Molecular characterization of multidrug-resistant Shigella species isolated from epidemic and endemic cases of shigellosis in India

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Shigella species represent one of the growing numbers of antimicrobial-resistant bacteria in developing countries. Fluoroquinolone-resistant strains of Shigella dysenteriae type 1 and Shigella flexneri type 2a emerged in India during 2002 and 2003, respectively. Sixty strains of Shigella from different parts of India were analysed for antimicrobial susceptibility, the presence of the qnr plasmid, mutations in the quinolone resistance determining regions (QRDRs), fluoroquinolone accumulation, and the presence of other genes encoding resistance to various antimicrobials. Fluoroquinolone-resistant strains had mutations in gyrA and parC genes and had an active efflux system. They were also resistant to several other antimicrobials but were susceptible to azithromycin and ceftriaxone. The majority of the strains harboured genes encoding resistance to ampicillin (97%), tetracycline (95%), streptomycin (95%) and chloramphenicol (94%). PFGE analysis revealed clonality among strains of S. dysenteriae types 1 and 5, S. flexneri type 2a and Shigella boydii type 12.

INTRODUCTION

Shigelllosis remains an important public health problem in developing countries with Shigella sonnei in Europe and the US and Shigella flexneri in Asian and African countries being of epidemiological importance. Antimicrobial therapy is advocated for shigellosis to shorten the duration of illness (Salam & Bennish, 1991). However, in Asia and Africa, antimicrobial resistance is an emerging problem among Shigella species (von Seidlein et al., 2006) and treatment options are becoming limited globally (Salam & Bennish, 1991; Kariuki & Hart, 2001). The World Health Organization has recommended that ciprofloxacin should be considered a first-line antibiotic for the treatment of shigellosis, and the use of nalidixic acid is not encouraged, even in areas where it is still effective against Shigella (WHO, 2004). Similar to the prevalence of different serotypes, antimicrobial-resistance patterns of strains also differ from country to country and even within the same country (Pazhani et al., 2005; von Seidlein et al., 2006), which may be due to the spread of resistant clones as found for multidrug-resistant strains of Shigella dysenteriae type 1 in eastern parts of India (Pazhani et al., 2004). In this study, we have investigated the mechanisms of antibiotic resistance and clonal relatedness of Shigella strains isolated from epidemic and endemic cases of shigellosis in different parts of India.

METHODS

Bacterial strains. We examined 60 strains of Shigella species (20 S. dysenteriae, 16 S. flexneri, 7 Shigella boydii and 17 S. sonnei) isolated from dysentery outbreaks from different parts of India and sporadic hospitalized cases of shigellosis in Kolkata and Goa between 2001 and 2004. Strains were confirmed as Shigella spp. by standard biochemical tests (WHO, 1987) and serotyped using commercially available antisera (Denka Seiken).

Antimicrobial susceptibility testing. Antimicrobial susceptibility tests were performed by a disc diffusion method in accordance with National Committee of Clinical Laboratory Standards guidelines (NCCLS, 2004) for ampicillin (10 µg), co-trimoxazole (25 µg),...
tetracycline (30 μg), chloramphenicol (30 μg), streptomycin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), ofloxacin (5 μg), ceftriaxone (30 μg) and azithromycin (15 μg) (Becton Dickinson). *Escherichia coli* ATCC 25922 was used for quality control in each batch of tests. MICs of nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin and azithromycin were determined for selected strains by the E-test (AB Biodisk).

**Plasmid isolation and Southern hybridization.** Plasmids were isolated from the representative quinolone- and fluoroquinolone-resistant *Shigella* strains using QIAGEN tip 100. Plasmids were transferred to Hybond-N+ nylon membrane (Amersham Pharmacia) by a capillary method (Sambrook et al., 1989). The membrane was hybridized with a DIG-labelled *qnr* probe, which was amplified from a *qnr*-positive strain (FJ3, *p*<ME23). With published primers (Wang et al., 2003). For hybridization, a DIG-labelling and detection kit was used (Boehringer Mannheim). All strains were also screened with the *qnr* probe by colony hybridization.

**PCR amplification**

**Amplification of the quinolone resistance determining regions (QRDRs).** QRDRs of *gyrA* and *parC* genes were amplified as reported previously (Chu et al., 1998). For each strain, 10 ng of the chromosomal DNA was used in the PCR assay.

