplify DNA sequences located between successive repetitive elements, such as the 126-bp enterobacterial repetitive intergenic consensus (ERIC) sequence, in gram-negative bacteria, and they suggested that this method may have the potential for subtyping gram-negative enteric bacteria. We have applied this technique successfully for typing strains of *Serratia marcescens* isolated in nosocomial infections (10).

In the present study, we compared the results of plasmid content analysis, restriction endonuclease digestion of plasmids, ribotyping, PFGE, and ERIC sequence-based PCR (ERIC-PCR) as applied in studies of the molecular epidemiology of *S. sonnei* isolated in Taiwan.

MATERIALS AND METHODS

Bacterial isolates. A total of 20 clinical isolates of *S. sonnei* from six different incidents of infection were examined in the study (Table 1). Incident 1 comprised five isolates (S1 to S5) collected during an outbreak that occurred in a primary school in Taichung City, Taiwan, in September 1993. Incident 2 comprised three isolates (S6 to S8) recovered from one household outbreak in Taichung City in February 1994. Two epidemiologically related isolates (S9 and S10) were collected during incident 3, which occurred in Hsinchu City, Taiwan, in November 1994. Incident 4 comprised eight isolates (S11 to S18) collected in Taipei City, Taiwan, during 1987 over a 3-month period (21 September to 19 December) when an unusually high frequency of isolations was noted. Two sporadic strains (S19 and S20) were isolated from Taichung City in March 1994 (incident 5) and December 1991 (incident 6), respectively. The type strain of the species, NCTC 9774, was also included in this study for comparison. All isolates were identified as *S. sonnei* by the analytical profile index procedure 20E system (API-Bio-Merieux, La Balme les Grottes, France) and serotyping.

Plasmid profile analysis. Lysates of *S. sonnei* isolates were prepared by the simplified alkaline lysis method of Kado and Liu (6). Plasmid DNA was detected by electrophoresis in 0.7% horizontal agarose gels containing 0.5 μg of ethidium bromide per ml and photographed with UV light illumination. Plasmid size was

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

Source and isolate[d]	Date of isolation (mo/day/yr)	Plasmid profile	REAP pattern[a]	rDNA RFLP[b] pattern with:			Macrorestriction genotype[c]	ERIC-PCR fingerprint
				*Eco*RI	*Sal*I	*Hinc*II		
Type strain								
NCTC 9774		A	A	a	a	a	1	I
Incident 1 (Taichung City)								
S1	9/13/93	B	B	a	b	a	2	II
S2	9/20/93	B	B	a	b	a	2	II
S3	9/21/93	B	B	a	b	a	2	II
S4	9/24/93	B	B	a	b	a	2	II
S5	9/15/93	B	B	a	b	a	2	II
Incident 2 (Taichung City)								
S6	2/28/94	C	C	a	b	a	3	III
S7	2/28/94	C	C	a	b	a	3	III
S8	2/28/94	C	C	a	b	a	3	III
Incident 3 (Hsinchu City)								
S9	11/9/94	D	D	a	b	a	4	IV
S10	11/9/94	D	D	a	b	a	4	IV
Incident 4 (Taipei City)								
S11	9/21/87	E	E	a	b	a	5	V
S12	10/9/87	E	E	a	b	a	5	V
S13	11/4/87	E	E	a	b	a	5	V
S14	11/17/87	E	E	a	b	a	5	V
S15	11/17/87	E	E	a	b	a	5	V
S16	11/25/87	E	E	a	b	a	5	V
S17	11/26/87	E	E	a	b	a	5	V
S18	12/19/87	E	E	a	b	a	5	V
Incident 5 (Taichung City)								
S19	3/1/94	F	F	a	a	a	6	VI
Incident 6 (Taichung City)								
S20	12/1/91	A	A	a	a	a	7	VII

[a] Determined by digestion with *Eco*RI.
[b] RFLP, restriction fragment length polymorphism.
[c] Determined by PFGE after digestion by *Xba*I.
[d] Each isolate was from the patient with the corresponding number.

determined with a supercoiled DNA ladder (GIBCO-BRL Life Technologies, Gaithersburg, Md.). Only small plasmids, which appeared as bright bands below the band of chromosomal DNA on the gel, were used in the analysis. Large plasmids were not further investigated because of their instability (9). Faint bands seen below the chromosomal DNA were interpreted as relaxed forms of the brighter bands.

