Characterization of Endemic *Shigella flexneri* Strains in Somalia: Antimicrobial Resistance, Plasmid Profiles, and Serotype Correlation

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One hundred twelve *Shigella flexneri* strains isolated from children with diarrheal disease in Somalia in 1983, 1984, 1988, and 1989 were analyzed for serotype, plasmid profile, and genetic location of antimicrobial resistance determinants. The prevalent serotypes were 4 (46% of the isolates), 1b (16%), 2a (16%), 3a (12%), and 6 (8%). Each serotype was associated with a characteristic predominant plasmid profile, whereas no specific correlation between antimicrobial resistance patterns and single serotypes was found. All but three of the strains were resistant at least to ampicillin, chloramphenicol, spectinomycin, and tetracycline. Of these resistant strains, 41 were resistant to sulfonamide and streptomycin and 14 were resistant to trimethoprim or trimethoprim and kanamycin. The genes for resistance to ampicillin, chloramphenicol, spectinomycin, and tetracycline formed a linkage group located on the chromosome of the strains of all serotypes. The genes for resistance to sulfonamide and streptomycin were located on a 6.3-kb plasmid in strains of serotypes 1b, 2a, and 4. Conjugal transfer of trimethoprim or trimethoprim and kanamycin resistance plasmids with lengths of 80 to 110 kb were present in strains of serotypes 1b, 2a, 3a, and 4. The systematic presence of a chromosomal component in this uncommon genetic plasmid-chromosome configuration may play a role in the emergence of increased genetic stability of resistance patterns in *S. flexneri*.

Shigellosis is one of the major causes of morbidity and mortality among children less than 5 years of age in developing countries. Furthermore, a great worldwide proportion of *Shigella* spp. strains of various serotypes (especially *S. dysenteriae* type 1 and *S. flexneri* serotypes) are now multiply drug resistant, and new, simple, and effective treatments of shigellosis have not yet been developed (9, 26).

From January 1983 to December 1990, selected samples from children with diarrheal disease were studied in five regions of Somalia. *Shigella* infections were frequently detected in urban areas and at high rates during the rainy seasons. On average, the annual isolation frequencies of *Shigella* spp. in children aged 1 to 4 years with diarrheal disease who presented for treatment at pediatric or regional hospitals in towns ranged from 10 to 15%. Of all *Shigella* spp. strains isolated, more than 60% belonged to *S. flexneri*. The *S. flexneri* strains were shown to be multiply resistant, and most of their resistance patterns appeared to be highly stable throughout the 8-year period between 1983 and 1990 (2, 14, 15). At the moment, little information is available on pathogenic *S. flexneri* strains present in Somalia (14, 17), and, to our knowledge, the plasmid content has never been examined.

Together with a large plasmid that is carried by all *Shigella* species, the genus *Shigella* has been reported to contain a heterogeneous population of small plasmids ranging in number from 2 to 10 (11). The determination of plasmid profiles can aid in the differentiation of isolates and has been shown to be a useful tool in investigating the epidemic and the histories of individual endemic strains (7, 8, 13, 22, 23).

To investigate the extent and genetic nature of high stability of drug resistance in *S. flexneri*, representative samples of 112 strains of *S. flexneri* isolated in Mogadishu, Somalia, in 1983, 1984, 1988, and 1989 have been characterized for serotypes, plasmid profiles, and the genetic locations of drug resistance determinants. The prevalent serotypes have been correlated with specific profiles of small plasmids, and the more stable cluster of resistance determinants has been localized on the chromosome of all serotypes identified.

**MATERIALS AND METHODS**

*S. flexneri* strains. The 112 strains of *S. flexneri* examined in this study were isolated from stool specimens or rectal swabs from children with diarrheal disease who presented for treatment at the Banaadir and Forlanini hospitals in Mogadishu, Somalia. Of these strains, 98 were isolated from January 1983 to December 1984 and 14 were isolated from August 1988 to April 1989. Details of the clinical and epidemiological features as well as the laboratory methods used to isolate *S. flexneri* and other enteric pathogens have been described previously (2).

