Genetic characteristics and changing antimicrobial resistance among Shigella spp. isolated from hospitalized diarrhoeal patients in Kolkata, India

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To study the prevalence pattern and trends in the phenotypic and genetic characteristics of shigellae, we tested 212 isolates isolated from diarrhoeal patients admitted to the Infectious Diseases Hospital, Kolkata, India, from November 2007 to October 2010. Prevalence of Shigella spp. was higher in the >5 years age group (69 %) than in children in the <5 years age group (31 %). Serotypes 2a, 3a and untypable isolates of Shigella flexneri were frequently detected. An increase in the isolation of Shigella sonnei (15 %) is a novel trend in this region. Fluoroquinolone resistance among S. flexneri serotypes 2a, 3a and other serogroups of shigellae is another evolving trend. The set gene was exclusively present in S. flexneri 2a, and the sen gene was detected in all serogroups. PFGE revealed the grouping of S. flexneri isolates according to their serotypes with approximately 80–100 % similarity, whilst Shigella dysenteriae type 2 and S. sonnei were clonal in nature. There was no demarcation in the prevalence of serotypes, antimicrobial resistance or clonality between the two age groups.

INTRODUCTION

Shigellosis remains a considerable public-health problem in many parts of the world. About 125 million cases of Shigella infection occur annually in Asia, of which approximately 14 000 are fatal (Bardhan et al., 2010). The distribution of serogroups of Shigella spp. differs from country to country: Shigella flexneri, Shigella sonnei and Shigella boydii serogroups are predominant in developing countries, whilst S. sonnei is frequently reported in industrialized countries. Prevalence of Shigella dysenteriae is mostly reported from South Asia and sub-Saharan Africa (Kotloff et al., 1999). Unlike other acute diarrhoeal illnesses that require adequate fluid replacement by either oral or intravenous rehydration, shigellosis needs antimicrobial therapy to reduce the duration of the illness and to prevent transmission to close contacts. Shigella spp. have developed perpetual resistance to ampicillin and trimethoprim–sulfamethoxazole, which have been used in paediatric cases during the past two decades. Fluoroquinolones, ceftriaxone and pivmecillinam are the antibiotics currently recommended by the World Health Organization for the treatment of dysentery in children, as these drugs are effective at reducing mortality caused by Shigella spp. (Traa et al., 2010). These drugs are also effective among immunocompromised children, including neonatal patients, suffering from infections caused by multi-drug-resistant strains of Salmonella and Shigella spp. (Leibovitz, 2006). The use of antimicrobials for the treatment of shigellosis varies from country to country. Pivmecillinam is not the drug of choice for the treatment of diarrhoea in many countries, but is being used in Bangladesh (Rahman et al., 2007). Ceftriaxone is an ideal drug for the treatment of shigellosis in regions where fluoroquinolone resistance is common. The American Academy of Pediatrics and the Infectious Diseases Society of America recommend azithromycin as an alternative drug for the treatment of shigellosis (Jain et al., 2005) and this drug was found to be effective against multi-drug-resistant Shigella spp. in many countries.

Due to the distribution of diverse serogroups/serotypes and the combination of resistance to several antimicrobial drugs, treatment failures are often seen in Shigella-mediated infections. Hence, it is very important to understand the serogroup(s) and antimicrobial-resistance patterns of Shigella spp. prevailing in different geographical regions for instituting effective treatment.

In Kolkata, multi-drug resistance in Shigella spp. has been increasingly reported since the emergence of fluoroquinolone-resistant strains of epidemic S. dysenteriae type 1

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(Pazhani et al., 2005). This trend has been rising among diverse serogroups of Shigella spp., not only in Kolkata but also in other parts of India (Srinivasa et al., 2009). In this study, we analysed the extent of antimicrobial resistance, distribution of virulence genes and clonality of diverse serogroups of Shigella isolates from hospitalized children and adult patients with acute diarrhoea.

