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# Identification of Broad-Spectrum Beta-lactamase *CTX-M-2*, *CTX-M-8*, and Ampc-dependent *CMY* Genes in *Shigella sonnei* Isolated from Pediatric Diarrhea Specimens by Multiplex-PCR and Antibiotic Resistance Pattern Determination

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## ABSTRACT

**Background:** *Shigella* species are one of the most common causes of bacillary dysentery and sometimes death especially in children and immune-compromised individuals. The diversity of disease-causing species and the emergence of drug resistance make it difficult to select the appropriate antibiotic to treat shigellosis. One of the important causes of resistance in *Shigella* isolates is the presence of genes encoding broad-spectrum beta-lactamase enzymes. The aim of this study was to determine the frequency of *Shigella sonnei* strains producing *CTX-M-2*, *CTX-M-8*, and *CMY* beta-lactamase genes by Multiplex PCR and to investigate their association with antibiotic resistance in *S. sonnei* strains.

**Materials & Methods:** This descriptive cross-sectional study was conducted in a period of 6 months from the beginning of June to the end of October 2016. A total of 200 diarrhea specimens were collected from the patients with suspected shigellosis from the Children's Medical Center (Tehran). The antibiotic susceptibility testing was performed using disk diffusion method on Müller-Hinton agar in accordance with CLSI instructions. After DNA extraction, the presence of *CTX-M-2*, *CTX-M-8*, and Ampc-dependent *CMY* genes was determined by Multiplex-PCR using specific primers.

**Results:** From all the samples, 60 (30%) *S. sonnei* strains were obtained using standard microbiological and biochemical tests. Majority of the strains were resistant to erythromycin (26 strains, 43.3%) and cefepime (24 strains, 40%). The molecular test results showed that 40 (66.6%) and 33 (55%) of the strains carried the *CTX-M-8* and *CMY* genes, respectively ($P$<0.05). The *CTX-M-2* gene was not detected in any of the samples.

**Conclusion:** The results indicate a high frequency of *CMY* gene among *Shigella sonnei* isolates and higher resistance of these strains was found against erythromycin and cefepime. Therefore, careful medical care and proper and timely use of appropriate antibiotics to prevent the spread of resistant isolates seems inevitable.

**Keywords:** Broad-spectrum beta-lactamase, Disk diffusion, Multiplex-PCR, *Shigella sonnei*

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## Introduction

*Shigella* species are members of the *Enterobacteriaceae* family with four groups: group A (*Shigella dysentery*), group B (*Shigella flexneri*), group C (*Shigella boydii*) and group D (*Shigella sonnei*) **(1)**. The infectious dose of *Shigella* is less than 200 bacilli; therefore, shigellosis can spread rapidly among communities with low health standards **(2)**. Bacterial dysentery (Bacillary dysentery or shigellosis) is considered as one of the most important acute gastrointestinal diseases, especially in children and immune-compromised patients which cause high number of fatalities in addition to economic losses and social problems **(3)**. Anorexia, fever, intestinal inflammation, bloody-purulent stools, abdominal pain, tenesmus, and feeling of incomplete bowel emptying with anal pain are the symptoms of this disease **(4)**. It is estimated that 165 million cases of bacillary dysentery and 1.1 million deaths occur annually worldwide, about 60% of which are in children younger than five years old **(5)**.

The appropriate and timely antibiotic treatment shortens the course of the disease and reduces its complications and prevents the transmission of the disease to the healthy people. Today, due to the widespread use of antibiotics, *Shigella* species have become resistant to many antibiotics, including the third generation of cephalosporins. This issue has made the treatment of this disease difficult **(6)**. The most important problem in the treatment of people with shigellosis is the development of multi-drug resistance (MDR) **(7)**. Production of the beta-lactamase enzymes is the main reason for the gram-negative bacteria resistance to beta-lactams **(8)**. These enzymes destroy the amide ring of beta-lactams. Beta-lactamase enzymes are divided into four groups A to D based on the amino acid sequences. Broad-spectrum beta-lactamase enzymes (ESBLs) are from class A beta-lactamases that hydrolyze broad-spectrum cephalosporins with a side chain of Oximino, causing bacterial resistance to penicillins, the first, second and third generations of cephalosporins and aztreonam. They are inhibited by beta-lactamase inhibitors such as clavulanic acid **(9)**.