**Media and chemicals.** *S. flexneri* strains were routinely grown at 37°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). LB broth (18) was used to grow bacteria for plasmid DNA extractions. Carbon sources (0.2%), amino acids (50 μg/ml), vitamins (2 μg/ml), and nicotinic acid
(10 μg/ml) were added to M9 minimal salt medium (18). After preliminary tests performed by the disk diffusion method, drug resistance patterns were determined on plates containing single antibiotics. The following antibiotics were used at the concentrations indicated: ampicillin (AP) 50 μg/ml; chloramphenicol (CM), 30 μg/ml; gentamicin (GM), 20 μg/ml; kanamycin (KM) 30 μg/ml; mercurochrome (HG), 150 μg/ml; nalidixic acid (NAL), 40 μg/ml; rifampin (RIF), 100 μg/ml; streptomycin (SM), 100 μg/ml; sulfonamide (SU), 600 μg/ml in M9 minimal medium; tetracycline (TC), 10 μg/ml; and trimethoprim (TP), 10 μg/ml in M9 minimal medium.

**Plasmid isolation.** Plasmid DNA was prepared by the method of Kado and Liu (12) and analyzed by 0.8% agarose vertical slab gel electrophoresis (27). A supercoiled DNA ladder (Bethesda Research Laboratories, Bethesda, Md.), which was made with 11 plasmids, was used as a molecular weight standard.

**Serotyping of S. flexneri.** Commercially available group factor and type-specific antiserum of S. flexneri (Wellcome Diagnostic, Dartford, England) were used in this study. Strains were grown at 37°C on Trypticase soy agar plates, and serotypes were determined by the slide agglutination test (19).

**Colicin assay.** The capacity of the S. flexneri strains to produce colicins was determined as described previously (6). The indicator strain used was ZM46 (5).

**Selection of TC-susceptible mutants.** Direct selection of TC-susceptible mutants of representative TC-resistant S. flexneri isolates was accomplished by a modification of the method described by Maloy and Nunn (16), by growing bacterial strains in LB broth supplemented with fusaric acid (12 μg/ml), chlorotetracycline hydrochloride (50 μg/ml), and ZnCl2 (0.1 mM) as described previously (6). TC-susceptible mutants were examined for their resistance patterns by replica plating.

**Genetic procedures.** S. flexneri strains were mated with Escherichia coli K-12 strain ZM46, a nalidixic acid-resistant mutant of CSH26 (5), in liquid or on filters as described previously (19). The frequency of a transfer of genetic marker was expressed as the number of recipient cells carrying the marker per donor cell in the mating mixture at the time of plating. Resistance plasmids were also detected by transforming E. coli K-12 strain 803 with plasmid DNA extracted from S. flexneri strains, as described previously (19). Selection of transconjugants or transformants was done on Trypticase soy agar plates supplemented with single antibiotics. One hundred colonies of transconjugants or transformants able to grow on the selection plates separately with each drug were tested for their resistance pattern by replica plating. Plasmid profiles of transconjugants or transformants were always compared with those of the S. flexneri donors.

Hfr derivatives of representative S. flexneri strains were constructed by the use of the conjugal temperature-sensitive plasmid F' ts114lac::Tn5 which codes for KM resistance and β-galactosidase activity (21). E. coli K-12 strain C600 (F' ts114lac::Tn5) was mated with different KM-susceptible S. flexneri strains overnight at 30°C. Independent Km-resistant, lactose-positive S. flexneri transconjugants were grown in Trypticase soy broth overnight at 30°C, plated on lactose-minimal plates prewarmed at 42°C, and then incubated overnight at the nonpermissible temperature of 42°C. Isolated lactose-positive colonies were checked for the stability of the Lac" phenotype by repeated streaking on lactose-indicator plates at 42°C. Stable expression of the Lac" phenotype at 42°C was assumed to be due to possible integration of the F' ts114lac::Tn5 plasmid into the S. flexneri chromosome (Hfr strain). S. flexneri Hfr strains were mated with the multiple auxotrophic E. coli K-12 strain ZM2000, a Rif-resistant derivative of strain AB1157 (lacY ara xyl gal thr leu proA2 his argB tsx supE rpsL) (25) for 3 h at 42°C.

