Characterization of Extended-spectrum β-lactamase-producing Clinical Isolates of Shigella flexneri

Sir,

Antimicrobial agents are the mainstay of therapy in severe shigellosis. Conventionally, the fluoroquinolones have been the main drugs of choice for the treatment of shigellosis. With the emergence of fluoroquinolone resistance among Shigella (1-5), the third-generation cephalosporins have been used for the treatment of severe shigellosis. However, recent reports of resistance to the third-generation cephalosporins from India (6-8) made the selection of antibiotics for empirical therapy, particularly in children, even more complicated. Here, we report the 2 Shigella flexneri isolates which were genotypically proven to produce extended-spectrum β-lactamase (ESBL).

The study was undertaken in the Department of Microbiology and Department of Pediatrics, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India. The three strains described in this study were isolated from the stool samples of paediatric patients coming to JIPMER hospital. One of the strains was isolated from a one-year old child with the manifestations of severe dysentery which had recurred more than three times while the second strain was isolated from a 10-month old infant who had been diagnosed to have acute gastroenteritis mimicking cholera. The stool samples from these children were plated directly onto MacConkey agar, Deoxycholate citrate agar, and Xylose lysine deoxycholate agar as well as into Selenite F broth for enrichment, which was subcultured after 18 hours in the abovementioned plates. The plates were incubated at 37 °C overnight under aerobic conditions. Following incubation, non-lactose-fermenting pale translucent colonies on MacConkey agar and Deoxycholate citrate agar (Himedia, Mumbai, India) and pink translucent colonies on Xylose lysine deoxycholate (Himedia, Mumbai, India) were selected for the biochemical tests (9). Further confirmation was done by slide agglutination using specific antisera (Denka-Seiken, Tokyo, Japan). Antibiotic susceptibility testing was done by Kirby Bauer method as per Clinical Laboratory Standards Institute (10) against ampicillin (A) 10 µg, ceftriaxone (Ci) 30 µg, ciprofloxacin (Cf) 5 µg, nalidixic acid (Na) 30 µg, furoxone (Fx) 300 µg, chloramphenicol (C) 30 µg, and cotrimoxazole (Co) 25 µg, which showed that the two strains were resistant to Ci. The MIC for ceftriaxone was determined by agar dilution method and E-test for those strains that were resistant to the drug. For the agar dilution method, ceftriaxone-sodium salt (Himedia, Mumbai, India) was used. Different dilutions of the antibiotics were used as per recommendations (10,11). ATCC Escherichia coli 25922 was inoculated on each plate as growth control. The E-test was performed as per the manufacturers’ instructions (Biomeriuex, India). Both the strains had an MIC value of >256 µg/mL for ceftiaxone. The ESBL detection was performed by combination disc method on the ceftriaxone-resistant isolates (10,12). These three strains, when phenotypically tested by the combination disc method, were positive for ESBL production. The DNA was extracted from the Shigella strains, using the boiling method (13). The DNA was subjected to PCR amplification targeting the known class of ESBL genes, using primers (Table 1) that would identify sequences encoding the ESBL genes TEM, CTX, and SHV.

The Multiplex PCR was done to identify blaSHV and blaCTX-M genes simultaneously (14). The reaction mixture consisted of 2 µL of DNA, 100 pmol (1 µL) concentration of each oligonucleotide primer of blaSHV, 30 pmol (1 µL) concentration of each oligonucleotide primer of blaCTX, master mix of 35 µL containing dNTPs, 1.25 U Taq polymerase and buffer with 1.5 mM MgCl₂ and sterile nuclease-free water of 9 µL, making a total volume of 50 µL. The cycling conditions included an initial denaturation step at 94 °C for 5 min and then 32 cycles with denaturation at 94 °C for 45 sec, primer annealing at 50 °C for 40 sec, and extension at 72 °C for 60 sec. A final extension step at 72 °C for 10 min was performed. The reaction mixture consisted of 4 µL of DNA, 2 µL of 100 pmol concen-
Mandal J et al.

ESBL-producing *Shigella flexneri*

Table 1. Primers used in the study for detecting the ESBL genes

| Primer | Sequence | Product-size (bp) | Reference |
|--------|----------|------------------|-----------|
| SHV-F  | 5’ATT TGT CGC TTC TTT ACT CGC-3’ | 1,018 bp | 14 |
| SHV-R  | 5’ TTG ATG GCG TTA CCT TTG ACC-3’ | | |
| CTXMU-1 | 5’ATG TGC AGY ACC AGT AAR GT 3’ | 544 bp | 14 |
| CTXMU-2 | 5’TGG GTR AAR TAR GTS ACC AGA 3’ | | |
| TEM F  | 5’ATA AAA TTC TTG AAG ACG AAA 3’ | 1,076 bp | 15 |
| TEM R  | 5’GAC AGT TAC CAA TGC TTA ATC 3’ | | |