Restriction endonuclease analysis of plasmids (REAP). The restriction endonuclease *Eco*RI was used for digestion of the isolated plasmids. The restriction enzyme was obtained from GIBCO-BRL Laboratories, and digestion was performed according to the manufacturer's instructions.

Ribotyping analysis. Total cellular DNA was extracted by guanidium thiocyanate as previously described (13). The optical densities at 260 and 280 nm were used to estimate the DNA concentration and purity. DNA (5 μg) was digested with 20 U of the restriction enzymes with the buffers and reaction conditions recommended by the manufacturer (GIBCO-BRL Laboratories). Restriction enzymes *Eco*RI, *Sal*I, and *Hinc*II were used, according to the suggestions of Hinojosa-Ahumada et al. (5) and Nastasi et al. (11). The digested DNA was subjected to horizontal electrophoresis in 0.8% agarose gels in Tris-borate-EDTA buffer (pH 8.3) (TBE buffer) for 16 h at a constant 25 V. Southern blotting to a nylon membrane (Biodyne A; Pall Corp., East Hill, N.Y.) was performed with a vacuum pump unit (Hoeffer Scientific Instruments, San Francisco, Calif.), and the DNA fragments were fixed to the membrane by exposure to UV light for 2 min. A biotin-labelled cDNA probe was made by reverse transcription of 16S plus 23S rRNA from *Escherichia coli* (Boehringer Mannheim Biochemicals, Mannheim, Germany) as described previously (12). The membrane filter was soaked in prehybridization solution for 4 h at 42°C and then in hybridization solution, containing 100 μl of the biotinylated probe, for 16 h at the same temperature. Hybridization bands on the Southern blot membrane which contained digested *Shigella* DNA were detected with the streptavidin-alkaline phosphatase system with the BluGene kit (GIBCO-BRL Laboratories), following the procedures of the manufacturer. Ribosomal banding patterns were confirmed by repeated runs. Ribotypes were considered to be identical if they exhibited similar numbers and matching positions of bands.

PFGE of total genome DNA. Genomic DNA was prepared as described previously (1, 16) but with some modifications. The bacterial suspension, prepared by scratching bacterial colonies directly from an overnight-incubated culture on blood agar, was adjusted to a concentration of 10^9 CFU/ml in SE buffer (75 mM NaCl and 25 mM EDTA, pH 7.5) with a VITEK colorimeter (Hach Company, Loveland, Colo.). A portion of this bacterial suspension was then mixed with an equal volume of 2% low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.), dispensed in a plug mold (Bio-Rad Laboratories), and allowed to solidify. For lysis, the resulting plugs were then placed in a mixture of 10 mM Tris-HCl (pH 7.6), 100 mM EDTA, 1 mM NaCl, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, and 0.5 mg of lysozyme per ml. Following overnight incubation at 37°C, the plugs were transferred into a solution which contained 1% sodium lauryl sarcosine, 0.5 M EDTA (pH 9.5), and 500 μg of proteinase K per ml and incubated for 2 days at 56°C under gentle shaking. The plugs were washed once for 1 h at room temperature in TE buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA), once for 1 h at 37°C in TE buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.), and twice for 1 h at 4°C in TE buffer. A slice of each plug (2.5 mm) was cut and incubated overnight with 20 U of *Xba*I (GIBCO-BRL Laboratories) with the buffers and the reaction conditions recommended by the manufacturer. The slices were then loaded into the wells of a 1.2% SeaKem GTG agarose plate (FMC Bioproducts, Rockland, Maine) in 0.5× TBE buffer. Electrophoresis was done in a Bio-Rad contour-clamped homogeneous electric field (CHEF-DRII) apparatus for 24 h at 14°C with an electric field of 6 V/cm, and the pulse time was increased from 5 to 35 s. A lambda ladder (Bio-Rad Laboratories) was used as the molecular size marker. Gels were stained with ethidium bromide (0.5 μg/ml) for 30 min and destained in distilled water for 3 h. DNA bands were visualized under UV light and photographed. More than three band differences in the PFGE profile must be present for two strains to be considered different. This definition is based on the possibility that minor differences in the restriction patterns may occur secondary to a single base pair mutation in the chromosomal DNA (7).

