Cephalosporin-resistant Shigella flexneri over 9 years (2001–09) in India

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Objectives: To determine the pattern and antimicrobial resistance genes of cephalosporin resistance in Shigella flexneri and Shigella dysenteriae over 9 years.

Methods: Isolates of Shigella (S. flexneri, n = 119 and S. dysenteriae, n = 24) were tested for resistance to ceftiraxone and cefepime by disc diffusion, for MIC by Etest and for extended-spectrum β-lactamase (ESBL) and AmpC production. The presence of antimicrobial resistance genes was investigated by PCR using specific primers for blaTEM, blaOXA-1, blaCTX-M-15, blaSHV and blaCMY-2 for all the isolates.

Results: Twenty (16.8%) S. flexneri isolates were resistant/intermediately susceptible to ceftiraxone/cefeplime, while all S. dysenteriae were susceptible. In S. flexneri isolates, the MIC50 values of ceftiraxone and cefepime were found to be 0.032 and 0.125 mg/L, respectively, while their MIC90 values were 12 and 8 mg/L, respectively. The MIC90 for S. dysenteriae was below 1 mg/L for ceftiraxone; however, for cefepime the MIC90 was found to be 4 mg/L. Of the 20 resistant/intermediately susceptible S. flexneri isolates, 9 were positive for ESBL production and 4 for AmpC production by phenotypic tests. All 20 isolates were found to be positive for blaTEM, 10 for blaCTX-M-15, 8 for blaOXA and 7 for blaCMY-2; none was positive for blaSHV.

Conclusions: We report a high level of cephalosporin resistance with high MICs and ESBL- and AmpC-mediated antibiotic resistance in Shigella from north India.

Keywords: multidrug resistance, extended-spectrum β-lactamases, ESBLs, AmpC

Introduction

Shigellosis is one of the most common causes of morbidity and mortality in children in developing countries. In a recent multicentre study from six Asian countries, China, Vietnam, Thailand, Bangladesh, Pakistan and Indonesia, the incidence of treated shigellosis was found to be 13.2 per 1000 per year in children under 60 months.1 Antibiotic treatment is usually recommended for shigellosis as it reduces the duration and severity of symptoms, reduces excretion of organisms2 and prevents potentially lethal complications.3 Increasing antimicrobial resistance of the Shigella spp. has been reported worldwide and this has emerged as a therapeutic challenge.4–8

In 1990, the WHO recommended ciprofloxacin as the drug of choice for empirical treatment of shigellosis in view of the existing high level of resistance to agents like ampicillin, chloramphenicol, nalidixic acid and co-trimoxazole.9 Ciprofloxacin has been highly effective in the treatment of shigellosis, but, most likely due to overuse and misuse, resistance has emerged for this as it has for the other agents.10–13 Ciprofloxacin resistance first emerged in Shigella dysenteriae serotype 1 in south-east Asia in 2001, with an epidemic of bacillary dysentery in tea gardens of north-east India.16 Fluoroquinolone resistance in Shigella flexneri type 2a emerged in December 2003; however, Shigella boydii and Shigella sonnei remain susceptible.15,16 At our tertiary care referral centre in northern India, an outbreak of ciprofloxacin-resistant S. dysenteriae serotype 1 occurred in 2003. Isolates were found to be resistant to ciprofloxacin, and S. dysenteriae serotype 1 emerged as the predominant serogroup after a decade. PFGE of the isolates performed at the National Institute of Cholera and Enteric Diseases, Kolkata, India, showed these strains to be same as those causing outbreaks in north-east India and those isolated from sporadic cases of shigellosis in Nepal and Bangladesh. A serogroup switch occurred in 2004, when S. flexneri again became the predominant serogroup. However, in 2005 ciprofloxacin resistance also emerged in S. flexneri and this became a therapeutic challenge in our region.16

Ceftiraxone is recommended for the treatment for such ciprofloxacin-resistant infections.17 To monitor antimicrobial resistance to cephalosporins we have enhanced our antimicrobial surveillance by determining the MIC for every clinical isolate of S. flexneri and S. dysenteriae and for our collection of Shigella...
isolates of the last 9 years, for resistance to ceftriaxone and ceftetrom, for extended-spectrum β-lactamase (ESBL) and AmpC production, and for antimicrobial-resistance genes of cephalosporin resistance.

