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Mutations in *gyrA* & *parC* genes of *Shigella flexneri* 2a determining the fluoroquinolone resistance

Sir,

Shigellosis or bacillary dysentery is caused by a group of facultative anaerobic Gram-negative rods of the genus *Shigella*. Worldwide, about 165 million cases of shigellosis were reported annually (99% occurring in the developing world) with one million associated deaths[1]. Approximately 60 per cent of deaths involve children younger than five years[2]. Four *Shigella* species, *S. dysenteriae, S. flexneri, S. boydii*, *S. sonnei* cause shigellosis in humans. Of these, *S. flexneri* is the most frequently isolated species in developing countries, which has six serotypes and two variants (X, Y) including subserotypes[3-6]. Antibiotic therapy lessens the risk of serious complications and death, shortens the duration of symptoms and hastens the elimination of *Shigella* from the stool. Multidrug resistance is widespread in *Shigella* and the current treatment of shigellosis is with ciprofloxacin and with three second-line antibiotics; pivmecillinam, azithromycin and ceftriaxone[7]. Since 2002, there has been an alarming increase in *S. flexneri* resistant to fluoroquinolones in India, thereby limiting the treatment options[8-11]. We report here two isolates of *S. flexneri* type 2a isolated from a hospital in Kerala in 2010 which were found to be resistant to fluoroquinolones and possessed mutations in *gyrA* and *parC* genes.

Two isolates of *Shigella* (SF1 and SF2) were isolated from a 62 yr old female and a 52 yr old male dysentery patients who presented with severe bleeding per rectum, fever and inflammatory bowel disease, admitted to Medical College Hospital, Thiruvananthapuram, Kerala, India in 2010. There was no history of travelling and they were taking protein powder as food supplement. Both patients responded well with injection of cefotaxime. The isolates were confirmed as *Shigella* species by biochemical tests and serotyped using commercially available antisera (Denka Seiken, Tokyo, Japan). These were stored in Luria–Bertani broth (BD, Difco, MD, USA) containing 50 per cent glycerol at -80°C.

Antibiotic susceptibility testing was performed using the Kirby–Bauer disc susceptibility method[12] according to Clinical Laboratory Standards Institute guidelines[13]. The antibiotic discs (µg) (Hi-Media Laboratories, Mumbai, India) used were ampicillin (10), ceftriaxone (30), nalidixic acid (30), ciprofloxacin (5), ofloxacin (5), norfloxacin (10), trimethoprim (5), tetracycline (30), chloramphenicol (30), streptomycin (10), gentamicin (10) and co-trimoxazole (1.25 + 23.75). *Escherichia coli* ATCC 25922 was used as control. The minimum inhibitory concentrations (MICs) of nalidixic acid, norfloxacin and ciprofloxacin were determined using E test (AB Biodisk, Solna, Sweden).

The bacterial cell lysate was used as a template for PCR analysis. The bacteria grown overnight at 37 °C in Luria-Bertani broth were boiled, snap-cooled and stored at -20°C until use. Quinolone resistance determining regions (QRDR) of *gyr*A, *gyr*B, *parC* and *par*E were amplified as described previously[14,15]. The amplified products were separated on a 1 per cent agarose gel, stained with ethidium bromide and visualized using a BIORAD Gel Doc EZ Imager (Bio Rad, USA). PCR products were purified using ExosapIT (USB) and sequencing reactions were carried out using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA). 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). Sequences were edited with BIOEDIT v7.1.3 (*http://www.mbio.ncsu.edu/bioedit/bioedit.html*) and compared in BLAST of the NCBI database (*www.ncbi.nlm.nih.gov/BLAST*).

The two *Shigella* isolates (SF1 and SF2) were identified as *S. flexneri* 2a by serotyping with specific antisera. Antibiogram revealed that both the isolates were resistant to ampicillin, co-trimoxazole, nalidixic acid, ciprofloxacin, ofloxacin, norfloxacin, trimethoprim, chloramphenicol, streptomycin and gentamicin. The isolates did not show resistance to ceftriaxone. Antimicrobial resistance has been increasing among *Shigella* and the choice of antibiotics becoming limited with the emergence of multidrug resistant strains. In the present investigation, the test isolates were resistant to two or more classes of antibiotics, including ampicillin and co-trimoxazole. Similar findings have been reported from different parts of the country[8-10]. Both isolates showed high level resistance to ciprofloxacin (MICs of 24 and 12 µg/ml) and for norfloxacin (MICs of 32 µg/ml for both). Both isolates possessed mutations in *gyrA* at position 87 with the replacement of D-aspartic acid with N-asparagine and at position 80 of *parC* with the replacement of serine by isoleucine. None of the isolates had any mutation in *gyrB* or *parE* genes. The mutation reported in *parC* gene is in accordance with the previous reports[9,15]. In *gyrA* gene, mutation initially happens at 83rd position (S83L) followed by D87N[16]. But in the present study, the isolates did not show any change at 83rd position of *gyrA* gene. The resistance to fluoroquinolones may be due to the acquisition of mutations in the QRDR of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*), plasmid-mediated quinolone resistance (PMQR) determinants, such as (*qnr*), aminoglycoside *aac(6')-Ib-cr* and active efflux of quinolones[17]. Since only the QRDR genes were sequenced, the possibility of other mechanisms in the observed resistance to fluoroquinolones was not ruled out.

Shigellosis is a major public health problem in India and several reports have identified *S. flexneri* to be the predominant circulating serotype in different parts of the country[8,10,11,18,19]. A study conducted in 1978 identified 16 shigellae from Calicut and Trivandrum, of which eight were *S. flexneri*. Three of these showed resistance to ampicillin, chloramphenicol, streptomycin, sulphadiazine and tetracycline[20]. The emergence of fluoroquinolone resistant *S. flexneri* type 2a is a therapeutic challenge in the treatment of shigellosis. Periodic monitoring and reporting of *Shigella* serotypes circulating in the country and their antibiotic susceptibility will help the clinicians in the proper selection of drugs and their judicious use for shigellosis.

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