ERIC-PCR analysis. ERIC-PCR was performed as previously described (10). Three colonies from a fresh 18-h culture on nutrient agar were harvested into 50 μl of sterile distilled water, boiled for 15 min, and then centrifuged for 5 min at 13,000 × *g*. The supernatant fluid (1 μl) was used as target DNA and added to a reaction mixture (final volume, 100 μl) containing 1 U of *Taq* polymerase (Super Taq; HT Biotechnology Ltd., Cambridge, England), 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 250 μM (each) deoxynucleoside triphosphates, and a 1 μM concentration of a single primer. The primer used was ERIC1 (5′-GTGAATCCCCAGGAGCTTACAT-3′). Amplification was

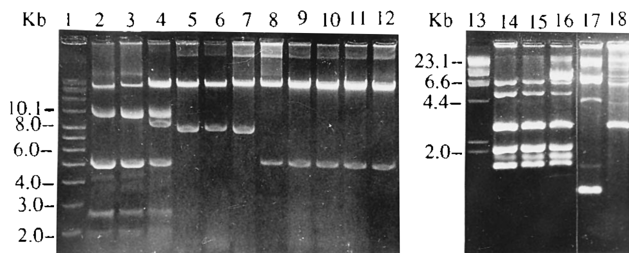

FIG. 1. Agarose gel electrophoresis of total plasmids and their *Eco*RI digests from *S. sonnei* isolates. Lane 1, supercoiled DNA size standard (GIBCO-BRL); lane 2, plasmid content of type strain NCTC 9774; lane 3, plasmid content of isolate S20; lane 4, plasmid content of isolate S19; lanes 5 to 7, plasmid contents of epidemiologically related isolates S6 to S8, respectively; lanes 8 to 12, plasmid contents of epidemiologically related isolates S1 to S5, respectively; lane 13, *Hin*dIII restriction fragments of phage lambda; lanes 14 to 18, restriction fragment patterns of *Eco*RI-digested total plasmid DNAs from type strain NCTC 9774 and isolates S20, S19, S6, and S1, respectively.

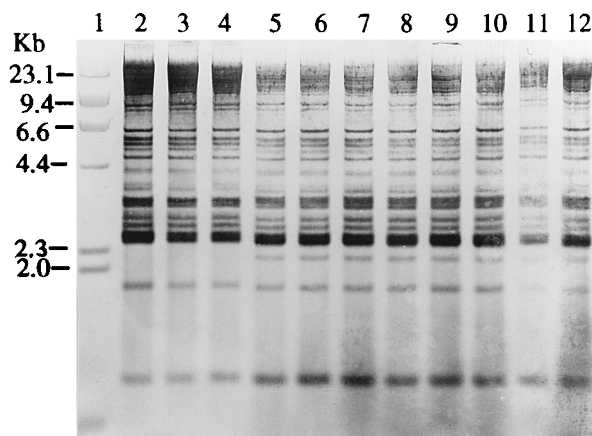

FIG. 2. Ribotyping of *S. sonnei* (*Sal*I digestion and Southern blotting). Lane 1, lambda DNA digested with *Hin*dIII; lane 2, DNA digest of type strain NCTC 9774; lane 3, DNA digest of isolate S20; lane 4, DNA digest of isolate S19; lanes 5 to 7, DNA digests of epidemiologically related isolates S6 to S8, respectively; lanes 8 to 12, DNA digests of epidemiologically related isolates S1 to S5, respectively.

performed in a PHC-3 thermal cycler (Techne, Princeton, N.J.), with temperature ramping as follows: 95°C for 5 min to denature the template; four low-stringency cycles of 94°C for 1 min, 26°C for 1 min, and 72°C for 2 min; 40 cycles of 94°C for 30 s, 40°C for 30 s, and 72°C for 1 min; and finally, 72°C for 10 min. Negative controls with no template DNA were included in each run. Amplified products (10 μl) were resolved by agarose gel electrophoresis in 1.6% agarose gels in TBE buffer containing ethidium bromide (1 μg/ml) at 30 V for 6 h and were visualized by UV transillumination. The PCR patterns were considered to be identical on the basis of similar numbers and matching positions of all major bands. Small differences in the intensities of faint bands were ignored.

RESULTS

The results with the different molecular typing techniques are summarized in Table 1.

Plasmid profile analysis and REAP. Some representative profiles and patterns from REAP are illustrated in Fig. 1. The clinical isolates and the type strain of the species exhibited six distinct profiles of one to three small plasmids ranging from 5.0 to 10.1 kb. The plasmid profiles were identical within each group of epidemiologically related isolates but were distinguishable from each other. Isolate S20 shared the same plasmid pattern with type strain NCTC 9774, and these two strains also could not be differentiated by REAP with *Eco*RI (Table 1; Fig. 1).