Materials and methods

The study was carried out at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, a tertiary care centre to which patients are referred from different parts of north India (mainly from seven states: Punjab, Haryana, Himachal Pradesh, Chandigarh, western parts of Uttar Pradesh, Jammu and Kashmir and some parts of Rajasthan). Stool specimens of patients suffering from diarrhoea/dysentery admitted to the wards or presenting at the outpatient departments of PGIMER from 2001 to 2009 were received in the Enteric Laboratory of PGIMER. The study was approved by the Institute Ethics Committee of PGIMER.

Specimen collection, transport, processing and identification

The specimens were collected in Cary Blair medium and transported to the laboratory where they were inoculated on MacConkey agar (MCA), xylose lysine deoxycholate agar (XLD) and into Selenite F broth for subculture after 6 h (Himedia Laboratories Pvt. Ltd, Mumbai, India). Non-lactose-fermenting colonies and red colonies from the MCA and XLD plates were picked and subjected to further analysis by biochemical tests for the identification and isolation of possible Shigella colonies.18 Isolates were then confirmed by serotyping (Denka-Seiken, Tokyo, Japan). Isolates that were identified as Shigella spp. were stored at −70°C in brain/heart infusion broth with 15% glycerol for further studies.

Bacterial strains

All S. flexneri and S. dysenteriae strains were revived from our culture collection for the study. The isolates were confirmed by biochemical testing and serotyping (Denka-Seiken, Tokyo, Japan). Stool specimens of patients suffering from diarrhoea/dysentery were cultured after 6 h (Himedia Laboratories Pvt. Ltd, Mumbai, India). Non-lactose-fermenting colonies and red colonies from the MCA and XLD plates were picked and subjected to further analysis by biochemical tests for the identification and isolation of possible Shigella colonies. Isolates were then confirmed by serotyping (Denka-Seiken, Tokyo, Japan). Isolates that were identified as Shigella spp. were stored at −70°C in brain/heart infusion broth with 15% glycerol for further studies.

Susceptibility and MIC testing

Antibiotic susceptibility was determined using the Kirby Bauer disc diffusion method for the following antibiotics (Oxoid Limited, Hampshire, UK): amoxicillin (10 μg), co-trimoxazole (25 μg), nalidixic acid (30 μg), furazolidone (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), ofloxacin (5 μg), gentamicin (10 μg), amikacin (30 μg), ceftaxime (30 μg), ceftriaxone (30 μg) and azithromycin (15 μg), according to CLSI guidelines, 2010.17 All the isolates were subjected to MIC determination for ceftriaxone and ceftetrom by an Etest (bioMérieux India Pvt. Ltd, New Delhi, India) following the manufacturer’s instructions. The isolates were classified as susceptible (MICs ≤1 and ≤8 mg/L), intermediate (MICs 2 and 16 mg/L), or resistant (MICs ≥4 and ≥32 mg/L), to ceftriaxone and ceftetrom, respectively.19

ESBL and AmpC detection

Isolates with MICs >1 mg/L for either ceftriaxone or ceftetrom were screened for ESBL production by the phenotypic confirmatory method of CLSI using the disc potentiation method for ceftetrom, cefpodoxime and ceftazidime, along with discs to which clavulanic acid had been added. AmpC detection was done by the EDTA disc method as described by Block et al.20

PCR

PCR was performed for blaTEM, blaOXA-1, blaCTX-M-15, blaSHV and blaCMY-2 for all the isolates to identify the presence of the resistance genes, using the primers described in Table 1.21

Statistical analysis

A χ2 test for linear trends was applied to assess the trends of resistance to cephalosporins in the Shigella isolates over 9 years. A one-way ANOVA test with Bonferroni correction was used to determine the significance in year-wise changes to MICs of ceftriaxone and ceftetrom. The data were analysed using SPSS software (version 15.0).

