Typing and Characterization of Mechanisms of Resistance of Shigella spp. Isolated from Feces of Children under 5 Years of Age from Ifakara, Tanzania

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Eighty-six strains of Shigella spp. were isolated during the dry season from stool samples of children under 5 years of age in Ifakara, Tanzania. The epidemiological relationship as well as the antimicrobial susceptibility and mechanisms of resistance to ampicillin, chloramphenicol, and co-trimoxazole were investigated. Four different epidemiological tools, pulsed-field gel electrophoresis (PFGE), repetitive extragenic palindromic (REP)-PCR, plasmid analysis, and antibiogram, were compared for typing Shigella strains. Seventy-eight (90%) strains were Shigella flexneri and were distributed into four groups, by either PFGE or REP-PCR, with 51, 17, 7, and 3 strains. The four strains of Shigella dysenteriae belonged to the same group, and the four strains of Shigella sonnei were distributed in two groups with three and one strain each. Plasmid analysis showed a high level of heterogeneity among strains belonging to the same PFGE group, while the antibiogram was less discriminative. REP-PCR provided an alternative, rapid, powerful genotyping method for Shigella spp. Overall, antimicrobial susceptibility testing showed a high level of resistance to ampicillin (81.8%), chloramphenicol (72.7%), tetracycline (96.9%), and co-trimoxazole (87.9%). Ampicillin resistance was related to an integron-borne OXA-1-type ß-lactamase in 85.1% of the cases and to a TEM-1-type ß-lactamase in the remaining 14.8%. Resistance to co-trimoxazole was due to the presence of a dhfr 1a gene in all groups except one of S. flexneri, where a dhfr VII gene was found within an integron. Chloramphenicol resistance was associated in every case with positive chloramphenicol acetyltransferase activity. All strains were susceptible to nalidixic acid, ciprofloxacin, ceftazidime, cefotaxime, and cefoxitin. Therefore, these antimicrobial agents may be good alternatives for the treatment of diarrhea caused by Shigella in Tanzania.

Acute infectious diarrheal disease is one of the most frequent causes of childhood deaths in the developing world. Diarrheal disease accounts for approximately 25% of all deaths in children younger than 5 years of age in these areas (21). Infections caused by Shigella species are an important cause of diarrheal disease, in both developing and developed countries. Worldwide, it is estimated that shigellosis causes around 600,000 deaths per year, two-thirds of the deceased being children under 10 years of age. Shigella dysenteriae and Shigella flexneri are the predominant species in the tropics, while Shigella sonnei is the predominant species in industrialized countries (18).

Shigellosis is one of the acute diarrheal diseases for which antimicrobial therapy is effective. However, today it also presents a pressing challenge, as Shigella spp. have progressively become resistant over the past decades to most of the widely used and inexpensive antimicrobials (21). Thus, the history of the genus suggests that resistance will emerge to any antimicrobial agent used intensively (25). Antimicrobial resistance in enteric pathogens is of the greatest importance in the developing world, where the rate of diarrheal diseases is highest and indiscriminate use of antimicrobial agents is a fact.

The comparative analysis of different epidemiological mark-

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MATERIALS AND METHODS

Bacterial strains. Eighty-six strains of Shigella spp. were isolated from stool samples of children under 5 years of age during the dry period (July to September) of 1997 in Ifakara, Tanzania. The children included in the study were seen at Saint Francis Designated District Hospital. Shigella spp. were identified by conventional methods (16) and by serotyping. All the strains with different plasmid patterns or antibiograms were investigated in detail to determine their mechanisms of resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole.

Antimicrobial susceptibility testing. Susceptibility testing was performed by an agar diffusion disk method as recommended by the National Committee for Clinical Laboratory Standards (17). Mueller-Hinton agar was obtained from Becton Dickinson (Cockeysville, Md.), and antimicrobial disks were obtained...
chloramphenicol (dissolved in water) was added to one well (test reaction), and coenzyme A, and one part 10 mM 5,5-dithio-bis-(2-nitrobenzoic acid) in 0.1 M l of a solution containing two parts 0.2 M Tris-HCl (pH 8), 2 mM acetyl modifications (6). Briefly, the strains were grown overnight on MacConkey agar. A transferase activity assay was performed as described elsewhere (2), with slight visualized in 0.7% agarose gels stained with ethidium bromide.