Ribotyping analysis. For the 20 clinical isolates and the type strain of the species, analysis of rRNA gene (rDNA) restriction fragment length polymorphisms with *Eco*RI or *Hinc*II gave the same single profile. With *Sal*I, two distinct rDNA patterns were observed (Fig. 2; Table 1).

PFGE of total genome DNA. Some representative profiles and patterns from PFGE are shown in Fig. 3. *Xba*I digestion produced about 20 fragments; their sizes ranged from 32.4 to 582 kb. Seven distinct macrorestriction patterns were generated among the 20 clinical isolates and the type strain of the species. The epidemiologically related isolates were found to have identical PFGE patterns, and the pattern was distinct for each incident of infection (Table 1).

ERIC-PCR analysis. The ERIC-PCR technique successfully typed all isolates examined and produced bands in the 300- to 2,000-bp size range (Fig. 4). Seven different patterns were observed among the 20 clinical isolates and the type strain of *S. sonnei* (Table 1). The pattern was distinct for each incident of infection.

DISCUSSION

Several molecular methods have been applied previously to study the epidemiology of *S. sonnei* isolates. Plasmid profile analysis is the method used widely for the investigation of outbreaks of shigellosis (8, 9, 19, 20, 21, 26). Previous reports have shown that *S. sonnei* usually harbors a heterogenous population of plasmids, which may range in number from 2 to as many as 10 (4). Tacket and Cohen (19) successfully used plasmid analysis to identify the epidemic strain which caused two sequential outbreaks in two neighboring counties in Florida. The plasmid profile of this outbreak strain was distinguished from the profiles of three recent sporadic isolates from the same place. In contrast, Prado et al. (14) performed plasmid profile analysis on isolates of *S. sonnei* from Mexico and from Houston, Tex., and found that the majority of strains had identical or almost identical plasmid profile patterns. They

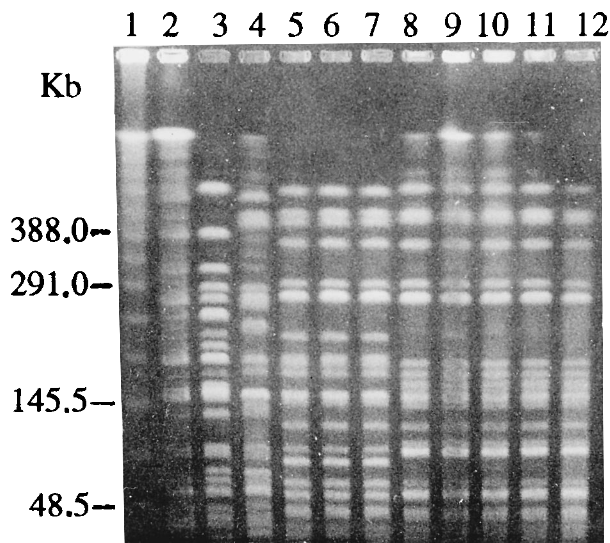

FIG. 3. PFGE of *Xba*I-digested genomic DNAs from *S. sonnei* isolates. Lane 1, lambda ladder (Bio-Rad) which served as a molecular size marker; lane 2, DNA digest of type strain NCTC 9774; lane 3, DNA digest of isolate S20; lane 4, DNA digest of isolate S19; lanes 5 to 7, DNA digests of epidemiologically related isolates S6 to S8, respectively; lanes 8 to 12, DNA digests of epidemiologically related isolates S1 to S5, respectively.

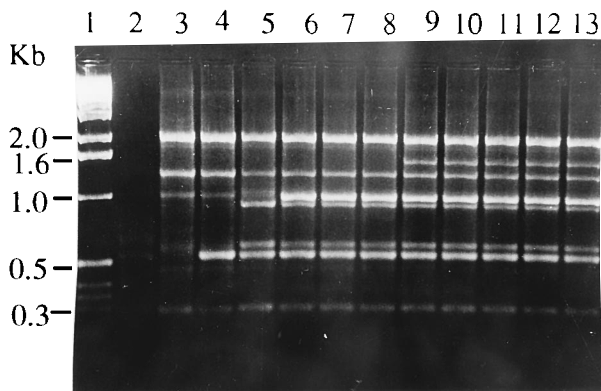

FIG. 4. ERIC-PCR products of *S. sonnei* analyzed by 1.6% agarose gel electrophoresis. The primer was ERIC1 (see text). Lane 1, 1-kb molecular size marker (GIBCO-BRL); lane 2, negative control; lane 3, product of type strain NCTC 9774; lane 4, product of isolate S20; lane 5, product of isolate S19; lanes 6 to 8, products of epidemiologically related isolates S6 to S8, respectively; lanes 9 to 13, products of epidemiologically related isolates S1 to S5, respectively.

