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Restriction Fragment Length Polymorphisms in rRNA Operons for Subtyping *Shigella sonnei*

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***Shigella sonnei* is the most frequent cause of shigellosis in the United States. Epidemiologic studies of this organism have been hampered by the lack of adequate typing procedures. Ribosomal DNA analysis (ribotyping), a method which analyzes restriction fragment length polymorphisms in the chromosomal genes that encode rRNA, has recently been shown to be useful for microbial species identification and subtyping. To determine whether ribotyping could be used to distinguish between *S. sonnei* isolates, we conducted Southern hybridization studies on isolates from 16 different geographic locations and from four recent outbreaks. *S. sonnei* genomic DNA fragments generated following digestion with *SalI* hybridized with *Escherichia coli* 16S and 23S rRNAs to produce six distinct patterns; strains with patterns 1, 2, and 3 were each further subdivided into two additional patterns by using *PvuII*, *SmaI*, and *SstI*, respectively. Epidemiologically related strains had identical patterns. Ribotyping appears to be a useful tool for epidemiologic studies of shigellosis caused by *S. sonnei*.**

Shigella sonnei is the most frequent cause of shigellosis in the United States: it accounts for about 60% of all *Shigella* isolates reported to the Centers for Disease Control (7). The lack of antigenic variation among strains of *S. sonnei* has hindered the development of a serologic typing schema. As a result, other typing procedures, such as colicin typing (1, 18) and phage typing, have been used (22, 24). Since a significant number of strains do not produce colicins, colicin typing is limited in its ability to type or differentiate between strains. Phage typing methods successfully discriminate between strains; however, these methods are difficult to standardize, and problems with reproducibility and interpretation of results are not uncommon. Plasmid profile analysis and antimicrobial susceptibility patterns have also been used to discriminate among strains (15, 17, 28); however, a potential disadvantage of these techniques is that they rely, in general, on the detection of extrachromosomal genetic information that can readily be lost. Characterization of strains according to stably maintained traits, such as essential genes located on the chromosome, should offer an advantage over existing typing procedures. Since all organisms have highly conserved genes coding for rRNA, we investigated the use of ribosomal DNA (rDNA) analysis (ribotyping), a technique that allows comparison of restriction fragment length polymorphisms in the chromosomal genes coding for rRNA, as a method for distinguishing between strains of *S. sonnei*.

MATERIALS AND METHODS

Bacterial strains. Included in the study were 100 *S. sonnei* strains from sporadic cases of shigellosis in 16 states in the United States during 1985 and 1986 and 45 isolates from four different outbreaks of shigellosis. The outbreak strains consisted of 8 isolates from an outbreak in Texas between 29 June and 21 July 1986 associated with contaminated oysters (23), 5 isolates from an outbreak in Texas between 30 August and 7 October 1986 associated with contaminated shredded lettuce (8), 10 isolates from an outbreak in Michigan in August 1988, and 22 isolates from an outbreak in Minnesota in October 1988. The *S. sonnei* type strain, ATCC 29930, and *Escherichia coli* K-12 (University of Washington Culture Collection) were kindly provided by A. G. Steigerwalt, Centers for Disease Control, Atlanta, Ga.

DNA isolation, restriction enzyme digestion, and separation of restriction fragments. Total cellular DNA was isolated from overnight broth cultures of *Shigella* and *E. coli* by previously described procedures (9, 21). Purified DNA (2 μg) was digested with excess amounts of *Acc*I, *Bam*HI, *Bcl*I, *Dpn*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Mbo*I, *Pst*I, *Sal*I, *Sau*3AI, *Sma*I, or *Sst*I (New England BioLabs, Beverly, Mass.) for 4 h under the conditions recommended by the manufacturer. DNA fragments were separated electrophoretically through 1% (wt/vol) horizontal agarose gels as described previously (16). Lambda DNA (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and 1-kb ladder DNA (Bethesda Research Laboratories) were used on each gel as molecular mass markers.