The *CTX-M* beta-lactamase genes were first reported in 1989 from Japan and spread to the other parts of the world since then **(10)**. Today, these beta-lactamase enzymes are divided into five groups: *CTX-M1* was reported from Germany in 1989 (11), *CTX-M-2* was reported from Japan in 1986, *CTX-M-8* was reported from Brazil in 1996 (12), *CTX-M-9* was reported from Spain in 1994, and the *CTX-M-25* was reported from Canada in 2000 **(10)**. These beta-lactamase genes have little association with the *TEM* and *SHV* beta-lactamase members, and instead there is a high similarity between the chromosomal enzymes *Amp-C*, especially *KLU-1* and *KLU2*, and the *CTX-M* enzymes. There are theories based on the derivation of these enzymes from one species **(13)**.

Broad-spectrum beta-lactamase-producing bacteria are placed in the human and animal intestines which are very difficult to control and eradicate because ESBL genes can be transmitted between different genus, species, and different strains of intestinal bacteria **(7)**. There are some reports of *Shigella sonnei* multiple-resistance to antibiotics, especially cephalosporins third-generation such as cefotaxime and ceftriaxone.

In recent years, the emergence of the antibiotic resistance phenomenon has raised many concerns in the medical communities due to the failure of the treatment process **(8)**. Due to the development of broad-spectrum beta-lactam antibiotic resistance genes in *Shigella sonnei* strains and the high speed and accurate detection of molecular methods and simultaneous identification of several genes, this study aimed to identify broad-spectrum beta-lactamase genes *CTX-M-2*, *CTX-M* and Ampc-dependent *CMY* gene in *Shigella sonnei* strains isolated from diarrheal specimens by Multiplex-PCR method and the antibiotic resistance pattern of these strains.

## Materials and Methods

In this descriptive cross-sectional study, diarrheal stool samples of the patients with suspected shigellosis were randomly collected from the Children's Medical Center (Tehran) over a period of 6 months from the beginning of June to the end of October 2016. A total of 200 diarrhea stool samples were collected in disposable sterile containers specified for stool collection, including 94 male and 106 female samples. The samples were examined macroscopically for the consistency, mucus and blood presence and microscopically for the red and white blood cells. The inclusion criteria included the presence of blood and mucus in diarrheal stool, tenesmus and no antibiotic use and the exclusion criteria were the absence of *Shigella* in the positive culture samples or arbitrary use of antibiotics before referral. The stool samples were transferred to the laboratory in Kerry-Blair (Merck, Germany) medium at the earliest opportunity. The Selenite-F (SF) medium was used to enrich the samples. After incubation at 37°C for 8 hr, the samples were transferred from the SF medium to Salmonella-Shigella agar (SS) and McConkey agar (Merck, Germany) and incubated for 24 to 48 hr at 37°C.

Finally, to differentiate and confirm Shigella species the biochemical tests (oxidase, catalase, SIM, MRVP, citrate consumption, TSI test, urease production, phenylalaninidaminase, lysine decarboxylase, sodium malonate, and decarboxylation of the amino acids ornithine and mannitol) were conducted. The isolates with biochemical properties: lactose negative, gas production negative, immobilized, lysine decarboxylation negative, citrate negative, urea hydrolysis negative and methyl red positive were considered as Shigella isolate. Serotyping tests were performed on the slides of fresh *Shigella* cultures using Baharafshan Co. kits by agglutination method **(1, 2)**.

### Disk Diffusion

The antibiotic susceptibility test was performed using disk diffusion method and according to the instructions of the Laboratory and Clinical Standards Institute (14) on Müller-Hinton agar medium (Merck, Germany) for the antibiotics trimethoprim-sulfamethoxazole (25 µg), erythromycin (30 µg), amoxicillin (25 µg), ceftriaxone (30 µg), cefepime (30 µg), amoxiclav (25 µg) (produced by Himedia Co, India).

After bacterial DNA extraction using gram-negative bacterial genomic DNA extraction kit of Iranian Biological Resource Center (Cat no. MBK0041) and confirmation of the extracted DNA purity using biophotometer (Bio-Rad, USA), the genes *CTX-M-2*, *CTX-M-8* and *CMY* were amplified using M-PCR method and specific primer sequences (Table 1) in thermocycler (Eppendorf, Germany) in the final volume of 25 µL including 10.5 µL PCR master mix 5X (Sinaclon, Iran) containing Taq DNA polymerase (0.05 U/µL), MgCl2 (3 mM) and dNTPs (0.4 mM), 0.7 µL of each primer, 1 µL of template DNA (10 ng) and 12.1 µL of double-distilled water for 33 cycles. The primer BLAST was performed on the website https://www.ncbi.nlm.nih.gov/tools/primer-blast/.