**Screening of antimicrobial-resistance genes.** PCR was performed to detect genes encoding resistance to ampicillin (*bla<sub>TEM</sub>*, gentamicin (*aadB*), streptomycin (*aadA1*, *strA*), kanamycin (*aphA1*-*ia*), chloramphenicol (*cata1*), tetracycline [*tet(A),* *tet(B),* *tet(C),* *tet(D)*], *tet(E)* and *tet(Y)*) and *β*-lactams (*bla<sub>OXOXA-1</sub>, *bla<sub>OXOXA-7</sub>, *bla<sub>SEPIA</sub>, *bla<sub>SEPE</sub>-4* and *bla<sub>CTX-M-1</sub>*) and plasmid mediated quinolone resistance (*qnr*) as published for other organisms (Maidhof et al., 2002; Maynard et al., 2003; Robicek et al., 2006). The newly designed primers FMEF (5'-GCA ACG CAA AAA CAA AGT TAG G-3') and FMER (5'-GTG TTT GAA CCA TGT ACA-3') were used to detect *aac(6')-Ib* variants. All assays were carried out as single PCR assays except that for the *qnr* gene, which was performed in a multiplex format, targeting all the three variants of *qnrA*, *qnrB* and *qnrS*. Template DNA was prepared by boiling the cultures grown in Luria–Bertani (LB) broth medium (Diço) for 10 min, rapidly cooled on ice followed by brief centrifugation at 5000 r.p.m.; the supernatant was retained for PCR assays.

**Nucleotide sequencing of the PCR products.** PCR products were purified with a QIAquick PCR purification column (Qiagen), and sequencing reactions were carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Nucleotide sequencing was performed in both directions with the same PCR primers used for the amplification of the target genes in an automatic sequencer (ABI Prism 3200; Applied Biosystems). Contig sequences were edited with DNASTAR (Lasergene) and compared in BLAST of the NCBI database.

**Fluoroquinolone accumulation assay.** Fluoroquinolone-sensitive and -resistant strains of *Shigella* were grown to mid-exponential phase in LB (OD<sub>600</sub> 0.4), harvested, and suspended in 0.2 M MOPS/Tris buffer (pH 7.0) to an OD<sub>600</sub> of 20 ml<sup>-1</sup>. Cells were energized with 0.2% glucose for 20 min and fluoroquinolones were added at a concentration of 10 μg ml<sup>-1</sup>. Aliquots of this mixture were taken and suspended in 1 ml 100 mM glycine/HCl (pH 3.0), shaken for 1 h at room temperature, and the amount of released fluoroquinolone was determined spectrophotometrically with excitation at 277 nm and emission at 448 nm. Experiments were performed in triplicate after the addition of carbonyl cyanide m-chlorophenylhydrazone (CCCP) to the assay mixture, as an inhibitor of the proton-motive force, at a final concentration of 100 μM.

**PFGE.** DNA fingerprinting was carried out by PFGE with the restriction enzyme *XbaI* (Takara) according to a standard procedure (CDC, 2000). PFGE run conditions were generated by the autogrowth mode of the CHEF Mapper system with a size range of 30–600 kb (Bio-Rad). After gel electrophoresis, gels were stained with ethidium bromide for 30 min and destained for 30 min with distilled water. The gel images were digitalized for computer-aided analysis (Gel Doc 2000; Bio-Rad).

**Cluster analysis.** PFGE gel images were retrieved and aligned to generate composite images containing the banding profiles of all the strains. The images were analysed with Diversity Database fingerprinting software (version 2.2.0; Bio-Rad) to determine the relatedness of the strains. Bands ranging from 48.5 to 600 kb were considered for the construction of dendrograms. Degrees of homology were determined by comparison of the Dice coefficient, and clustering correlation coefficients were calculated by an unweighted pair-group method with arithmetic averages (UPGMA). A dendrogram showing the hierarchical representation of the level of linkage between the strains was drawn to predict the degree of clonal relatedness.