Preparation of nucleic acid probes and Southern hybridization analysis. The 16S and 23S rRNAs from *E. coli* (Boehringer Mannheim, Indianapolis, Ind.) were purified by high-performance liquid chromatography to remove contaminating 5S rRNA as described previously (2) and were end-labeled by using polynucleotide kinase with [γ-^{32}P]ATP by standard procedures (2). Lambda DNA and the 1-kb ladder DNA were labeled by nick translation with [α-^{32}P]dCTP by using a commercially available kit (Bethesda Research Laboratories). The separated DNA fragments were transferred to nitrocellulose or nylon membranes (26) and were hybridized first with ^{32}P-labeled rRNA and then with a solution

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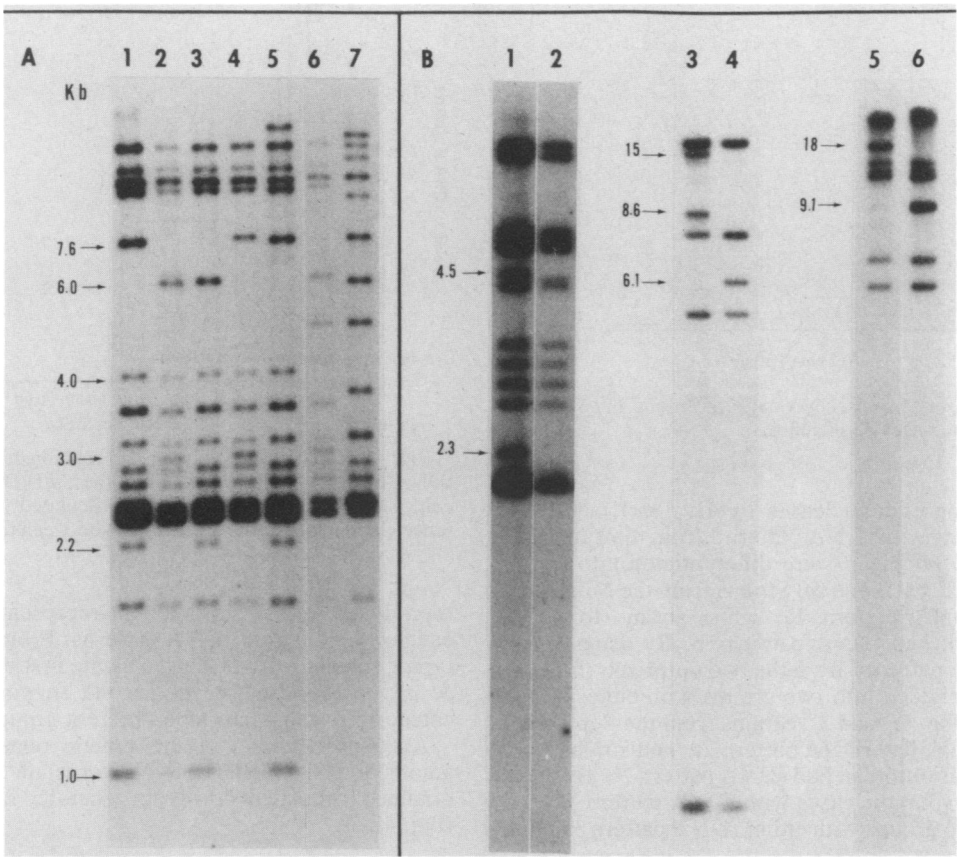