Thermal cycling included denaturation step at 95°C for 1 min, primer annealing at 60°C for 30 sec and elongation step at 72°C for 1 min. Finally, the PCR products were run on 1% agarose gel containing ethidium bromide (Figure 1). In the molecular study, *Shigella sonnei* ATCC 25931 and *Escherichia coli* ATCC 25922 (prepared from the Microbial Bank of Pasteur Institute of Iran) were used as negative and positive controls, respectively.

### Statistical Analysis

Data were collected and analyzed using SPSS software version 16. The statistical analysis was performed using Pearson-Chi Square test. The P-value<0.05 was considered as significant level.

**Table 1.** Sequence of the primers

| Target gene | Primer sequence (3´ to 5´) | Product length (bp) |
|---|---|---|
| *CTX-M-2* | F: TTAATGATGACTCAGAGCATTC<br>R: GATACCTCGCTCCATTTATTG | 901 |
| *CTX-M-8* | F: CGCTTTGCCATGTGCAGCACC<br>R: GCTCAGTACGATCGAGCC | 307 |
| CMY | F: TGGCCAGAACTGACAGGCAAA<br>R: TTTCTCCTGAACGTGGCTGGC | 462 |

## Results

From a total of 200 diarrheal fecal samples obtained from children, 60 isolates of *Shigella sonnei* were obtained, of which 28 isolates (46.6%) were from boys and 32 isolates (53.3%) belonged to female fecal samples. All the strains obtained in this study were *Shigella sonnei* confirming by the slide agglutination test. The results of antibiotic susceptibility test showed that all (100%) *Shigella sonnei* isolates were sensitive to cotrimoxazole. Also, only eight isolates (13.4%) were resistant to ceftriaxone (Table 2). In the other words, the highest percentage of resistance among the isolates of this study was related to erythromycin (43.3%) and cefepime (40%). From 60 *Shigella* isolates in this study, 19 (31.6%) strains were resistant to three different classes of antibiotics and were considered as MDR.

**Table 2.** Antibiotic resistance pattern of the isolates under study

| Antibiotic | Sensitive (S) (%) | Intermediate (I) (%) | Resistant (R) (%) |
|---|---|---|---|
| Cotrimoxazole | 60 (100) | 0 (0.0) | 0 (0.0) |
| Erythromycin | 30 (50) | 4 (6.7) | 26 (43.3) |
| Amoxicillin clavulanic acid | 40 (66.6) | 8 (13.4) | 12 (20) |
| Cefepime | 30 (50) | 6 (10) | 24 (40) |
| ceftriaxone | 40 (86.6) | 0 (0.0) | 8 (13.4) |

The presence or absence of broad-spectrum beta-lactamase genes was studied on all 60 *Shigella sonnei* isolates of the children's diarrheal stool samples. The frequencies of *CMY* and *CTX-M-8* genes were 66.7% (n=40) and 55% (n=33), respectively (Figure 1). All the strains were negative regarding *CTX-M-2* gene (Figure 2).

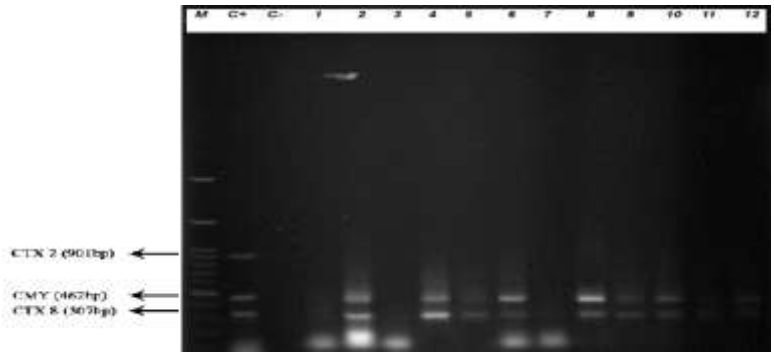
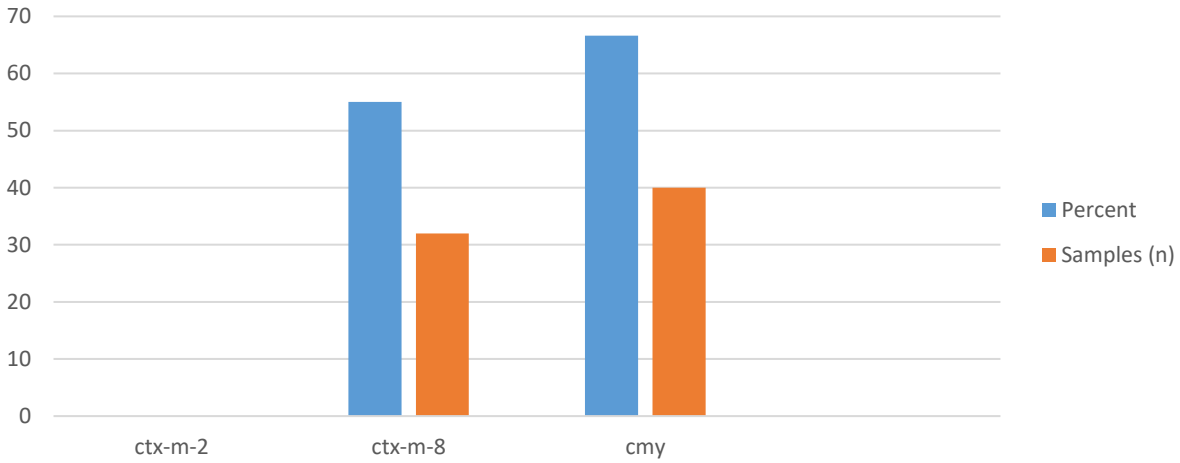