**METHODS**

**Bacterial strains.** From November 2007 to October 2010, 212 (6.5 %) Shigella isolates were isolated from 3262 enrolled diarrhoeal patients admitted to the Infectious Diseases Hospital, Beliaghata, Kolkata, India. Stool specimens collected from these patients were examined for common enteric pathogens within 2 h, using standard microbiological methods (Nair et al., 2010; WHO, 1987). Biochemically identified Shigella isolates were confirmed serologically by slide agglutination using commercially purchased antisera (Denka Seiken Co. Ltd). The untypable Shigella isolates were tested with a provisional monoclonal antisemum (# 88-893; a gift from N. Stockbine, CDC, Atlanta, GA, USA). Institutional ethical clearance was obtained to conduct this study.

**Antimicrobial-susceptibility testing and determination of MIC.** Antimicrobial-susceptibility testing was performed on all Shigella isolates by a disc-diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2010), using commercially available ampicillin (10 μg), azithromycin (15 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), co-trimoxazole (25 μg), furazolidone (100 μg), nalidixic acid (30 μg), norfloxacin (10 μg), ofloxacin (5 μg) and tetracycline (30 μg) discs (BD) on Mueller–Hinton agar (Difco). MICs of nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin and ceftriaxone were determined by using an E-test (AB bioMérieux), following the manufacturer’s instructions. *Escherichia coli* ATCC 25922 was used as the quality-control strain for each batch of the assay.

**Detection of virulence genes.** Virulence genes such as *stx* (ShET-1), *sen* (ShET-2), *ipaH*, *ial*, *sat* and *virF* were detected by a simplex PCR assay using published primer pairs (Frankel et al., 1989; Thong et al., 2005).

**PFGE.** PFGE of *NotI* or *XhoI*-digested genomic DNA was performed using a CHEF-Mapper (Bio-Rad Laboratories) according to the PulseNet standardized protocol for subtyping of *Shigella* species (Ribot et al., 2006). PFGE images were captured by using a Gel Doc XR system (Bio-Rad). The gel images were normalized by aligning the peaks of the *Salmonella braenderup* size standard and analysed by using the BioNumerics software version 5.0 (Applied Maths). Degree of banding similarity was determined by comparison of the Dice coefficient, and the clustering correlation coefficients were calculated by UPGMA.

**RESULTS**

During this study, 212 (6.5 %) Shigella isolates were identified from 3262 diarrhoeal patients. The numbers and proportions of different Shigella serogroups detected were 160 (75.5 %) *S. flexneri*, 33 (15.6 %) *S. sonnei*, 14 (6.6 %) *S. boydii* and 5 (2.3 %) *S. dysenteriae*. Throughout the study period, *S. flexneri* was the most common serogroup, with a predominance of serotype 2a during 2009. After serotype 2a, prevalence of *S. flexneri* 3a was the next highest in this study. Interestingly, 19 (11.8 %) *S. flexneri* isolates agglutinated only with polyvalent type B antisera; complete serotyping of these isolates was not accomplished with commercially available Shigella antisera and none of them reacted with the provisional *S. flexneri* monoclonal antisemum (# 88-893). These isolates were designated untypable. *S. dysenteriae* types 1 and 3 (one isolate each) and three isolates of type 2 were isolated in this study. Among 14 *S. boydii* isolates, type 12 was relatively common (four isolates) and the others were represented by serotypes 1, 11 and 15 (two isolates each) and serotypes 9, 18 and 10 (one isolate each). One strain agglutinated with only polyvalent *S. boydii* antisemum, but not with any of the monovalent antisera.