FIG. 1. Southern hybridization analysis of genomic DNA from *S. sonnei* and *E. coli* digested with *Sal*I, *Pvu*II, *Sma*I, or *Sst*I and probed for the 16S and 23S rRNA genes. (A) Lanes 1 through 6, *Sal*I rDNA patterns 1 through 6, respectively, from *S. sonnei*; lane 7, *Sal*I rDNA pattern of *E. coli* K-12. (B) Lanes 1 and 2, rDNA patterns 1a and 1b, respectively, obtained by using *Pvu*II to digest DNA from strains with *Sal*I rDNA patterns 1; lanes 3 and 4, rDNA patterns 2a and 2b, respectively, obtained by using *Sma*I to digest DNA from strains with *Sal*I rDNA pattern 2; lanes 5 and 6, rDNA patterns 3a and 3b, respectively, obtained by using *Sst*I to digest DNA from strains with *Sal*I rDNA pattern 3. The sizes of the restriction fragments that vary between patterns are indicated (in kilobases) at the left of each panel.

containing ^{32}P-labeled lambda DNA and ^{32}P-labeled 1-kb ladder DNA by previously published procedures (12). DNA fragments homologous with the radiolabeled probes were identified and sized following autoradiography. Membranes were exposed to X-ray film (X-Omat AR; Eastman Kodak Company, Rochester, N.Y.) in the presence of intensifying screens (X-Omatic regular; Eastman Kodak Company) for 16 to 24 h.

Relatedness analysis. Visual examination of the *Sal*I rDNA fragment patterns from autoradiograms of samples run together on one gel, as well as in various combinations on several other gels, revealed 25 different fragments among the 100 *S. sonnei* isolates and *E. coli* K-12. The fingerprint of each isolate was visually scored for the presence or absence of each of the 25 fragments (0, absence of a fragment; 1, presence of a fragment). In this manner, a numerical profile was developed for each unique *Sal*I rDNA pattern. Relatedness values between the *Sal*I rDNA patterns were calculated as the proportion of weighted mismatches of fragments, and a dendrogram showing the clustering or relatedness of the rDNA patterns was generated by the unweighted pair group method for arithmetic averages (25) by using the techniques developed by Jacobs (11).

RESULTS

To determine the optimal enzymes for discriminating between strains, Southern blot analysis with ^{32}P-labeled *E. coli* rRNA was performed on five *S. sonnei* strains by using the following restriction endonucleases: *Acc*I, *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Sal*I, *Sma*I, and *Sst*I. Of the enzymes tested, *Sal*I provided the greatest strain discrimination (data not shown). Using *Sal*I to test 100 *S. sonnei* strains from sporadic cases of shigellosis in 16 different states, six distinct rDNA patterns, designated 1 through 6, were observed (Fig. 1A). The distributions of these strains among the six rDNA patterns are shown in Fig. 2. The rRNA genes of 89% of the strains were organized like rDNA patterns 1 or 2. Like the majority of strains from sporadic cases, strains from the four different outbreaks exhibited rRNA genes organized like patterns 1 or 2. Strains from the outbreaks in Minnesota and Michigan had rDNA pattern 1, while strains from the two different outbreaks in Texas had rDNA pattern 2. All strains from a given outbreak had identical rDNA patterns.

To determine whether further discrimination among outbreak strains could be achieved, we performed Southern hybridization analysis of the rRNA genes following digestion

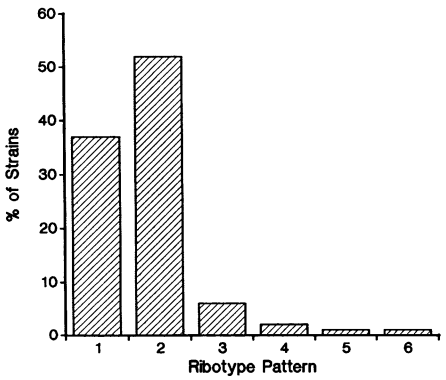

FIG. 2. Distribution of *Sal*I rDNA patterns among 100 *S. sonnei* strains from sporadic cases of shigellosis.