Results

Of 8790 stool specimens from 2001 to 2009, 265 Shigella isolates were obtained (170 S. flexneri, 52 S. dysenteriae, 20 S. sonnei, 13 S. boydii and 10 non-agglutinable shigellae). Of these, 119 S. flexneri and 24 S. dysenteriae isolates were available in our culture collection for the present study; the rest of the isolates could not be revived. Out of the 119 S. flexneri isolates, 20 (16.8%) were found either to be resistant or to have intermediate susceptibility to at least one of the cephalosporins tested, while all the S. dysenteriae strains were susceptible to these drugs. Of the S. flexneri isolates, 18 (15.1%) were resistant

<table>
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<th>Sequence</th>
<th>Size (bp)</th>
<th>Origin/reference</th>
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<td>TEM-F</td>
<td>S'-AGAGATTTCAACATTTCCGC-3'</td>
<td>859</td>
<td>Peirano et al.21</td>
</tr>
<tr>
<td>TEM-R</td>
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<td>Peirano et al.21</td>
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<tr>
<td>OXA-1-F</td>
<td>S'-AGTAAAAACACAGAAAATATCAACGTCGC-3'</td>
<td>318</td>
<td>this study (GenBank AF282921.1)</td>
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<tr>
<td>OXA-1-R</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SHV-F</td>
<td>S'-AGCCGCTTGAGCAAATAAA-3'</td>
<td>820</td>
<td>Peirano et al.21</td>
</tr>
<tr>
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<td>CTX-M-15-F</td>
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<td>CMY-2-F</td>
<td>S'-AAATGCCTGTATGCTGCTTAC-3'</td>
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<td>CMY-2-R</td>
<td>S'-CCGTCTTATCCGCGGCGCACG-3'</td>
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to at least one of the third-generation cephalosporins tested (ceftriaxone/cefotaxime), while 7 (5.9%) isolates were resistant and 3 (2.5%) had intermediate susceptibility to cefepime. Cephalosporin resistance was first seen in 2001, and has been more frequently observed since 2005 (Figure 1). The trend in cephalosporin resistance was not found to be significant by the $\chi^2$ test for linear trends over 9 years ($\chi^2=11.3$, df=8, $P=0.184$); this may be due to the small number of isolates per year. A retrospective analysis of the disc diffusion susceptibility data from 1990 to 2000 showed absence of any resistance to third-generation cephalosporins during that period; the pre-2001 pool of isolates were, however, not available in our culture collection. In S. flexneri isolates, the MIC$_{50}$ values of ceftriaxone and cefepime were found to be 0.032 and 0.125 mg/L, respectively, while their MIC$_{90}$ values were 12 and 8 mg/L, respectively (Table 2). We applied a one-way ANOVA test with Bonferroni correction to determine the year-wise difference in MICs of ceftriaxone and cefepime and found no statistical significance in the rise in MICs over the 9 years ($P=0.301$ and 0.237 for ceftriaxone and cefepime, respectively). The MIC$_{50}$ and MIC$_{90}$ for S. dysenteriae remained below 1 mg/L of ceftriaxone; however, of cefepime the MIC$_{90}$ was found to be 4 mg/L. Figure 1 shows the year-wise distribution and MIC values for the isolates.

Of the 119 S. flexneri isolates, a total of 25 isolates with MICs $\geq$1 mg/L for either ceftriaxone (17/119) or cefepime (20/119) were screened for the presence of ESBLs and AmpC. Of these, 20 were either resistant or had intermediate susceptibility to at least one of the cephalosporins, and 5 were cephalosporin susceptible (they did not show production of ESBLs or AmpC enzymes). Of these 20 isolates, 9 were found to be positive for ESBL and 4 were positive for AmpC production by phenotypic tests (Table 3). All 9 ESBL-positive isolates had very high MICs of ceftriaxone ($\geq$256 mg/L). Out of the 18 isolates resistant to third-generation cephalosporins, 9 and 2 were positive for ESBL and AmpC production, respectively, while all 7 cefepime-resistant isolates were found to be positive for ESBL production, but none was positive for AmpC. All the isolates were found to be positive for bla$_{TEM}$-10 for bla$_{CTX-M-15}$, 8 for bla$_{OXA}$, and 7 for bla$_{CMY-2}$; none was positive for bla$_{SHV}$ (a laboratory strain of uropathogenic Escherichia coli producing SHV was used as a positive control for bla$_{SHV}$). On sequencing the ampli- cobs of bla$_{TEM}$, bla$_{OXA}$, bla$_{CTX-M-15}$ and bla$_{CMY-2}$, these were found to be 99%, 98%, 100% and 99% identical to the corresponding NCBI GenBank sequences (accession numbers HQ877616.1, EU752483.1, GO345158.1 and HQ680723.1, respectively). The sequences have been submitted to the NCBI GenBank database.