stated that *S. sonnei* with the same plasmid pattern can be found within a wide geographic distribution, which will limit the usefulness of the plasmid fingerprinting technique for outbreak investigation. A similar result was also found by Yagupsky et al. (26). They suggested that REAP can distinguish more strains than the plasmid profile. In our study, all of the epidemiologically unrelated strains except isolate S20 and the type strain of the species were found to be different by both plasmid profile analysis and REAP. Both of these techniques are simple and easy to perform. However, they cannot be used for isolates without plasmids. Instability of profiles or patterns of digestion caused by the acquisition or loss of plasmids represents another disadvantage of these techniques.

Ribotyping has been applied for discriminating strains of *S. sonnei* (5, 11). Hinojosa-Ahumada et al. (5) identified six distinct rDNA patterns by the use of *Sal*I as the restriction endonuclease among 100 isolates from sporadic cases and 45 isolates from four different outbreaks of shigellosis which occurred in the United States. Nastasi et al. (11) have tried different enzymes for digestion of whole-cell DNA from *S. sonnei* isolates, and they found that rDNA restriction patterns generated by *Hin*cII provided the greatest strain differentiation: 13 ribotypes among 432 isolates were identified after digestion with this enzyme. However, 95% of the sporadic strains were assigned to only four different ribotypes. Our results showed the poor discriminatory power of ribotyping for *S. sonnei* isolates. By using *Eco*RI, *Sal*I, and *Hin*cII, we identified only two distinct ribotypes among 20 clinical isolates and the type strain of the species, and this technique could not discriminate the epidemiologically related isolates from the epidemiologically unrelated strains. The fact that ribotyping is both labor intensive and time-consuming further discourages its use in clinical laboratories.

PFGE has been used successfully to identify genetic subtypes among *S. sonnei* strains, and in one study, seven distinct subtypes among nine epidemiologically unrelated strains were identified by this technique (16). In addition, Brian et al. (1) used PFGE to differentiate between outbreak-associated and non-outbreak-associated isolates during an outbreak of shigellosis in a day-care center. In our present study, we showed the usefulness of this technique for discrimination of epidemiologically related isolates from epidemiologically unrelated strains. However, this technique is as labor intensive and time-consuming as ribotyping.

PCR-mediated genome fingerprinting based on ERIC has been applied successfully to the epidemiologic typing of methicillin-resistant *Staphylococcus aureus* (22), *Acinetobacter baumannii* (17), and *S. marcescens* (10). A similar approach was successfully used in the present study to characterize epidemiologically associated isolates of *S. sonnei*. To our knowledge, this is the first report of the application of the PCR technique for studying the epidemiology of *S. sonnei*. The discriminatory power of ERIC-PCR was good. It provided a result which agreed for all isolates examined to date with those given by the more established PFGE technique. The reproducibility of this technique is good if the PCR protocol and parameters described above are strictly followed each time. In comparison with ribotyping and PFGE, ERIC-PCR is relatively easy to perform and less time-consuming. The results can be obtained within 24 h. However, the intensities and the patterns of the bands can be affected if there is any variation in the parameters of PCR or conditions of gel running. Therefore, it is much better to have all of the samples processed at the same time and running on the same gel so that the banding patterns of different isolates can be compared confidently.

Incident 4 comprised eight isolates that were collected from Taipei City within a 3-month period. They were thought to be temporally related sporadic isolates, but the frequency of isolations was unusually high during this period. All of the isolates showed identical plasmid profiles, rDNA restriction fragment length polymorphism patterns, PFGE patterns, and ERIC-PCR fingerprints. Brian et al. (1) also found that plasmid analysis and PFGE sometimes could not differentiate common-source isolates from sporadic isolates in the same location during the same period of time. It is possible that these temporally related isolates belonged to a single bacterial clone and circulated obscurely through the community.

In conclusion, we found that PFGE and ERIC-PCR have the highest discriminatory power for differentiation of strains of *S. sonnei*. ERIC-PCR fingerprinting is particularly useful because of its simplicity and represents a less time-consuming procedure. It provides a degree of discrimination equivalent to that of PFGE but higher than those of plasmid profile analysis, REAP, and ribotyping. However, the validation of the discriminatory powers of REAP, ribotyping, PFGE, and ERIC-PCR would be best established by analysis of strains which cannot be differentiated by plasmid profiles, so future work on such a comparison is necessary.

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