The plasmids obtained were visualized and analyzed by 0.8% agarose gel electrophoresis. The plasmids were excised from the gel, and the DNA was recovered with a GeneClean kit (Bio 101, Inc., La Jolla, Calif.) and cloned into pCRII vector (Invitrogen BV, Leek, The Netherlands). DNA sequencing. Plasmid extraction was performed as described above. The sequencing of the plasmids with the cloned inserts was done with a Thermosequenase dye terminator sequencing kit in an automatic DNA sequencer (mod-377; Applied Biosystems, Perkin-Elmer, Emeryville, Calif.) following the manufacturer's instructions.

**RESULTS**

The eighty-six strains of *Shigella* spp. that were isolated were distributed as follows: 78 (90%) were *S. flexneri*, 4 (4.6%) were *S. dysenteriae*, and 4 (4.6%) were *S. sonnei*. No *Shigella boydii* strains were isolated. The 78 *S. flexneri* strains were grouped into four epidemiological groups by PFGE or REP-PCR (Fig. 1 and 2). The distribution of strains according to these epidemiological markers was as follows: 51 strains in group F-I, 17 strains in group F-II, 7 strains in group F-III, and 3 strains in group F-IV. However, the four major *S. flexneri* groups were subdivided into nine different subgroups based on antibiogram and plasmid analysis (Table 1). Eight different plasmid patterns were obtained among *S. flexneri* strains (Fig. 3). These patterns contained from three to six different plasmids each, although in some cases the difference between two patterns was due to the gain or loss of only one plasmid.

The basis of antibiotic susceptibility, six phenotypes were defined: phenotype I (Amp<sup>r</sup> Cm<sup>r</sup> Tet<sup>r</sup> Sxt<sup>r</sup>), phenotype II (Amp<sup>r</sup> Cm<sup>r</sup> Tet<sup>r</sup> Sxt<sup>r</sup>), phenotype III (Amp<sup>r</sup> Cm<sup>r</sup> Tet<sup>r</sup> Sxt<sup>r</sup>), phenotype IV (Amp<sup>r</sup> Cm<sup>r</sup> Tet<sup>r</sup> Sxt<sup>r</sup>), phenotype V (Amp<sup>r</sup> Cm<sup>r</sup> Tet<sup>r</sup> Sxt<sup>r</sup>), and phenotype VI (Amp<sup>r</sup> Cm<sup>r</sup> Tet<sup>r</sup> Sxt<sup>r</sup>). In spite of belonging to the same clone by PFGE, REP-PCR, or plasmid analysis (Fig. 1 to 3), the four strains of *S. sonnei* were distrib-

**FIG. 1.** PFGE. Lanes 1, 2, and 3, *S. flexneri* strains belonging to group F-I; lanes 4, 5, and 6, *S. flexneri* strains belonging to group F-II; lanes 7, 8, and 9, *S. flexneri* strains belonging to group F-III; lanes 10 and 11, *S. flexneri* strains belonging to group F-IV; lanes 12 and 13, *S. dysenteriae* strains; lanes 14 and 15, *S. sonnei* strains.

TABLE 1. Characterization of Shigella spp. by four different epidemiological markers

<table>
<thead>
<tr>
<th>Species</th>
<th>Groupa (no.)</th>
<th>Subgroupb (no.)</th>
<th>PFGE Antibio-gramc</th>
<th>Plasmid profile</th>
<th>REP-PCR group</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. flexneri</td>
<td>F-I (51)</td>
<td>F1 (46)</td>
<td>A I b 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F2 (4)</td>
<td>A I a 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F3 (1)</td>
<td>A I I c 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-II (17)</td>
<td>B (14)</td>
<td>B I d 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-III (7)</td>
<td>F1 (5)</td>
<td>C I f 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-IV (3)</td>
<td>F1 (1)</td>
<td>D IV h 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2 (2)</td>
<td>D III h 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. sonnei</td>
<td>S-I (4)</td>
<td>S1 (3)</td>
<td>F IV j 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S2 (1)</td>
<td>F V j 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. dysenteriae</td>
<td>D-I (4)</td>
<td>D1 (4)</td>
<td>E I i 6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Distribution of strains based on PFGE and REP-PCR.
b Distribution of groups according to antibiogram and plasmid analyses.
c See text for phenotypes.