On clinical chart review of the 20 cases, there was an equal male (10) to female (10) distribution. There were 12 children (2 were <1 year, 8 were 1–5 years and 2 were 5–14 years of age) and 8 adults (age range of 20 cases: 2 months to 65 years with a mean age of 19.4 years); 16 patients were admitted and 4 were outpatients; 9 patients presented with acute dysentery, 3 with chronic dysentery of more than 1 month duration, 3 with chronic diarrhoea, 2 with post-renal transplant-associated diarrhoea, and 1 each with haemolytic uremic syndrome, carcinoma rectum and ulcerative colitis-associated diarrhoea.

Discussion

This study reports resistance of S. flexneri and S. dysenteriae to cephalosporins, and the molecular analysis of cephalosporin resistance genes in isolates from North India collected over a period of 9 years. Overall, 16.8% of the S. flexneri were found to be either resistant or had intermediate susceptibility to at least one of the cephalosporins tested, while all the S. dysenteriae strains were susceptible to these drugs. Among the S. flexneri isolates, 15.1% were found to be resistant to at least one of the third-generation cephalosporins (ceftriaxone/cefotaxime). Studies from South-East Asia have reported the resistance of Shigella spp. to cephalosporins at 2.0%–5.2%; however, there was a higher percentage of cephalosporin-resistant isolates (15.1%) in our study, compared with these reports. In a recent report from Kolkata, Nair et al. observed an emerging resistance to ceftriaxone (to 5.2%) in Shigella isolated from 2001 to 2009. In neighbouring regions, Khan et al. have reported 2.4% resistance to ceftriaxone in Pakistan in 1573 isolates of Shigella spp. from 1996 to 2007. Similarly, in Bangladesh 2% of Shigella isolates were found to be resistant to ceftriaxone/cefotaxime during 2001–02 as compared with 1991–92. A large multicentre study in eight Asian countries (Korea, Taiwan, Singapore, Thailand, Vietnam, Philippines, Hong Kong and Sri Lanka) from 2001 to 2004 also observed increased resistance to ceftriaxone (5%) in Shigella isolates. The high resistance observed in our isolates may not reflect the true prevalence of cephalosporin resistance in Shigella spp. in the community as ours is a tertiary care referral centre that receives severe and complicated cases that are often multiply treated before presenting to our hospital.

The first isolate showing cephalosporin resistance was obtained in 2001, and we have observed an increase in the number of S. flexneri isolates resistant to third-generation cephalosporins since 2005. Although the pre-2001 pool of isolates was not available in our culture collection, the disc diffusion susceptibility data available from 1990 onwards showed the absence of any resistance to third-generation cephalosporins. We did not observe significant linear trends of increasing resistance to cephalosporins over 9 years ($P=0.184$), which may be due to the small number of isolates per year. There was a loss of isolates from our pool of S. flexneri (all isolates could not be revived), though this did not produce a statistical bias in the analysis as the loss was uniform across the years. Moreover, the resistance seems to be fluctuating, which may be due to plasmid-mediated carriage of the resistance determinants within members of the Enterobacteriaceae family. We observed high MIC values of ceftriaxone and cefepime, the MIC$_{90}$ values being 12 and 8 mg/L, respectively. However, the rise in MICs of ceftriaxone and cefepime was not found to be statistically significant over 9 years ($P=0.301$ and 0.237 for ceftriaxone and cefepime, respectively). MIC values for S. dysenteriae remained below 1 mg/L for ceftriaxone; however, of cefepime the MIC$_{90}$ was found to be 4 mg/L. Although the number of isolates (24) of S. dysenteriae was too small to determine the exact MIC trends, nevertheless it can be taken as an indicator of rising resistance. The WHO now recommends ceftriaxone, pivme-