![Image](image-url)

**DISCUSSION**

Shigellosis is the major cause of dysentery in children. *S. flexneri* is the most predominating pathogen in developing countries, and the same is reflected in our study also. Antibiotics are indicated in the treatment for shigellosis. However, the alarming rise in antimicrobial resistance is a great threat to mankind. In the recent decades, majority of the members of the family Enterobacteriaceae were shown to produce extended-spectrum β-lactamase. This fact did not spare the genus *Shigella* where an SHV-11 ESBL-producing *S. dysenteriae* strain was reported in India in 1999 (19). There are several other reports of ESBL-producing *Shigella* from India (6-8). Third-generation cephalosporin-resistant *S. flexneri* isolate was first reported from a stool sample of a 16-month old child in Paris in 1995 (20). In the recent years, various ESBL-producing *Shigella* were also reported from Korea (CTX-M-14) (21), Argentina (CTX-M-2) (22), Viet Nam (CTX-M-15 and CTX-M-24) (23), and Turkey (24). In India, CTX-M-3 type of ESBL was reported in *S. sonnei* from Andaman and Nicobar islands (8). A novel CTX-M-64, a hybrid of CTX-M-15 and CTX-M-14, was reported by Nagano *et al.* from a shigellosis patient infected with *S. sonnei* after returning to Japan from China (25).

Conjugation experiments showed the transfer of the plasmid-encoded blaCTX-M-15 gene from the ESBL-producing *S. flexneri* isolate to *E. coli* J53. Transconjugants demonstrated resistance to third-generation cephalosporins mediated by transfer of a >35.5 kb plasmid. CTX-M-15 is a major CTX-M subtype which has spread all over the world and has been found in many members of the Enterobacteriaceae (26-28).
Conclusions

Cephalosphorins are the antibiotics of choice for severe and hospitalized cases, particularly in children where quinolones are not considered as treatment options by most clinicians. ESBL production in Shigella has complicated the situation, limiting the treatment options. Further, Shigella has the ability to carry multiple plasmids in relation to virulence and the antimicrobial resistance. In this study, we have described three Shigella flexneri strains which were resistant to ceftriaxone and were shown to produce ESBL by phenotypic and molecular methods. The major concern regarding these strains is that these were isolated from the stool samples of the children, in which case, most of the clinicians do not prefer using quinolones and cephalosphorins. However, with the emergence of these strains showing resistance to the third-generation cephalosphorins, the protocols for therapy of shigellosis need to be re-analyzed with emphasis on close continuous monitoring. This imparts that continued surveillance is needed to identify the ESBL-producers in patients with shigellosis which, in turn, will assist us in developing effective strategies in controlling the current situation.

REFERENCES

1. von Seidlein L, Kim DR, Ali M, Lee H, Wang X, Thiem VD et al. A multicentre study of Shigella diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. PLoS Med 2006;3:e353.

2. Niyogi SK, Pazhani GP. Multiresistant Shigella species isolated from childhood diarrhea cases in Kolkata, India. Jpn J Infect Dis 2003;56:33-4.

3. Srinivasa H, Baijayanti M, Raksha Y. Magnitude of drug resistant shigellosis: a report from Bangalore. Indian J Med Microbiol 2009;27:358-60.

4. Kosek M, Yori PP, Pan WK, Olortegui MP, Gilman RH, Perez J et al. Epidemiology of highly endemic multiply antibiotic-resistant shigellosis in children in the Peruvian Amazon. Pediatrics 2008;122:e541-9.

5. Pazhani GP, Niyogi SK, Singh AK, Sen B, Taneja N, Kundu M et al. Molecular characterization of multidrug-resistant Shigella species isolated from epidemic and endemic cases of shigellosis in India. J Med Microbiol 2008;57(Pt 7):856-63.

6. Mandal J, Mondal N, Mahadevan S, Parjia SC. Emergence of resistance to third-generation cephalosphorin in Shigella—a case report. J Trop Pediatr 2010;56:278-9.

7. Varghese SR, Aggarwal A. Extended spectrum beta-lactamase production in Shigella isolates—a matter of concern. Indian J Med Microbiol 2011;29:76-8.

8. Bhattacharyya D, Bhattacharjee H, Ramanathan T, Sudharma SD, Singhana M, Sugunan AP et al. Third-generation cephalosphorin resistance in clinical isolate of Shigella sonnei in Andaman & Nicobar Islands, India. J Infect Dev Ctries 2011;5:674-6.

9. Bopp CA, Ries AA, Wells JG. Laboratory methods for the diagnosis of epidemic dysentery and cholera. Atlanta, GA: Centers for Disease Control and Prevention, 1999. 108 p. (WHO/CDS/CSR/EDC/99.8).

