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Differentiation of *Shigella flexneri* Strains by rRNA Gene Restriction Patterns

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We studied the restriction endonuclease cleavage patterns of rRNA genes (ribotypes) of 72 clinical isolates of *Shigella flexneri* representing eight serotypes to determine whether ribotyping could be used to distinguish *S. flexneri* strains and to compare the discriminating ability of the method with that of serotyping. By using a cloned *Escherichia coli* rRNA operon as the probe, Southern blot hybridization of restriction endonuclease-digested total DNA was carried out. Ribotyping of the isolates with each of the five restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sal*I generated reproducible restriction patterns. However, *Hin*dIII produced the optimum digestion pattern of the rRNA genes and was more useful than the other enzymes used in differentiating strains. Analysis of the 72 isolates showed 11 different *Hin*dIII cleavage patterns of their rRNA genes. Four of these *Hin*dIII-generated ribotypes could be further differentiated into two to four subribotypes by using *Pst*I. The results indicate that ribotyping has an application for differentiation of *S. flexneri* strains and can complement serotyping. Definition of strains in terms of both serotype and ribotype may be of greater use in epidemiological studies.

Shigellosis is an acute gastrointestinal illness that represents a major health problem in developing countries (9). In Bangladesh, *Shigella flexneri* is the most prevalent among the four *Shigella* species, with serotype 2 being predominant. Shigellae have been typed by biochemical profiles and by using serotype-specific antisera (3–6, 9). These typing methods rely on phenotypes that may not be stably expressed; the necessary reagents are not always reliable (7), and the sensitivity is not sufficient to differentiate between all strains of a species. Analysis of plasmid profiles and antimicrobial susceptibilities have also been used to differentiate strains of different bacterial species, including *Shigella* species (9, 12), but these techniques have limitations because they rely on the existence and expression of extrachromosomal genetic elements which can readily be lost. A recent typing method based on developments in DNA analysis techniques is the use of rRNA gene probes to study restriction fragment length polymorphisms of rRNA genes in different strains (10, 18). The probing of chromosomal DNA with an *Escherichia coli* rRNA probe provides a widely applicable system for investigating the molecular epidemiology of bacteria (18). The present study was designed to determine the applicability of a cloned *E. coli* rRNA operon as a probe for differentiating between strains of *S. flexneri*.

Shigella strains isolated from either stool specimens or rectal swabs of 2,915 patients included in the diarrhea surveillance system of the International Centre for Diarrhoeal Disease Research Bangladesh (ICDDR,B) between January and December 1984 were used. More elaborate descriptions of the surveillance system have been published previously (9, 17). The identities of all isolates were confirmed biochemically and serologically (6), and serotyping was done by the slide agglutination method with both polyclonal and monoclonal antibody reagents specific for all *S. flexneri* type- and group-factor antigens as described previously (3–5, 9). A single colony of each confirmed *Shigella* strain was grown in trypticase soy broth (GIBCO Ltd., Paisley, Scotland) with 0.3% Bacto Yeast Extract (TSBY; Difco Laboratories, Detroit, Mich.) containing 15% glycerol and was stored at −70°C until required for further analysis. Of a total of 166 *S. flexneri* strains isolated during the period, 72 *S. flexneri* isolates belonging to eight different serotypes were included in the present study (see Table 1).

Southern blot hybridization with the rRNA gene probe was performed on all 72 *S. flexneri* isolates by using the enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sal*I. High-molecular-weight chromosomal DNA was isolated from strains grown overnight in TSBY at 37°C with aeration, as described by Stull et al. (18). Approximately 5 μg of purified whole-cell DNA was digested with the appropriate restriction endonucleases as instructed by the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.) by using 5 U of enzyme per μg of DNA. The digested DNAs were electrophoresed in 0.8% agarose gels in Tris-borate-EDTA buffer for 15 h at 60 V by using an H4 horizontal gel electrophoresis apparatus (Bethesda Research Laboratories). A molecular mass marker, either a 1-kb ladder DNA or *Hin*dIII fragments of bacteriophage lambda DNA, was also run in an adjacent slot of each gel. DNA fragments from the gels were transferred onto nylon membranes (Hybond-N; Amersham International plc., Aylesbury, United Kingdom) by Southern blotting (16) by using a blot transfer apparatus (Bethesda Research Laboratories).

The probe DNA was a 7.5-kb *Bam*HI fragment of pKK3535 (2), which is a pBR322-derived plasmid containing an *E. coli* rRNA operon consisting of one copy each of the genes coding for 5S rRNA, 16S rRNA, 23S rRNA, and $tRNA^{Glu}$. The recombinant plasmid pKK3535 was prepared and digested with restriction enzymes, and the insert was separated by electroelution from agarose gels as described by Maniatis et al. (13). The probe DNA was labeled by random priming (8) by using [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) and a random primer DNA labeling system