with the restriction endonucleases *Pvu*II, *Sma*I, and *Sst*I. Using *Pvu*II, strains with rDNA pattern 1 from the outbreaks in Minnesota and Michigan were differentiated into rDNA patterns 1a or 1b (Fig. 1B and 3). Strains from the Minnesota outbreak had rDNA pattern 1a, while strains from the Michigan outbreak had rDNA pattern 1b. By using *Sma*I, strains with rDNA pattern 2 from the two outbreaks in Texas could be further divided into two groups with either rDNA pattern 2a or 2b (Fig. 1B and 3). Strains from the September 1986 Texas outbreak had rDNA pattern 2a, and strains from the July 1986 Texas outbreak had rDNA pattern 2b. By using *Sst*I, strains from sporadic cases with rDNA pattern 3 could be divided into two groups with either rDNA pattern 3a or 3b (Fig. 1B and 3).

Strains from sporadic cases of shigellosis that were of rDNA patterns 1 and 2 were also tested by using *Pvu*II and *Sma*I, respectively. Interestingly, all 37 isolates from sporadic cases with rDNA pattern 1 were found to be of pattern 1a, and all 52 isolates from sporadic cases with rDNA pattern 2 were found to be of pattern 2b. This suggests that the Michigan and Texas (September 1986) outbreak strains, with patterns 1b and 2a, respectively, are relatively uncommon.

With *Sal*I, *Pvu*II, *Sma*I, and *Sst*I, nine distinct rDNA patterns were observed. The initial rDNA patterns were

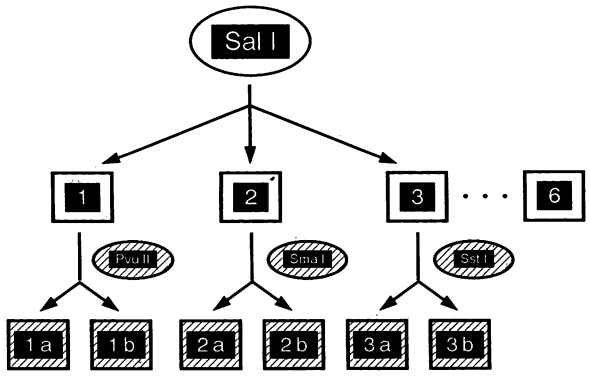

FIG. 3. Two-step flow diagram to differentiate *S. sonnei* strains by ribotyping. Digestion of genomic DNA from *S. sonnei* with *Sal*I and subsequent hybridization with radiolabeled 16S and 23S rRNAs yielded six patterns. Further digestion of DNA from strains with the indicated *Sal*I patterns with *Pvu*II, *Sma*I, or *Sst*I differentiated *Sal*I patterns 1 through 3, respectively, into two additional patterns each.

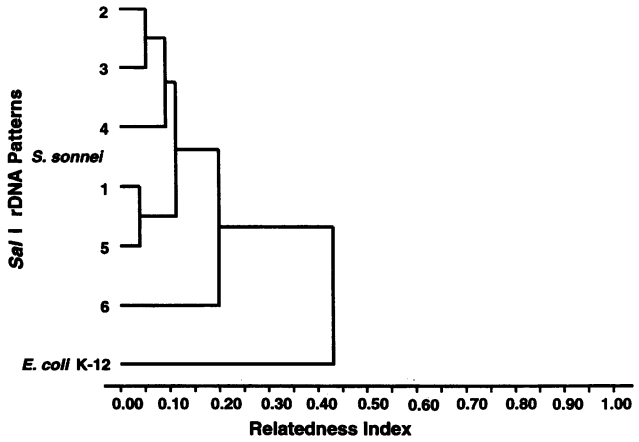

FIG. 4. Dendrogram of genetic relationships among six *Sal*I rDNA patterns from *S. sonnei* and the *Sal*I rDNA pattern from *E. coli*. The relatedness values were calculated as the proportion of unweighted mismatches of fragments between the different patterns.

reproduced four to six times for representative strains with each of the different rDNA patterns. From these patterns, a typing schema with *Sal*I used as the first enzyme and *Pvu*II, *Sma*I, or *Sst*I used as the second enzyme was devised to categorize strains into nine different groups (Fig. 3).