**RESULTS**

**Epidemiological background.** In our general surveys of diarrheal diseases in Mogadishu, more than 2,500 children with diarrheal disease were screened for enteric pathogens between January 1983 and December 1990. Shigella spp. were isolated in 10% of the patients and, taken together, represented the third most frequent enteric pathogen identified after rotavirus and enterotoxigenic E. coli. The detection frequency of Shigella spp. in the age group of 1 to 4 years was 12%. Of 256 Shigella sp. isolates, 51 (20%) were S. dysenteriae (43 were type 1), 156 (61%) were S. flexneri, and 49 (19%) were S. sonnei. Interestingly, no strain of S. boydii was isolated.

**S. flexneri serotypes.** Of the 156 S. flexneri isolates, 112 (isolated in 1983, 1984, 1988, and 1989) were available for further characterization. The serotypes of these 112 strains were 4 (52 strains), 1b (18 strains), 2a (18 strains), 3a (13 strains), and 6 (9 strains). Two strains were untypeable and were probably antigen-variant of serotype 4 as inferred from their plasmid profiles (see below and Table 1). The serotype distribution of the 1988 and 1989 isolates was very similar to that observed among the 1983 and 1984 isolates.

**Plasmid profiles.** The plasmid profiles of the 112 (isolated in 1983, 1984, 1988, and 1989) S. flexneri strains are shown in Table 1. The large virulence plasmid, which is unstable during storage or subculturing of strains (13, 20), was not considered in this study for epidemiological correlations between plasmid patterns and serotypes.

The analysis of plasmid profiles showed that strains of each serotype had a characteristic plasmid profile (Table 1; Fig. 1). Although plasmids with an apparently identical size were present in different serotypes (plasmids with sizes of 6.3, 4.1, 3.2, 2.7, 1.9, and 1.6 kb [Table 1]), strains belonging to the different serotypes show different prevalent plasmid profiles that characterize each serotype, with minor variations due to the acquisition or the loss of one or a few plasmids.

The serotype-specific plasmid patterns of the strains isolated in 1988 and 1989 were consistent with those of the strains isolated in 1983 and 1984.

**Antimicrobial resistance patterns.** The antimicrobial resistance patterns of the 112 S. flexneri strains are shown in Table 1. All but three (one susceptible strain of serotype 4 and two TC-resistant strains of serotype 6) were resistant at least to AP, CM, SP, and TC. Forty-one of the 112 strains examined were also resistant to SU and SM, and 14 were resistant to TP alone or in combination with KM and HG. All strains were susceptible to GM. There was no evidence of increased resistance in strains isolated in 1988 and 1989 compared with the strains isolated in 1983 and 1984.

The most common patterns were resistance to AP, CM, SP, and TC (63 strains) and AP, CM, SP, TC, SU, and SM (28 strains), which taken together accounted for 81% of the 112 strains tested. There was no correlation between resistance patterns and serotypes.

Interestingly, only the 13 S. flexneri strains of serotype 3a produced a colicin.

**Genetic location of antimicrobial resistance genes.** To ascertain whether resistance determinants were located on conjugational plasmids, 25 multiply resistant S. flexneri strains, representative of the different serotypes, were mated with E. coli ZM46 in liquid or on filters. Only the six TP- and TP-KM-resistant strains (serotypes 1b, 2a, 3a, and 4) were able
TABLE 1. Serotypes, plasmid profiles, and antimicrobial resistance of 112 strains of endemic S. flexneri in Somalia