More than 75 % of Shigella-positive patients were infected with *S. flexneri*, followed by *S. sonnei* (15.6 %). About 31 and 69 % of Shigella-infected patients were children of <5 years and >5 years of age, respectively. Ninety per cent of the Shigella isolates in this study were resistant to multiple drugs and the prevalence of different resistance patterns is shown in Table 1. Overall, the majority of the isolates were resistant to trimethoprim–sulfamethoxazole (94.3 %), nalidixic acid (93.4 %), tetracycline (88.7 %), ciprofloxacin, norfloxacin, ofloxacin (85.8 % each), furazolidone (79.2 %) and chloramphenicol (62.7 %). Resistance to ampicillin (53.8 %) and azithromycin (34.4 %) was moderate, whilst much lower resistance was observed to the third-generation cephalosporin ceftriaxone (1.9 %). Among *S. flexneri*, serotype 2a was totally resistant to nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin and streptomycin. Ninety-six per cent of isolates were resistant to co-trimoxazole and tetracycline and 90 % of isolates were resistant to ampicillin and chloramphenicol. Resistance to azithromycin was detected among 32 % of isolates, whilst 1.3 % of isolates were resistant to ceftriaxone. MIC ranges of *S. flexneri* type 2a for nalidixic acid, norfloxacin, ciprofloxacin and ofloxacin were >256, 20–24, 5–8 and 8–16 μg ml⁻¹, respectively. This trend was more or less similar to the results for the other *S. flexneri* serotype, 3a. *S. sonnei* isolates, on the other hand, were susceptible (100 %) to ampicillin and ceftriaxone. The majority of the *S. sonnei* isolates were resistant to ciprofloxacin (94 %), norfloxacin (94 %), ofloxacin (94 %), co-trimoxazole (94 %) and tetracycline (85 %), but resistance to azithromycin was lower (27 %). The MIC ranges of *S. sonnei* for nalidixic acid, norfloxacin, ciprofloxacin and ofloxacin were >256, 12–20, 4–6 and 8–12 μg ml⁻¹, respectively. Fluoroquinolone resistance was lower in *S. boydii*, with the exception of one isolate (serotype 18) that was highly resistant to all antimicrobial agents tested and its MICs for nalidixic acid, norfloxacin, ciprofloxacin, ofloxacin and ceftriaxone were >256, >256, >32, >32 and >32 μg ml⁻¹, respectively. MIC ranges of other *S. boydii* isolates for nalidixic acid, norfloxacin, ciprofloxacin and ofloxacin were 7 to >256, 0.38–6, 0.22–1.5 and 0.38–4 μg ml⁻¹, respectively. Only one *S. dysenteriae* type 1 isolate was identified in this study during 2010, and it was resistant to all antimicrobials tested.
except ceftriaxone and azithromycin. All *S. dysenteriae* type 2 isolates exhibited reduced susceptibility to fluoroquinolones. MIC ranges of *S. dysenteriae* for nalidixic acid, norfloxacin, ciprofloxacin and ofloxacin were 2.5–14, 0.125–1.5, 0.109–0.315 and 0.047–0.5 μg ml⁻¹, respectively, and no *S. dysenteriae* isolates were resistant to ceftriaxone.

All isolates tested were positive for the invasive plasmid antigen H (*ipaH*) gene, and 75% of *Shigella* isolates harboured a virulence plasmid (*virF*). The secreted autotransporter toxin Sat, encoded by the *sat* gene harboured by 73.5% of the *Shigella* isolates (156 of 212), was present in three of five (60%) *S. dysenteriae*, 146 of 160 (91.2%) *S. flexneri* and seven of 14 (50%) *S. boydii* isolates. None of the *S. sonnei* isolates harboured this gene.

### Table 1. Antimicrobial-resistance patterns of *Shigella* species and their serotypes

<table>
<thead>
<tr>
<th>Resistance profile</th>
<th>Species/serotype (n)</th>
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<tr>
<td>AM, AZM, C, CIP, CRO, FX, NA, NOR, OFX, SXT, TE</td>
<td>Sb 18 (1), Sf UT (1)</td>
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<tr>
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<td>Sf 1b (1), Sf 2a (32), Sf 3a (2), Sf Y (1), Sf 2b (1), Sd 1 (1)</td>
</tr>
<tr>
<td>AZM, C, CIP, FX, NA, NOR, OFX, SXT, TE</td>
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<td>Sf 2a (11), Sf 3a (1), Sf 4 (1)</td>
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<td>Sd 2 (1)</td>
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<td>AM, SXT, SXT</td>
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<td>FX, NA, SXT</td>
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<td>AM, AZM, FX</td>
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<td>AM, FX</td>
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<td>AM, FX</td>
<td>Sf UT (1)</td>
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variant Y [agglutinated with polyvalent B and group 3(4) sera] harboured only the ShET1 subunit A gene. The sen gene encoding ShET2 was present in 194 of 212 (91.5%) Shigella isolates, more specifically in 144 of 160 (90.0%) S. flexneri, 32 of 33 (96.9%) S. sonnei, 13 of 14 (92.8%) S. boydii and all S. dysenteriae (100%). The invasive-associated locus, encoded by the ial gene, was present in 164 of 212 (77.3%) Shigella isolates, and was most predominant among S. flexneri (62.2%), followed by S. sonnei (8.4%), S. boydii (4.2%) and S. dysenteriae (2.3%). One S. dysenteriae type 1 isolate in this study harboured the stxl gene encoding Shiga toxin.