**RESULTS AND DISCUSSION**

**Antimicrobial resistance**

Table 1 shows the breakdown of the serotypes of the 60 *Shigella* strains, their antimicrobial-resistance profiles and resistance gene complement. *S. dysenteriae* type 1 strains (*n* = 17) were uniformly resistant to all the tested antimicrobials, except for azithromycin and ceftriaxone. *S. dysenteriae* type 1 strains HU8 and BCH518 isolated during 1988 and 1995 from a dysentery outbreak and sporadic infections, respectively, had similar resistance profiles. The two *S. dysenteriae* type 5 strains were susceptible to ampicillin, fluoroquinolone, azithromycin and ceftriaxone and were resistant to co-trimoxazole, tetracycline, chloramphenicol, nalidixic acid and streptomycin. Except for two strains (NK2685 and NK2683), all the tested *S. flexneri* strains (*n* = 16) were resistant to co-trimoxazole, tetracycline and streptomycin. Three strains of *S. boydii* serotype 12 and the majority (94%) of the *S. sonnei* strains had an identical resistance pattern (co-trimoxazole, tetracycline, nalidixic acid and streptomycin). The MIC for azithromycin-resistant *S. flexneri* type 3b (NK2788) and *S. boydii* type 1 (G24371) was 192 and 128 μg ml<sup>-1</sup>, respectively. None of the other *Shigella* strains proved to be resistant to azithromycin and ceftriaxone, in contrast to a recent report from Bangladesh of resistance to these agents among *Shigella* species (Rahman et al., 2004).

**Fluoroquinolone resistance and resistance mechanisms**

Ciprofloxacin, norfloxacin and ofloxacin are broad-spectrum fluoroquinolone agents that have excellent activity against most enteric pathogens. Clinical studies have underlined their safe use in adults and children (Bhattacharya et al., 1997; Salam et al., 1998). In this study, 30% of the *Shigella* strains were resistant to...
fluoroquinolones and a S. boydii serotype 1 strain (G24371) was resistant to each of the four compounds tested (Table 2). Due to the unrestricted use of fluoroquinolones in Kolkata for the treatment of diarrhoea and other infectious diseases, resistance to these drugs has been reported among enteric pathogens (Garg et al., 2003; Talukder et al., 2004). In India, this trend has been increasing year on year since 2002 among Shigella species (Pazhani et al., 2005). Fluoroquinolone resistance has also been identified in Shigella isolates in many Asian countries (MoezArdalan et al., 2003; Talukder et al., 2004; von Seidlein et al., 2001). At present, fluoroquinolone-resistant S. dysenteriae type 1 and S. flexneri 2a strains remain susceptible to azithromycin and ceftriaxone, which have been reported to be effective against shigellosis in many countries (Khan et al., 1997; Ashkenazi et al., 2003), although cephalosporin resistance has been reported from Spain (Vila et al., 1994) and Argentina (Radice et al., 2001).

S. dysenteriae type 1 strains isolated from sporadic cases of dysentery from Kolkata (BCH518, NK2678 and H16576) and Goa (12567) and outbreak cases from Kolkata, Siliguri, Aizwal and Chandigarh (D2, 21, AZ21 and 115, respectively) were tested for mutations in the gyrA and parC genes. For comparison, we included nalidixic acid-resistant and fluoroquinolone-susceptible S. dysenteriae type 1 strains isolated during 1988 (HU8) and 1995 (BCH518). All fluoroquinolone-resistant strains had a uniform mutation in GyrA at position 83 (replacement of serine with leucine), and the majority of strains had a second mutation in GyrA at position 83, while strain HU8, isolated in Tripura during 1988, had no mutation in GyrA and parC. However, the S. dysenteriae type 1 strain BCH518, isolated during 1995, had a single mutation in GyrA at position 83, while strain HU8, isolated in Tripura during 1988, had no mutation in gyrA and parC and showed reduced susceptibility to nalidixic acid although it was susceptible to fluoroquinolones (Table 2).
Accumulation of norfloxacin and ciprofloxacin in clinical isolates of Shigella strains

<table>
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<tr>
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<td>&gt;256</td>
<td>0.064</td>
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*Na, Nalidixic acid; Cf, ciprofloxacin; Nf, norfloxacin; Of, ofloxacin.

Fluoroquinolone-resistant strains of Shigella strains

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<th>Strain</th>
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<td>4</td>
</tr>
<tr>
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<td>6</td>
</tr>
<tr>
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<td>3</td>
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<td>&gt;256</td>
<td>0.064</td>
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*Data represent the means ± standard deviations of three determinations.