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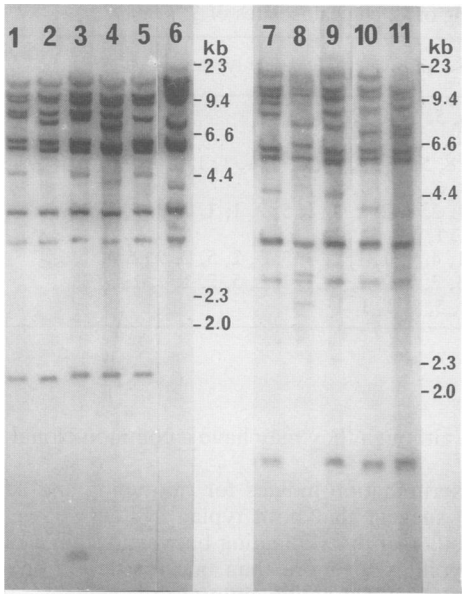

FIG. 1. Southern hybridization analysis of genomic DNA from *S. flexneri* digested with *Hin*dIII and probed with a 7.5-kb *Bam*HI fragment of the *E. coli* rRNA clone pKK3535. Lanes 1 through 11, the patterns corresponding to 11 different ribotypes of *S. flexneri*. Numbers on the right indicate the molecular sizes of the bands and correspond to *Hin*dIII fragments of bacteriophage lambda DNA used as molecular size markers.

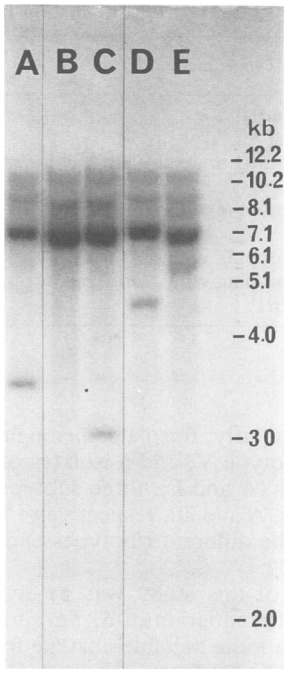

FIG. 2. Southern hybridization analysis of genomic DNA from *S. flexneri* digested with *Pst*I and probed with the rRNA gene probe derived from pKK3535. Lanes A through E, the patterns corresponding to five different *Pst*I-based ribotypes of *S. flexneri* identified in 72 clinical isolates. Numbers on the right indicate molecular size markers and correspond to 1-kb DNA ladder (Bethesda Research Laboratories).

(Bethesda Research Laboratories). Southern blots were hybridized and washed under stringent conditions as described by Stull et al. (18). Autoradiographs were developed from the hybridized filters, using Kodak X-Omat AR film (Kodak, Rochester, N.Y.), at −70°C for 24 h. The discriminatory abilities of ribotyping with different enzymes and that of serotyping were compared by calculating numerical discrimination indices as described by Hunter and Gaston (11).

Of the five different restriction enzymes used, *Hin*dIII showed the optimum digestion pattern of rRNA genes, producing 10 to 12 bands (Fig. 1). *Bam*HI produced only four high-molecular-mass bands ranging in size from 20 to 8.1 kb, whereas both *Eco*RI and *Sal*I produced bands of both high and very low molecular masses (i.e., 10 to 12 bands between 15 and less than 0.5 kb; data not shown). *Pst*I produced four high-molecular-mass bands (11.5 to 7.0 kb), in addition to one low-molecular-mass band, in most but not all strains (Fig. 2).

Eleven different reproducible rRNA gene restriction patterns (ribotypes I to XI) were obtained by ribotyping the 72 isolates with *Hin*dIII (Fig. 1). The sizes of the bands ranged from 19.3 to 1.8 kb in all ribotypes except ribotype III, which showed an additional band of less than 1.0 kb (Fig. 1); the size distribution was optimum for discrimination between strains. Of the 72 isolates, 26 (36.1%) belonged to ribotype I, 12 (16.6%) belonged to ribotype V, and 8 (11.1%) were of ribotype III. Ribotypes II and VI had six isolates (8.3%) each, five isolates (6.9%) belonged to each of ribotypes IV and VII, whereas ribotypes VIII through XI each had one isolate. *Pst*I produced a total of five ribotypes, ribotypes A through E (Fig. 2), and the *Pst*I ribotypes further differentiated the *Hin*dIII ribotypes into subtypes. The 26 ribotype I isolates fell into four different *Pst*I ribotypes (subtypes): 7 isolates in subtype A, 2 in subtype B, 6 in subtype C, and 11

TABLE 1. Correlation between ribotypes and serotypes of 72 clinical isolates of *S. flexneri*

Ribotype[a]	No. of *S. flexneri* isolates with the following serotype:											
	1a	1b	2a	2b	3a	3b	4a	5a	6	X	Y	Total
IA	3		2	2								7
IB						1		1				2
IC		1		3							2	6
ID			7				2			2		11
IIA	2									1	1	4
IIE		2										2
IIID	5	2			1							8
IVC			1	1				3				5
VA					3	4						7
VB						3						3
VD			2									2
VIA							4					4
VID										1	1	2
VIIC									5			5
VIIIA											1	1
IXA											1	1
XD										1		1
XIA											1	1
Total	10	5	12	6	4	8	6	4	5	5	7	72

[a] Ribotypes I through XI were determined by using the restriction enzyme *Hin*dIII, whereas ribotypes A through E were determined by using *Pst*I. Some isolates belonging to particular *Hin*dIII-based ribotypes could be further differentiated to more than one *Pst*I-based ribotype.