Cephalosporin resistance in Shigella
Figure 1. Year-wise distribution of MICs of (a) ceftriaxone and (b) cefepime for 119 S. flexneri isolates from 2001 to 2009.
fluoroquinolone-resistant shigellae. At our centre, the infections caused by ceftriaxone-resistant *S. flexneri* were successfully treated with azithromycin.

The molecular analysis of antimicrobial resistance genes in *Shigella* spp. in our study revealed the production of *bla*<sub>TEM</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>OXA-1</sub> and *bla*<sub>CMY-2</sub>. While *bla*<sub>SHV</sub> was not found in any of the strains, *bla*<sub>TEM</sub> was the most common ESBL gene (this closely resembled *bla*<sub>TEM-116</sub>), followed by *bla*<sub>CTX-M-15</sub>, which is the most common ESBL gene found in the Enterobacteriaceae family in India. The first isolate of *S. flexneri* producing an ESBL was reported from France in 1995 and harboured a plasmid that encoded the *bla*<sub>SHV-2</sub> gene. Subsequently, there have been other reports of *Shigella* spp. harbouring different types of ESBL genes, from Israel, Argentina, Canada, Lebanon, Turkey, Korea, Japan, China and various other regions in Asia.

Molecular characterization of the identified ESBLs has revealed the production of CTX-M-2, CTX-M-3, CTX-M-9, CTX-M-14, CTX-M-15, CTX-M-39, OXA, TEM-1, TEM-15, TEM-17, TEM-19, TEM-20, TEM-52, TOHO-1, SHV-2 and PER-2 ESBL enzyme types in these isolates.

There is serious concern over the high ESBL prevalence in members of the Enterobacteriaceae family in India, and the prevalence of ESBLs in *E. coli* and *Klebsiella pneumoniae* have been reported to be as high as 78.9% and 72.7%, respectively, in hospital-acquired strains and 79% and 61.8%, respectively, in community-acquired infections.

Infections of *Shigella*, a member of the Enterobacteriaceae family, are foodborne, so are usually acquired from the community. *bla*<sub>CTX-M-15</sub> is widespread in India by horizontal transfer and/or mobilization of genetic mobile elements, and faecal carriage of *bla*<sub>CTX-M</sub>-producing bacteria has also been described. It has been suggested that conditions of overcrowding and poor sanitation, and the selective pressure created by overuse of antibiotics, has enabled such widespread dispersal of *bla*<sub>CTX-M-15</sub>. The finding of a high prevalence of ESBL-producing genes in *Shigella* isolates in our study has serious implications for the further spread of resistance to third-generation cephalosporins strains to other regions. This calls for a stronger surveillance by microbiologists to detect such trends earlier in order to implement timely interventions, and reemphasizes the need for the implementation of strong infection-control practices and judicious use of antibiotics by physicians so as to minimize selection pressure of antibiotics on bacteria in hospitals as well as in the community.

### Table 2. MICs for *S. flexneri* (Sf, *n* = 119) and *S. dysenteriae* (Sd, *n* = 24) isolates

<table>
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<tr>
<th>Drug</th>
<th>MIC (mg/L)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>range tested (mg/L)</td>
<td>SF</td>
<td>Sd</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.016–256</td>
<td>0.032</td>
<td>0.023</td>
</tr>
<tr>
<td>Cefepime</td>
<td>0.016–256</td>
<td>0.125</td>
<td>0.064</td>
</tr>
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</table>

DD, disc diffusion; I, intermediate susceptible; N, negative; P, positive; P+M−, *S. flexneri* polytype reactive monotype non-reactive; R, resistant; S, susceptible; var, variant.
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Transparency declarations
None to declare.

References