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Plasmid pattern†</th>
<th>No. of strains</th>
<th>Resistance and colicinogenic‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>4.1 3.2 2.7 2.3</td>
<td>1.6 10</td>
<td>AP CM SP TC</td>
</tr>
<tr>
<td>6.3</td>
<td>4.1 3.2 2.7 2.3</td>
<td>1.6 2</td>
<td>AP CM SP TC</td>
</tr>
<tr>
<td>6.3</td>
<td>4.1 3.2 2.7 2.3</td>
<td>1.6 2</td>
<td>AP CM SP TC SU SM</td>
</tr>
<tr>
<td>6.3</td>
<td>4.1 3.2 2.7 2.3</td>
<td>1.6 1</td>
<td>AP CM SP TC SU SM TP</td>
</tr>
<tr>
<td>6.3</td>
<td>4.1 3.2 2.7 2.3</td>
<td>1.6 1</td>
<td>AP CM SP TC TP</td>
</tr>
<tr>
<td>2a</td>
<td>6.3 4.1 3.2</td>
<td>1.6 14</td>
<td>AP CM SP TC SU SM</td>
</tr>
<tr>
<td>6.3</td>
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<td>2.1 10</td>
<td>AP CM SP TC Col</td>
</tr>
<tr>
<td>5.2</td>
<td>3.2</td>
<td>2.1 1</td>
<td>AP CM SP TC SU SM TP Col</td>
</tr>
<tr>
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<td>2.1 1</td>
<td>AP CM SP TC SU SM TP KM HG Col</td>
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<td>7.0 4.1 3.2</td>
<td>1.9 1.6 28</td>
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</tr>
<tr>
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<td>6.0</td>
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<td>AP CM SP TC</td>
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<tr>
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<td>1.9 2</td>
<td>TC</td>
</tr>
<tr>
<td>NT†</td>
<td>7.0 4.1</td>
<td>1.9 1.6 2</td>
<td>AP CM SP TC</td>
</tr>
</tbody>
</table>

† Plasmid sizes (in kilobases) are given; boldface values denote the prevalent plasmid pattern for each serotype. Plasmids with large sizes are not shown. A plasmid larger than 200 kb was detected in a majority of the strains. Conjugal TP and TP-KM resistance plasmids with sizes of 80 to 110 kb were present in the TP- and TP-KM-resistant strains, respectively. The 6.3-kb plasmids in serotypes 1b, 2a, and 4 coded for resistance to SU and SM. The 6.0-kb plasmids in serotype 6 coded for resistance to SU.

‡ All 112 strains were GM susceptible. Strains resistant to SP were also resistant to small amounts of SM (10 μg/ml) (10); Col, production of colicin.

NT, not typeable; most likely antigenic variants of serotype 4.

To transfer resistance markers. The resistance patterns of transconjugants, the frequencies of transfer, the results of successive conjugal transfer, and physical analysis of plasmid DNA from donors and transconjugants identified two classes of R plasmids: conjugal TP or TP-KM resistance plasmids with sizes of 80 to 110 kb and a nonconjugal SU-SM resistance plasmid with a size of 6.3 kb. The conjugal TP or TP-KM resistance plasmids (transfer frequency of 10^{-3} to 10^{-2} in 1-h S. flexneri-E. coli matings) were present in all four serotypes examined and mobilized the SU-SM resistance plasmid at a frequency of 10^{-6} to 10^{-5}. No SU- or SM-resistant transconjugant was isolated from matings with donor strains of serotype 3a. No AP-, CM-, SP-, or TC-resistant transconjugant was isolated in any conjugation experiments.

To identify possible resistance plasmids undetectable by conjugation, we used plasmid DNA from five AP-, CM-, SP-, and TC-resistant and four AP-, CM-, SP-, TC-, SU-, and SM-resistant S. flexneri strains of different serotypes to transform E. coli 803. Plasmid profiles of SU-SM-resistant transformants obtained with plasmid DNA from serotypes 1b, 2a, and 4 contained only one plasmid with a size of 6.3 kb. No SU- or SM-resistant transconjugant was isolated after transformation by plasmid DNA from serotype 3a. No AP-, CM-, SP-, or TC-resistant transformant was isolated in any transformation experiments. Finally, spontaneous SU-SM-susceptible derivatives of SU-SM-resistant S. flexneri strains of serotypes 1b, 2a, and 4 and SU-susceptible derivatives of SU-resistant strains of serotype 6 lacked the 6.3-kb plasmid (Fig. 1, lanes a and b, d and e, and i and j) and the 6.0-kb plasmid (Fig. 1, lanes 1 and m), respectively. These data confirmed that the 6.3-kb plasmid alone was responsible for resistance to SU and SM in serotypes 1b, 2a, and 4 and indicated that the 6.0-kb plasmid in strains of serotype 6 determined resistance to SU.