10. Cockrell FR, Wikler MA, Bush K, Dudley MN, Eliopoulos GM, Hardy DJ et al. Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement. Wayne, PA: Clinical and Laboratory Standards Institute, 2011. 165 p.

11. Jorgensen JH, Turnidge JD. Susceptibility test methods: dilution and disk diffusion methods. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Volken RH, editors. Manual of clinical microbiology. 9th ed. Washington, DC: American Society for Microbiology Press, 2007:1108-27.

12. Jarlier V, Nicolas MH, Fournier G, Philippon A. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev Infect Dis 1988;10:867-78.

13. Ahamed J, Kundu M. Molecular characterization of SHV, TEM, CTX-M and extended-spectrum β-lactamase produced by Escherichia coli, Acinetobacter baumannii and Klebsiella isolates in a Turkish hospital. Afr J Microbiol Res 2010;4:650-4.

14. Jemima SA, Verghese S. Multiplex PCR for bla (CTX-M) & bla(SHV) in the extended spectrum beta lactamase (ESBL) producing gram-negative isolates. Indian J Med Res 2008;128:313-7.

15. Kim S, Kim J, Kang Y, Park Y, Lee B. Occurrence of extended-spectrum β-lactamases in members of the genus Shigella in the Republic of Korea. J Clin Microbiol 2004;42:5264-9.

16. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 1979;7:1513-23.

17. Shiraki Y, Shibata N, Doi Y, Arakawa Y. Escherichia coli producing CTX-M-2 β-lactamase in cattle, Japan. Emerg Infect Dis 2004;10:69-75.

18. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. New York, NY: Cold Spring Harbor Laboratory Press, 2001. Chapter 1: protocol 27.

19. Ahamed J, Kundu M. Molecular characterization of the SHV-11 β-lactamase of Shigella dysenteriae. Antimicrob Agents Chemother 1999;43:2081-3.

20. Fortineau N, Naas T, Gaillot O, Nordmann P. SHV-type extended-spectrum β-lactamase in a Shigella flexneri
clinical isolate. J Antimicrob Chemother 2001;47:685-8.

21. Pai H, Choi E-H, Lee H-J, Hong JY, Jacoby GA. Identification of CTX-M-14 extended-spectrum β-lactamase in clinical isolates of *Shigella sonnei*, *Escherichia coli*, and *Klebsiella pneumoniae* in Korea. J Clin Microbiol 2001;39:3747-9.

22. Radice M, González C, Power P, del Carmen Vidal M, Gutkind G. Third-generation cephalosporin resistance in *Shigella sonnei*, Argentina. Emerg Infect Dis 2001;7:442-3.

23. Nhu NTK, Vinh H, Nga TVT, Stabler R, Duy PT, Vien LTM et al. The sudden dominance of *bla*CTX-M harbouring plasmids in *Shigella* spp. circulating in Southern Vietnam. PLoS Negl Trop Dis 2010;4:e702.

24. Acikgoz ZC, Gulay Z, Bicmen M, Gocer S, Gamberzade S. CTX-M-3 extended-spectrum β-lactamase in a *Shigella sonnei* clinical isolate: first report from Turkey. Scand J Infect Dis 2003;35:503-5.

25. Nagano Y, Nagano N, Wachino J-i, Ishikawa K, Arakawa Y. Novel chimeric β-lactamase CTX-M-64, a hybrid of CTX-M-15-like and CTX-M-14 β-lactamases, found in a *Shigella sonnei* strain resistant to various oximino-cephalosporins, including ceftazidime. Antimicrob Agents Chemother 2009;53:69-74.

26. Vien LTM, Baker S, Thao LTP, Tu LTP, Thuy CT, Nga TTT et al. High prevalence of plasmid-mediated quinolone resistance determinants in commensal members of the *Enterobacteriaceae* in Ho Chi Minh city, Vietnam. J Med Microbiol 2009;58(Pt 12):1585-92.

27. Woerther P-L, Angebault C, Jacquier H, Hugede H-C, Janssens A-C, Sayadi S et al. Massive increase, spread, and exchange of extended spectrum β-lactamase-encoding genes among intestinal *Enterobacteriaceae* in hospitalized children with severe acute malnutrition in Niger. Clin Infect Dis 2011;53:677-85.

28. Smet A, Van Nieuwerburgh F, Vandekerckhove TTM, Martel A, Deforce D, Butaye P et al. Complete nucleotide sequence of CTX-M-15-plasmids from clinical *Escherichia coli* isolates: insertion events of transposons and insertion sequences. PLoS One 2010;5:e11202.

Jharna Mandal, V. Sangeetha, Nivedithadivya, Ankita Das, Subhash Chandra Parija

Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India