Table 2. Fluoroquinolone resistance and amino acid substitutions in the QRDR of Shigella strains

Table 3. Accumulation of norfloxacin and ciprofloxacin in clinical isolates of Shigella strains

mutation in gyrA. All the fluoroquinolone-resistant strains had a single mutation in ParC at position 80 (replacement of serine with leucine). In a nalidixic acid-resistant S. sonnei strain (NK2017), a mutation was identified at position 83 (replacement of serine with leucine). Fluoroquinolone-resistant strains of S. boydii (G24371), S. flexneri (NK2744) and a representative S. dysenteriae type 1 (12567) strongly exhibited fluoroquinolone efflux (Table 3). The steady state accumulation of norfloxacin and ciprofloxacin was two- to fourfold lower in the resistant strains compared to that in the case of the sensitive strain C152 (Table 3). This suggests that the lower accumulation of fluoroquinolones can also account for the resistance of these strains. After the disruption of the efflux pump with the proton-motive force uncoupler CCCP, the accumulation was almost at the same level in all the tested strains. This clearly suggests that efflux pumps are one of the factors responsible for the development of resistance.

S. dysenteriae type 1 strains from South Asia and Canada had uniform mutations in gyrA and parC (Talukder et al., 2004; CCDR, 2005). A S. dysenteriae strain (BCH518) which was susceptible to fluoroquinolones but resistant to nalidixic acid had an identical mutation in gyrA, similar to that of a 1995 strain isolated in Kolkata (Ahamed et al., 1999). Similarly, mutations in gyrA of S. flexneri and S. sonnei were identical to those reported from other studies (Jeong et al., 2003; Navia et al., 2005). In shigellae, nalidixic acid resistance is not only due to mutations in the QRDR region, but also to an active efflux system (Ahamed et al., 1999). Novel mechanisms for quinolone and fluoroquinolone resistance in members of the Enterobacteriaceae are emerging all the time (Perichon et al., 2007; Yamane et al., 2007), but high-level fluoroquinolone resistance in S. dysenteriae due to a proton-motive force-dependent efflux system was reported almost a decade ago in strains from Kolkata which were devoid of any gyrA mutations (Ghosh et al., 1998).
Plasmid-mediated quinolone resistance due to DNA gyrase protection by a protein from the pentapeptide repeat family called Qnr has recently been described in many clinical isolates of several species (Martinez-Martinez et al., 2003; Jonas et al., 2005). Indeed, clinical strains of S. flexneri type 2b from Japan were found to carry a transferable plasmid, which had 56% amino acid identity with Qnr (Hata et al., 2005). Based on the amino acid sequence, three subtypes of qnr, i.e. qnrA, qnrB and qnrS, and six variants each in qnrA and qnrB and two in qnrS have also been reported (Nordmann & Poirel, 2005; Cattoir et al., 2004). A novel ciprofloxacin-modifying enzyme qnr has recently been described in many clinical isolates of several species (Martinez-Martinez et al., 2005). Indeed, clinical strains of S. flexneri (G24371) and S. dysenteriae I PFGE profile of three belonging to serotype 12 were closely related in DNA profile. Of the seven strains of type 2b. The remainder of the strains showed extensive variation in PFGE profile (Pazhani et al., 2004). Sixteen strains representing different serotypes of S. flexneri showed multi-antibiotic resistance locus of S. flexneri type 2a (Rajakumar et al., 1997) was identified, supporting its common occurrence among several serotypes of S. flexneri (Casalino et al., 1994; Thong et al., 2002). We found 97% and 3% of ampicillin-resistant Shigella strains harbouring blaOXA-1 and blatem-1 genes, respectively. The predominance of blaOXA-1 in Shigella has been reported from many countries (Maraki et al., 1998; Siu et al., 2000; Huang et al., 2005). Similar to the reports from Mexico and Brazil (Martinez-Salazar et al., 1986; Peirano et al., 2005), we found a high frequency of tet(B) among S. flexneri strains, and in common with some South American Shigella strains (Peirano et al., 2005), the presence of the chloramphenicol-resistance gene catA1, which encodes chloramphenicol O-acetyltransferase, and streptomycin resistance was confirmed in S. flexneri strains with either strA or aadA1 genes or both (Table 1).