**Figure 1.** The M-PCR test result on some isolates. C+: Positive control (*E. coli* ATCC 25922), C: Negative control (*Shigella sonnei* ATCC 25931). The numbers 1-12 are clinical strains collected from diarrhea (M: DNA marker: 100 bp, Fermentas).

**Figure 2.** Number and results of beta-lactamase genes in Sony Shigella samples




## Discussion

Shigellosis is endemic worldwide and is the most common cause of bacterial dysentery **(1)**. The disease heals spontaneously in adults, while it is very dangerous in infants and children which can lead to death **(3)**. In developing countries, this disease still remains an important health issue **(4)**.

Due to the increase in antibiotic resistance in intestinal pathogens, it seems that opting a suitable drug to treat these infections has become difficult, thus, determining the drug resistance pattern on a regional and periodic basis seems necessary for this bacterium. In accordance with the present study, Abbaspour *et al.*, **(16)** in 2014 showed that 23.3% and 24.4% of *Shigella sonnei* isolates were resistant to ceftriaxone and cefixime, respectively. In the present study, 31.6% of the strains were MDR and the highest resistance (40%) was related to cefepime. The results showed a significant association ($P$<0.02) between the presence of ESBL genes and the incidence of resistance to cephalosporins such as cefepime. In a study conducted by Bonnet *et al.*, in 2007 in Brazil, the beta-lactamase genes *CMY-2* and *AmpC* were examined. In line with our study, all the strains with multiple resistance in the Bonnet *et al.*, study contained *CMY* gene **(17)**.

Like our study, Hussain *et al.*, in Pakistan in 2011 showed that the frequencies of *CTX-M* and *CMY* genes were 57.7% and 50%, respectively (18). Contrary to the present study, Mendonça *et al.*, in 2007 in Portugal showed that *blaCTX-M-2* had the highest abundance among other genes in the isolates **(19)**. This difference can be due to differences in the year of the study, geographical distance, variety of strains and sample size. Again, contrary to the present study, the broad-spectrum beta-lactamase gene *CTX-M-2* in cefotaxime-resistant *Shigella sonnei* was detected in Argentina by molecular method in the study conducted by Radice *et al.*, in 2001. The strains containing this gene were reported to be resistant to commonly used cephalosporins **(20)**. This gene was not detected in any of the samples in our study. This difference could be due to the geographical distance and genetics of the strains.

More comprehensive studies are needed to identify different types of beta-lactamase classes and other

resistance genes in *Shigella* strains in the country. Also, by determining the antibiotic resistance pattern and using the appropriate antibiotics when treatment is needed, antibiotic resistance and the spread of resistant strains in the community among human populations can be prevented. One of the salient features of the present study is the simultaneous study of resistance genes using M-PCR method. It is suggested that, the presence of other resistance genes and determining the genetic relationship of these strains should be considered in the future studies.

## Conclusion

Based on the present study results, it was found that the highest resistance in *Shigella sonnei* strains was against cefepime and the most common broad-spectrum beta-lactamase gene in these strains was *CMY*. There was a statistically significant association between the presence of *CMY* gene and resistance to cefepime ($P<0.05$). For the high prevalence of beta-lactam antibiotic resistance genes in *Shigella sonnei* strains, careful medical care and proper and timely use of appropriate antibiotics are essential to prevent the spread of resistant strains.

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## Conflict of Interest

Authors declared no conflict of interests.