TABLE 2. Discrimination indices for ribotyping and serotyping of 72 clinical isolates of *S. flexneri*

Typing method	No. of types	No. of isolates in different types	Discrimination index
Ribotyping with *Hin*dIII	11	26, 6, 8, 5, 12, 6, 5, 1, 1, 1, 1	0.816
Ribotyping with *Pst*I	5	25, 5, 16, 24, 2	0.723
Ribotyping with *Hin*dIII and subtyping with *Pst*I	18	7, 2, 6, 11, 2, 4, 8, 5, 7, 3, 2, 4, 2, 5, 1, 1, 1, 1,	0.929
Serotyping alone	11	10, 5, 12, 6, 4, 8, 6, 4, 5, 5, 7	0.909
Serotyping and ribotyping with *Hin*dIII	30	3, 1, 9, 5, 1, 2, 1, 2, 2, 2, 2, 1, 1, 5, 2, 1, 1, 1, 3, 2, 3, 7, 4, 1, 1, 5, 1, 1, 1, 1	0.957
Serotyping and ribotyping with *Pst*I	23	5, 2, 2, 3, 4, 4, 1, 4, 4, 1, 1, 1, 4, 3, 5, 2, 5, 2, 9, 2, 4, 1, 2	0.955
Serotyping and ribotyping with *Hin*dIII and subtyping with *Pst*I	33	3, 2, 2, 1, 1, 1, 3, 2, 7, 2, 2, 2, 1, 1, 2, 5, 2, 1, 1, 1, 3, 3, 4, 3, 2, 4, 1, 1, 5, 1, 1, 1, 1	0.969

in subtype D. Similarly, 6 isolates belonging to ribotype II, 12 isolates of ribotype V, and 6 isolates of ribotype VI fell into two subtypes (A and E), three subtypes (A, B, and D), and two subtypes (A and D), respectively. Table 1 shows the distributions of the different ribotypes and subtypes among the serotypes.

The objective of this study was to evaluate the use of ribotyping for differentiation of *S. flexneri* strains. *Hin*dIII was found to be a more suitable enzyme for ribotyping than the other four restriction enzymes used. *Pst*I produced only five ribotypes, and the discriminating ability of ribotyping with *Pst*I was low compared with that with *Hin*dIII (Table 2). *Bam*HI digestion of rRNA genes produced a low number of high-molecular-mass fragments, and small variations in size among comparable fragments derived from different strains were difficult to differentiate (data not shown). Although both of the enzymes *Eco*RI and *Sal*I produced a higher number of fragments, some were too small in size (<0.5 kb) and ran off the gel if electrophoresis was continued long enough to achieve resolution of the larger fragments. Hence, ribotypes produced by *Bam*HI, *Eco*RI, or *Sal*I were difficult to interpret. This showed that the selection of optimum restriction enzymes was important for obtaining useful results by ribotyping. This also suggested that the degree of restriction site heterogeneity among rRNA genes in different strains may vary for different restriction enzymes. In a previous study with *Shigella sonnei*, the optimum enzyme was found to be *Sal*I (10), but in the present study, *S. flexneri* strains could not be effectively differentiated with *Sal*I.

Comparison of serotypes and ribotypes (Table 1) showed that different ribotypes belonged to the same serotype, and again, there were isolates that had the same ribotypes but that belonged to different serotypes (Table 1). This was probably because it is possible for different strains to have similarities in portions of their genomes that encode serotype-specific antigens but have differences in other portions of their genomes, e.g., highly conserved rRNA genes. Similar relationships between ribotypes and serotypes have previously been reported (15) for *Pasteurella multocida* isolated from turkeys dying from fowl cholera.

The occurrence of isolates with the same ribotypes but different serotypes can be explained by the fact that the rRNA genes of strains originating from the same ancestral clone were conserved, while genes for serotype-specific antigens have undergone changes, since phenotypic characteristics could be under the control of environmental influences (1, 14). These results thus show that all *S. flexneri* strains with similar serotype designations are not necessarily identical. Similarly, all strains of the same ribotype are not identical, although they may have a common clonal relationship.

The discrimination indices for ribotyping and serotyping (Table 2) suggest that both typing systems have a nearly similar ability of discriminating between strains; ribotyping and subtyping with more than one restriction enzyme can increase the discriminating ability of the system, and typing of *S. flexneri* strains on the basis of the combination of both ribotypes and serotypes has a higher discriminating ability (Table 2). The results thus suggest that since serotyping and ribotyping are different approaches to the typing of strains, one system cannot replace but can complement the other system, resulting in finer discrimination between strains that cannot be achieved by either serotyping or ribotyping alone (Table 2). Hence, for epidemiological purposes, *S. flexneri* strains may be defined simultaneously in terms of both serotypes and ribotypes.

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