An approximation of the genetic relatedness of strains among the six *Sal*I rDNA patterns of *S. sonnei* can be obtained from the dendrogram generated from these patterns (Fig. 4).

DISCUSSION

Ribotyping has been determined to be useful for subtyping a wide range of bacterial pathogens, including *E. coli*, *Providencia stuartii*, *Haemophilus influenzae*, *Yersinia enterocolitica*, and *Salmonella typhi* (3, 4, 10, 14, 20, 27). Ribotyping has several advantages over other molecular typing methods. Because the ribosomal genes are located on the chromosome, they are not readily lost. In addition, the rDNA patterns are not sensitive to antibiotic pressure, and they are not dependent on susceptibility to phages or production of colicins. The high degree of conservation in the ribosomal genes allows the use of a single universal probe to subtype all prokaryotes. Most bacteria contain multiple copies of the ribosomal operons; therefore, an acceptable number of fragments (3 to 15) that hybridize with the probe are generated.

Among the majority of strains tested, we observed 15 *Sal*I fragments with homology to 16S and 23S rRNAs. *E. coli* contains seven highly conserved operons for 16S and 23S rRNA genes distributed throughout the chromosome (5). Restriction analysis of these genes has shown that *Sal*I cuts the rRNA genes once within the 16S gene and once within the 23S gene. By the manner in which the 16S and 23S genes are organized, an internal *Sal*I fragment of approximately 2.7 kb containing parts of the 16S and 23S genes would be obtained from each operon after *Sal*I digestion (13). Hybridization of *Sal*I-restricted DNA from *E. coli* with 16S and 23S rRNA genes would therefore produce 15 fragments: 14 fragments representing the left and right halves of each of the seven operons and the common internal fragment from each operon.

With respect to the number of *Sal*I fragments carrying

sequences homologous to rRNA, the restriction patterns we observed for *S. sonnei* were similar to those observed in *E. coli* (Fig. 1A). These findings suggest that, like *E. coli*, *S. sonnei* also carries seven rRNA operons. This seems likely since both organisms are so closely related that they are phylogenetically indistinguishable by DNA reassociation analysis (6). Although rDNA analysis (i.e., detection of restriction fragment length polymorphisms in uncharacterized regions of DNA that flank the rRNA genes) is not a definitive measure of true genetic relatedness, it is interesting that the relatedness index value of 0.43 for *S. sonnei* and *E. coli* K-12 (Fig. 3) also supports the close genetic relationship of these two organisms. The *S. sonnei* strains with patterns 1 and 2, which represented 89% of the sporatic isolates tested, clustered remarkably closely, suggesting that most U.S. strains of *S. sonnei* in this 1-year sample are "clonally" related. The close relatedness of *S. sonnei* strains was also observed by Ochman et al. (19), using multilocus enzyme analysis, and Brenner et al. (6), using DNA reassociation tests.

With *SalI* we were able to divide *S. sonnei* isolates into six different groups; however, by using multiple enzymes, we increased the discriminating capacity of the method to distinguish between nine groups. If we had used only *SalI* for ribotyping, we would not have been able to differentiate the Michigan outbreak strains from the Minnesota outbreak strains or the two Texas outbreak strains. Yogev et al. (29) made similar observations about the increased discrimination obtained by using more than one restriction enzyme to ribotype mycoplasmas. However, ribotyping is a time-consuming and labor-intensive procedure. Therefore, we have proposed the two-step ribotyping schema for *S. sonnei* to maximize discriminating ability while reducing the work load.

The ribotyping results for isolates from the Texas (July 1986 and September 1986) and Minnesota outbreaks compared favorably with their colicin typing results. No colicin typing results were available for isolates from the Michigan outbreak, and no phage typing results were available for any of the isolates. All isolates from the July 1986 Texas outbreak, which were rDNA pattern 2b, were colicin type 9, and all isolates from the September 1986 Texas outbreak, which were rDNA pattern 2a, were colicin type 7.