PFGE analysis of representative serotypes of S. flexneri after digestion with NotI revealed serotype-specific clusters, with approximately 80–100% similarity within each serotype (Fig. 1). When XbaI-digested genomic DNAs of current S. dysenteriae type 1 isolates and...
epidemic strains isolated previously in this region were compared, the recent isolates were identified as belonging to a different clone. *S. dysenteriae* type 2 isolates were identified as closely related (>90% band similarity; Fig. 2) and the representative *S. sonnei* isolates were observed to be clonally related (less than three bands difference; Fig. 3).

**DISCUSSION**

Shigellosis remains a considerable public-health problem in many developing countries and one of the most common enteric bacterial infections in the USA (Wong et al., 2010). *Shigella* spp. are phenotypically variable, as evidenced by variations in serotypes and antimicrobial-resistance profiles in different endemic locations. Many studies have highlighted the heterogeneous distribution of *Shigella* sereo- groups (Pazhani et al., 2005; Taneja et al., 2004). In addition, an increasing number of *Shigella* isolates have been identified as untypable by serotyping in many countries (Isenberger et al., 2001; Roy et al., 2006; Zafar et al., 2009). Unusual isolates of *S. flexneri* were first reported from travellers in Japan with diarrhoea (Matsushita et al., 1992) and from a restaurant-associated outbreak in California (Trevejo et al., 1999). In Bangladesh, about 20% of *S. flexneri* isolates were untypable and some of them cross-agglutinated with *S. boydii*-specific antisera (Talukder et al., 2001). Emergence of *S. flexneri* serotype 1c was identified in Bangladesh, Egypt, Indonesia and Pakistan. In the province of Son Tay, Vietnam, this serotype accounted for about 40% of *S. flexneri* isolates (Stagg et al., 2008). Interestingly, the untypable *S. flexneri* isolates in this study did not react with provisional serovar *S. flexneri* 1c monoclonal antiserum (*S. flexneri* serotype 1c and 88-893 antiserum) and also did not cross-react with other *Shigella* serogroup/serotype-specific antisera. As reported previously (Pazhani et al., 2005), *S. flexneri* type 2a continued to dominate the other serotypes. As seen in this study, prevalence of *S. flexneri* type 3a is increasing along with Y variants. The X variant of *S. flexneri* [agglutinated with polyclonal B and group 7(8) sera] appeared in China during 2001, replaced the predominant serotype 2a in 2003 and spread to several other provinces in 2007 (Ye et al., 2010). In Thailand, this variant was the second-most predominant serotype after *S. flexneri* type 2a and reduced over a period of time (Bangtrakulnonth et al., 2008).

After the dysentery outbreaks in eastern India in the early 1980s, *S. dysenteriae* type 1 re-emerged in 2002 as epidemics in several parts of India (Pazhani et al., 2004). Since 2002, isolation of this serotype has steadily declined compared with *S. flexneri*, and *S. dysenteriae* type 1 was not isolated from 2005 in Kolkata or other locations such as Bangladesh (Talukder et al., 2006a). We have identified one *S. dysenteriae* type 1 isolate in 2010, indicating the existence of an isolate that has epidemic potential in our setting. Prevalence of other serotypes of *S. dysenteriae*, including serotypes 2 and 3, was very low. *S. dysenteriae* type 2 occurred in Bangladesh between 1999 and 2002 and caused an unusual outbreak (Talukder et al., 2006b). In India, except for *S. dysenteriae* type 1, other *Shigella* serotypes have rarely been involved in outbreaks/epidemics. Shigellosis caused by *S. sonnei* is an emerging trend in India and has been increasingly detected during the past two decades. A similar increase in the incidence of this serotype with greater epidemiological impact has been reported from many Asian countries (Kuo et al., 2008; Vrints et al., 2009).