Since no spontaneous loss of AP, CM, SP, or TC resistance markers in isolates of every serotype could be detected at a frequency higher than 10^{-4} from any cultures and subcultures routinely tested by replica plating, TC-susceptible mutants of 10 (2 for each serotype) AP-, CM-, SP-, and TC-resistant S. flexneri strains were selected in the presence of 12 μg of fusaric acid per ml. All one hundred TC-susceptible isolates examined from each experiment were susceptible to AP, CM, and SP. The susceptible mutants that had undergone simultaneous loss of the entire AP-CM-SP-TC resistance component exhibited the same plasmid content as the parental resistant strains (Fig.
linkage group formed by AP, CM, SP, and TC resistance determinants.

DISCUSSION

Plasmid profile analysis is a useful tool in epidemiological studies dealing with enteric infections (1, 8, 13, 23). In this study a typical predominant plasmid profile has been associated with each serotype of S. flexneri in Somalia, and this association has remained uniform for 8 years. The profiles identified may be helpful serotype–specific reference patterns for detecting and tracing individual strains with possible variations in plasmid content that are acquiring new epidemiological importance in Somalia.

A few correlations of plasmid profiles with serotypes in S. flexneri and other Shigella spp. have been reported from other geographical regions (1, 8, 13, 23, 24), and the sizes of some plasmids resemble those of plasmids found in the Somali strains. However, direct comparisons between plasmids of different origin (including restriction enzyme analysis and DNA homology) are still needed to establish a combined reference frame.

All but 3 of the 112 S. flexneri strains examined in this study were multiply resistant (at least to AP, CM, SP, and TC), and the antimicrobial resistance patterns did not correlate with serotypes. The patterns revealed that, by the early 1980s, the limited multiple drug resistance first reported from Somalia in 1976 (17) had become widely disseminated in the S. flexneri population, with a high rate (41%) of resistance to five or more drugs. The components of the resistance patterns mediated by plasmids were the resistance to SU and SM and the resistance to TP or TP and KM. On the other hand, conjugation and transformation experiments as well as comparisons between the plasmid contents of resistant strains and susceptible mutants indicated that the AP, CM, SP, and TC resistance determinants were closely linked but not plasmid associated. Genetic analysis of hybrid recombinants isolated from matings of S. flexneri Hfr derivatives with E. coli recipient cells provided strong genetic evidence that the AP-CM-SP-TC resistance DNA region was located on the chromosome of all serotypes. The final picture displays that the genetic configurations for the determinants of resistance patterns in all multiply resistant strains of S. flexneri are very similar, irrespective of serotype and year of isolation. This finding has two direct implications. The chromosomal location of the AP-CM-SP-TC resistance region with its specific genetic stability appears to be completely dominant among the isolates and is likely to represent a significant selective advantage. In fact, as in most developing countries, in urban Somalia, the use of antibiotics has been massive and indiscriminate for many years. Second, the chromosomal location of the AP-CM-SP-TC resistance region in strains of all serotypes must result from some independent recombination events. Integration of the resistance region, presumably caused by insertion of an entire R plasmid or a part of it (e.g., a transposon-like resistance element) into the chromosome must have occurred several times in different individual strains, at least once in each serotype.

A chromosomal locus, designated mar, which controls multiple antibiotic resistance by mechanisms linked to decreased uptake or increased efflux of the antibiotics or both has recently been characterized in E. coli (3) and detected in various enteric species (4). However, there were important differences between resistance phenotypes expressed by the chromosome of the S. flexneri strains which we studied and those described for the mar locus. The TC resistance in the S.
flexneri isolates was stable at 42°C, the fusaric acid resistance was systematically associated with TC susceptibility, and the CM resistance proved to be selectively associated with the presence of chromosomal DNA sequences homologous with those of the cam gene of Tn9 (unpublished results). These features typical of plasmid-encoded resistances made the possible involvement of mar functions very remote.

In conclusion, our study shows that an uncommon genetic plasmid-chromosome organization for multiple drug resistance in S. flexneri has become predominant in an area of endemicity and the chromosomal component may play a role in determining increased genetic stability of resistance patterns (or a part of them) in this invasive enteric pathogen.

To our knowledge, this is the first report on the correlation of serotypes, plasmid profiles, and antimicrobial resistance genes in pathogenic S. flexneri strains isolated in the Horn of Africa, and we hope that it will be useful for future surveillance of the distribution and evolution of this species in this geographical area.

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