Occasionally, isolates from cases that are epidemiologically considered part of an outbreak have different, but related, plasmid profiles and antibiotic susceptibility patterns. Findings such as these are not surprising since enteric bacteria exchange genetic information readily, and plasmid profile and antibiotic susceptibility results are particularly sensitive to environmental or antibiotic pressures. In the July 1986 Texas outbreak, two related plasmid profiles and antibiotic susceptibility patterns were seen among strains from cases that were epidemiologically identified as being associated with the outbreak. Strains differed from each other only by the presence of a 2.8-MDa plasmid that appeared to mediate sulfisoxazole and streptomycin resistance. The same rDNA patterns and colicin types of these isolates provide further evidence of their relatedness.

Strains from the September 1986 Texas outbreak had identical plasmid profiles; however, one of five strains tested had acquired resistance to several additional antibiotics (trimethoprim, trimethoprim-sulfamethoxazole, ampicillin, carbenicillin, and streptomycin). All strains were colicin type 7 and rDNA pattern 2a. The relatively uncommon colicin types and rDNA patterns of these isolates provide additional evidence for their relatedness.

Outbreak-associated isolates from Minnesota had essentially the same antibiotic suspectibility patterns (all isolates were resistant to ampicillin and carbenicillin, with some isolates having an intermediate response to cephalothin or streptomycin) and one of three related plasmid profiles. All isolates had plasmids of 90, 23, and 5 MDa, and some isolates had an additional plasmid of either 36 or 45 MDa. Evidence from rDNA analysis and colicin typing further supported the relatedness of isolates epidemiologically associated with the outbreak: all outbreak-associated isolates were rDNA pattern 1a and had identical but unclassifiable colicin types (slight lysis of indicator strain 11 only).

Ten control strains that were collected during the Minnesota outbreak, but that were not related to the outbreak epidemiologically, were also evaluated. Ribotyping and colicin typing performed equally well for excluding these strains: six strains were correctly excluded by ribotyping, and six strains were correctly excluded by colicin typing. One strain was excluded by ribotyping that was not excluded by colicin typing, and one strain was excluded by colicin typing that was not excluded by ribotyping. A combination of colicin typing and ribotyping would have excluded 7 strains; however, all 10 strains were excluded by a combination of plasmid profile analysis and antibiotic susceptibility testing.

Because of the large variety of patterns generated by plasmid profile analysis and antibiotic susceptibility testing (e.g., 28 different antibiotic susceptibility patterns were detected among 60 of the sporadic isolates from this study; data not shown), these assays are usually the first methods used to discriminate between strains during outbreaks. Other techniques are usually used as needed to clarify or supplement the plasmid or antibiotic susceptibility results.

The advantage of ribotyping over colicin typing is that all strains are typeable by ribotyping, while a significant number of isolates may not be typeable by colicin typing. Morris and Wells (18) reported that 40% of strains from 33 different outbreaks of *S. sonnei* infection in the United States were untypeable by the colicin typing method. Since colicin genes are frequently located on plasmids, loss of extrachromosomal information can affect colicin typing results. In addition, uncharacterized factors, which can be influenced by such things as the age of a culture (18), can affect the expression of colicin genes and make interpretation of results problematic.

With ribotyping, the rDNA patterns were reproducible, and no differences between patterns were observed for rough and smooth variants of the same strain (data not shown). We propose the method outlined in Fig. 3 for typing *S. sonnei*, especially strains that are untypeable by colicin typing. The ability to type all strains by ribotyping makes it an attractive technique to complement traditional methods for investigating outbreaks of *S. sonnei* infection. For characterizing strains collected over extended periods of time, ribotyping may provide more dependable discrimination than plasmid profile analysis or antibiotic susceptibility testing since the genes encoding rRNA are chromosomally located and are not readily lost.

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