In India, antimicrobial resistance in the genus *Shigella* is more common than in any other enteric bacteria (Taneja et al., 2004). Subsequent to the emergence of nalidixic acid-resistant isolates all over the world during the 1990s, fluoroquinolones, especially ciprofloxacin, have been used for the treatment of shigellosis. However, in 2002, the appearance of *Shigella* isolates resistant to fluoroquinolones was reported, first with *S. dysenteriae* type 1. From that time, fluoroquinolone resistance has increased in developing countries, and also among other serogroups of *Shigella*. After *S. dysenteriae* type 1 acquired fluoroquinolone resistance, its incidence began declining and finally
this serotype was isolated rarely, if at all. The first appearance of ciprofloxacin-resistant *S. flexneri* type 2a was identified in Kolkata, India, in 2003 and in the following year this resistance was 41% (Pazhani et al., 2005). During 2007–2009, this resistance increased to 91.6%. The same trend has also been reported in many South Asian countries (Kuo et al., 2008; von Seidlein et al., 2006). The incidence of fluoroquinolone resistance is rising year by year in other serogroups of *Shigella* spp. Six of 19 untypable *S. flexneri* isolates encountered in this study showed reduced susceptibility to fluoroquinolones and all were resistant to nalidixic acid. In contrast, *S. flexneri* isolates from north-west Ethiopia were found to be resistant to fluoroquinolones, but susceptible to nalidixic acid (Tiruneh, 2009). An upsurge in fluoroquinolone resistance among *S. flexneri* and *S. sonnei* in developed countries has been reported, but these serogroups were isolated more frequently from travellers who had a history of visiting developing countries (Drews et al., 2010). In a previous study, *S. sonnei* isolates were resistant to nalidixic acid, but none were resistant to ciprofloxacin (Pazhani et al., 2005). During 1999–2003, >60% of Bangladeshi *S. sonnei* isolates were resistant to nalidixic acid, but none to ciprofloxacin (Talukder et al., 2006a). In developed countries, although nalidixic acid resistance was common among *S. sonnei* isolates, the trend of fluoroquinolone resistance is slowly increasing (Kim et al., 2008; Vrints et al., 2009). It is worthwhile mentioning that all *S. sonnei* isolates in this study were susceptible to ampicillin, which is not used in the treatment of shigellosis in Kolkata. Since 2002–2004, quinolone-resistant *S. boydii* strains have not been detected in this region. In the current study, 71.4% of isolates were resistant to nalidixic acid and 42.8% of isolates showed reduced susceptibility to fluoroquinolones. We have encountered few *S. boydii* isolates resistant to both fluoroquinolones and ceftriaxone in 2001 and 2008. The *set* gene (ShET-1) was exclusively present in *S. flexneri* serotype 2a, which was similar to other findings (Roy et al., 2006). In this study, one *S. flexneri* serotype variant Y harboured the *sen* gene, indicating the spread of this gene to other serotypes. As reported in the Andaman Islands, India (Roy et al., 2006), the *sen* (ShET-2) gene was detected in all isolates of this study. Only the *S. dysenteriae* type 1 strain carried the stx1 gene, which is a universal trend. PFGE analysis revealed that *S. flexneri* isolates were grouped into separate clusters according to serotypes. *Shigella* isolates belonging to each serotype had approximately 80–100% similarity (Fig. 1), in accordance with other such findings (Ahmed et al., 2006; Sirisiriro et al., 2006). *S. dysenteriae* type 1 isolated in this study was a different clone from the previous isolates from this region (Fig. 2). The three serotype 2 isolates were clonal in nature. The *S. sonnei* isolates seem to be genetically related, as the majority of them are highly similar, with many subclones. A comparable observation was reported in China (Zhang et al., 2011). The trend in antimicrobial resistance changes year by year, especially as the previously susceptible *Shigella* serogroups are now resistant to most of the effective antimicrobials used in the treatment of shigellosis. The current antimicrobial resistance towards fluoroquinolones indicates that these drugs will no longer be effective and emphasizes the need for using alternative drugs for the treatment of shigellosis.

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