**PFGE analysis**

It was necessary to determine whether the frequency of antimicrobial resistance and its determinants was due to the widespread occurrence of specific clones. Two S. dysenteriae type 5 strains (NK2440 and NK2454) had identical XbaI restriction patterns by PFGE, but were different from serotype 1 strains, which had similar patterns (Fig. 1), which are akin to the previously reported PFGE profile (Pazhani et al., 2004). Sixteen strains representing different serotypes of S. flexneri showed extensive variation in PFGE profile (Fig. 2) but six strains of type 2a were identical and clustered closest to two strains of serotype 2b. The remainder of the S. flexneri serotypes were distinct in DNA profile. Of the seven S. boydii strains, three belonging to serotype 12 were closely related in DNA profile while the remainder were distinct (Fig. 3). Eleven of the 17 S. sonnei strains were identical in DNA pattern and two strains clustered closely to this group, being different by two to three bands (Fig. 4). This underlines the findings by others of the clonal nature of S. sonnei (Alcoba-Florez et al., 2005; Mammina et al., 2005).

Although PFGE has proved to be a powerful tool for the discrimination of strains and identification of clonal lineages in several bacterial species, in some S. boydii serotypes it may not be as indicative of absolute strain relatedness. Woodward et al. (2005) reported that strains of S. boydii serotypes 1, 18, 19 and 20 from Canada gave highly similar XbaI macrorestriction patterns, which suggests that some strains that express different lipopolysaccharide antigen epitopes share a common genetic background. Other serotypes were genetically heterogeneous. The population structure of this species therefore

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**Resistance to other antimicrobials and resistance genes**

All the S. dysenteriae type 1 strains harboured blaOXA-1, catA1, tet(B) and strA genes, encoding resistance to ampicillin, chloramphenicol, tetracycline and streptomycin, respectively (Table 1). tet(B) was more common (90%) than tet(A) (10%) in S. dysenteriae. Irrespective of serotypes, S. flexneri strains harboured tet(B) as well as blaOXA-1, and in strain NK2788, blaOXA-1, blaTEM-1 and tet(A) genes were detected (Table 1). Group 9 CTX-M β-lactamase has been reported in S. boydii (Vasilev et al., 2007). In this study, blactx-M-3 was found in a S. boydii type 1 strain (G24371) and, to our knowledge, this is the first report of this enzyme in this serotype. The majority of S. sonnei strains harboured strA (88%) and tet(A) (76%) genes rather than aadA1 and tet(B) (6% each). Genes encoding resistance to kanamycin (aphIa), gentamicin (aadB) and tetracycline [tet(C), tet(D), tet(E) and tet(Y)] were not found (data not shown). The chromosomal multi-antibiotic resistance locus of S. flexneri type 2a (Casalino et al., 1994; Thong et al., 2002). We found 97% and 3% of ampicillin-resistant Shigella strains harbouring blaOXA-1 and blatem-1 genes, respectively. The predominance of blaOXA-1 in Shigella has been reported from many countries (Maraki et al., 1998; Siu et al., 2000; Huang et al., 2005). Similar to the reports from Mexico and Brazil (Martinez-Salazar et al., 1986; Peirano et al., 2005), we found a high frequency of tet(B) among S. flexneri strains, and in common with some South American Shigella strains (Peirano et al., 2005), the presence of the chloramphenicol-resistance gene catA1, which encodes chloramphenicol O-acetyltransferase, and streptomycin resistance was confirmed in S. flexneri strains with either strA or aadA1 genes or both (Table 1).

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![Fig. 1. XbaI PFGE profile of S. dysenteriae strains and dendrogram with percentage similarity.](image-url)
Antimicrobial-resistant Shigella strains

**Fig. 2.** XbaI PFGE profile of *S. flexneri* strains and dendrogram with percentage similarity.

**Fig. 3.** XbaI PFGE profile of *S. boydii* strains and dendrogram with percentage similarity.

**Fig. 4.** XbaI PFGE profile of *S. sonnei* strains and dendrogram with percentage similarity.
warrants further investigation with complementary molecular tools such as multilocus sequence typing.

In conclusion, we have identified multidrug resistance among several serotypes of *Shigella* species isolated from acute diarrhoeal patients. Irrespective of the serogroup/serotype, most of the strains carried similar genes encoding resistance to specific antimicrobials. It is evident that fluoroquinolone resistance is spreading across different serogroups of *Shigella* species as they all carried mutations in the gyrA gene. With few exceptions, multidrug-resistant *Shigella* strains belonged to distinct clones.

**ACKNOWLEDGEMENTS**

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