The presence of an OXA-1-type β-lactamase might be explained in 75% of the cases by an integron or transposon carrying the resistance gene integrated in the chromosomes. While S. flexneri strains also had the dhfr Ia gene, S. dysenteriae strains showed phenotype IV (group S1), and one strain showed phenotype V (group S2). The four strains of S. dysenteriae were all the same clone (Table 1).

Fourteen S. flexneri strains, three S. sonnei strains, and three S. dysenteriae strains were used for detailed investigations of the mechanisms of resistance to ampicillin, chloramphenicol, and co-trimoxazole. The MICs of ampicillin, chloramphenicol, tetracycline, co-trimoxazole, nalidixic acid, ciprofloxacin, cefazidime, cefotaxime, and cefoxitin (Table 2). The ampicillin-resistant S. dysenteriae strains also had the dhfr Ia gene and S. dysenteriae strains also had the dhfr Ia gene.

As long as the protocol is strictly followed and conditions are kept constant, this technique provides a degree of discrimination equivalent to that of PFGE with the advantages of speed, simplicity, and economy. To our knowledge, this is the first time that such a technique has been used in comparison with PFGE and plasmid profiles to type different species of Shigella.

Antimicrobial susceptibility testing showed a high degree of resistance to antibiotics most commonly used in the area (tetracycline, ampicillin, co-trimoxazole, and chloramphenicol). No resistance to quinolones and cephalosporins was observed.

**DISCUSSION**

The predominant species of Shigella during the studied period of time was S. flexneri, which is usually the predominant species in areas of endemicity, accounting for 50% of culture-positive disease (25). S. sonnei and S. dysenteriae were found in the same proportions. The most common typing procedures currently used with Shigella spp. are plasmid analysis and PFGE (7, 8, 12, 13, 24). Shigella species usually harbor a heterogeneous population of plasmids, ranging in number from 2 to more than 10 (9). Plasmid analysis has proven to be a useful typing technique (7, 8). Moreover, it is inexpensive and quick to perform, but it can be limiting if we take into account the fact that many plasmids are unstable and may be easily gained and/or lost. PFGE has a high discriminatory power, although it is cumbersome and expensive. However, it has been widely used for typing Shigella spp. (13, 24). Taking PFGE as a reference epidemiological tool, strains belonging to the same PFGE group but having different plasmid profiles and different antibiograms were observed (for instance, subgroups F4 and F5). Therefore, the mechanisms of resistance are probably carried in the missing plasmid. The contrary is also true; two strains belonging to the same PFGE group with the same plasmid profile showed different antibiograms (for instance, subgroups F9 and F10). This is probably due to an integron or transposon carrying the resistance gene integrated in the chromosome.

Recently, Liu et al. (13) compared plasmid profiles, PFGE, and enterobacterial repetitive intergenic consensus PCR for typing 20 clinical isolates of S. sonnei. PCR-based techniques have the advantages of being quick and easy to perform, and in this case they proved to be as good at discriminating epidemiologically related strains as PFGE. We found something similar with REP-PCR, another PCR-based technique, in which the amplification of the regions between REP sequences gives a good fingerprinting pattern valid for epidemiological typing. As long as the protocol is strictly followed and conditions are kept constant, this technique provides a degree of discrimination equivalent to that of PFGE with the advantages of speed, simplicity, and economy. To our knowledge, this is the first time that such a technique has been used in comparison with PFGE and plasmid profiles to type different species of Shigella.

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Antimicrobial susceptibility testing showed a high degree of resistance to antibiotics most commonly used in the area (tetracycline, ampicillin, co-trimoxazole, and chloramphenicol). No resistance to quinolones and cephalosporins was observed.

**FIG. 3. Plasmid patterns.** Lane 1, S. flexneri strain belonging to subgroup F2; lanes 2 and 3, S. flexneri strains belonging to subgroup F1; lane 4, S. flexneri strain belonging to subgroup F3; lanes 5 and 6, S. flexneri strains belonging to subgroup F4; lane 7, S. flexneri strain belonging to subgroup F5; lane 8, S. flexneri strain belonging to subgroup F6; lane 9, S. flexneri strain belonging to subgroup F7; lanes 10 and 11, S. flexneri strains belonging to subgroups F5 and F6; lane 12, S. dysenteriae; lanes 13 and 14, S. sonnei strains belonging to subgroups S1 and S2.
which can be explained by the fact that they are not used as alternative therapies in this area due to their high cost and lack of availability. However, a trend to quinolone resistance has been observed by Ries et al. (20) in S. dysenteriae strains isolated in Burundi. S. dysenteriae is considered the most resistant of the Shigella spp. (21). However, in our study S. flexneri showed the same level of resistance as S. dysenteriae. This pattern of resistance and susceptibility is commonly seen in developing countries, in contrast with strains from developed countries, which are less resistant to these antimicrobial agents (4, 27). In this study, the antimicrobial resistance pattern is not a useful epidemiological marker, due to the lack of variability in susceptibility patterns (i.e., the high level of resistance shown by most isolates). Resistance to ampicillin in S. flexneri groups F1 and F2 and S. dysenteriae (group D) is explained by the presence of an OXA-1-type ß-lactamase within an integron. Group F3, S. flexneri and the one ampicillin-resistant S. sonnei (group S2) isolate had a TEM-1-type ß-lactamase. Both genes have been previously described in Shigella strains isolated in Denmark and Greece (14, 22). Therefore, this is the most frequent mechanism of ampicillin resistance found in Shigella.

Besides ampicillin, the drug of choice for treating shigellosis is co-trimoxazole. Eighty-eight percent of the strains studied showed resistance to this drug, and in most cases this resistance could be explained by the presence of a dhfr Ia gene previously described in Shigella and considered the most common dihydrofolate reductase gene in the genus. In one group of S. flexneri, however, the dhfr gene found was dhfr VII, first described in E. coli (1). These genes were found inserted in an integron. Both genes were detected with specific primers to amplify the entire gene, which was further sequenced, showing in both cases 100% homology with the dhfr Ia and dhfr VII genes previously described (19, 23). Chloramphenicol resistance was explained in every case by a positive chloramphenicol acetyltransferase activity generating a high level of resistance. The use of this antibiotic has rapidly declined in many countries. However, due to the fact that it is inexpensive and presents a broad-spectrum activity it is extensively employed in developing countries, thereby ensuring strong selection pressure for the maintenance of chloramphenicol resistance.

In this study, we suggest that antibiotic resistance determinants are carried by plasmids, as well as in integrons which contain resistance genes, such as blaOXA or dhfr genes. The spread of multiresistant Shigella strains among a population in which diarrheal disease is one of the major causes of child morbidity and mortality requires greater attention to the appropriate use of antibiotics, the establishment of hygiene measures to prevent or decrease transmission, and the development of new effective drugs that can be safely used with children. Moreover, the guidelines for the treatment of shigellosis in developing countries should be updated, since in this study co-trimoxazole, one of the recommended antimicrobial agents for the treatment of shigellosis, has been shown to have little activity against Shigella spp.

TABLE 2. Antimicrobial susceptibility of Shigella spp.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
<th>F8</th>
<th>F9</th>
<th>S1</th>
<th>S2</th>
<th>D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>1</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<td>&gt;256</td>
<td>2</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>1</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<td>&gt;256</td>
<td>&gt;256</td>
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<td>&gt;256</td>
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<tr>
<td>Chloramphenicol</td>
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<td>1</td>
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<td>&gt;256</td>
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<td>&gt;256</td>
<td>&gt;256</td>
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<tr>
<td>Co-trimoxazole</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>0.032</td>
<td>&gt;32</td>
<td>0.38</td>
<td>&gt;32</td>
<td>0.125</td>
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<td>0.032</td>
<td>&gt;32</td>
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<td>Nalidixic acid</td>
<td>2</td>
<td>4</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1.5</td>
<td>3</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.008</td>
<td>0.016</td>
<td>0.006</td>
<td>0.006</td>
<td>0.008</td>
<td>0.008</td>
<td>0.016</td>
<td>0.016</td>
<td>0.012</td>
<td>0.008</td>
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<tr>
<td>Cefotaxime</td>
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<td>0.125</td>
<td>0.064</td>
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<td>0.064</td>
<td>&lt;0.016</td>
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<td>0.094</td>
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<tr>
<td>Cefoxitin</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
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a F1 to F9, subgroups of S. flexneri based on antibiogram and plasmid analysis.

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REFERENCES


