Antimicrobial-resistant Shigella infections from Iran: an overlooked problem?

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Objectives: In this study, we wanted to assess the level of antimicrobial resistance, the presence of genes encoding resistance to cephalosporins and plasmid-mediated quinolone resistance (PMQR), and genetic relatedness among Shigella isolates obtained from Iranian patients.

Methods: A total of 44 Shigella isolates were collected from Iranian patients admitted to Milad Hospital, Tehran, Iran, during 2008–10. Of these, 37 were serotyped and characterized by MIC determination. A subset of eight suspected extended-spectrum β-lactamase (ESBL) producers (six Shigella sonnei phase II and two Shigella flexneri type 1b) were examined for the presence of genes encoding cephalosporin resistance. The presence of PMQR was assessed in one S. flexneri isolate exhibiting low-level resistance to ciprofloxacin and susceptibility to nalidixic acid. PFGE was performed on 25 S. sonnei phase II isolates.

Results: Of the isolates, 25 (68%) were S. sonnei phase II, with 5 (14%) S. flexneri, 5 (14%) Shigella dysenteriae type 2, and 2 (5%) Shigella boydii type 2. Resistance to at least three classes of antimicrobials was detected in all species. The presence of blaCTX-M-15 and the AmpC β-lactamase producer blaCMY-2 was confirmed in five and one S. sonnei phase II isolates, respectively. One of the two S. flexneri type 1b that contained blaCTX-M-15 also harboured a qnrS1 gene. PFGE identified seven PFGE profiles; the main cluster included 15 of the strains, suggesting low genetic diversity between isolates or the presence of an endemic clone in Iran.

Conclusions: This is the first known description of ESBL-producing and AmpC β-lactamase-producing Shigella and of PMQR Shigella in Iran. The emergence of CTX-15, CMY-2 and qnrS1 genes may compromise the treatment of shigellosis. Strategies to minimize the spread of ESBL-producing and AmpC-β-lactamase-producing Shigella should be implemented.

Keywords: ESBLs, AmpC, PMQR, antimicrobial resistance, PFGE

Introduction

Shigella is an important cause of acute diarrhoeal disease. Worldwide, there are ~164.7 million cases yearly, and 1.1 million people are estimated to die from Shigella infections.1 A total of 69% of all episodes and 61% of all deaths attributable to shigellosis involve children <5 years of age.1 In Iran, shigellosis is one of the major causes of childhood morbidity associated with diarrhoea.2,3 All four species of Shigella (Shigella dysenteriae, Shigella flexneri, Shigella boydii and Shigella sonnei) can cause shigellosis. S. flexneri is endemic in many developing countries and causes higher mortality than other Shigella species.4 The most common serotype is S. flexneri serotype 2a, but other serotypes are also of major importance.1 In Iran, S. flexneri was the most frequent cause of shigellosis up to 2003,5,6 when a shift in highest prevalence towards S. sonnei was observed.

A range of antimicrobials is effective for the treatment of shigellosis, although the options have become limited due to the emergence of multidrug resistance. Thus, sulphonamides, tetracycline, ampicillin and co-trimoxazole are no longer recommended for empirical treatment.7 An increase in the occurrence
of antimicrobial resistance, including resistance to fluoroquinolones and third- and fourth-generation cephalosporins, among S. sonnei has been observed in several countries. 8–15 To date, blaCTX-M-15, blaCTX-M-14, blaCTX-M-3 and blaCMY-2 genes have been reported in S. sonnei isolates. A few articles have described the presence of plasmid-mediated quinolone resistance (PMQR) determinants mainly associated with S. flexneri, where the qnrS1 gene appears to be dominant. 16–18

The emergence of antimicrobial resistance is a matter of concern 19 and poses major difficulties in the determination of appropriate antimicrobial treatment due to shifts in the prevalences of the different serogroups and changes in the resistance patterns. 7 Knowledge of the occurrence of different serotypes in different countries and geographic regions may assist in the recognition and tracing of emerging pathogens and in the implementation of correct treatment and control strategies. 1

In this study, we have determined the serotype distribution and antimicrobial resistance profiles as well as the genes conferring resistance to quinolones, extended-spectrum β-lactamase (ESBL) and AmpC β-lactam antimicrobials among Shigella spp. collected from patients admitted to Milad Hospital, Tehran, Iran, during 2008–10. Additionally, we have investigated the molecular relatedness of S. sonnei isolates using PFGE.

Materials and methods

Sample collection

A total of 848 patients admitted to Milad Hospital, Tehran, Iran, between September 2008 and March 2010 suffered from acute diarrhoea and gastroenteritis. Only patients without a history of antimicrobial consumption submitted stool samples and were included in the study. From the 848 patients, 44 Shigella isolates were obtained and further analysed.

The strains were isolated directly from fresh stool samples following plating on selective agar and biochemical testing. The selective agar plates (MacConkey agar, Salmonella–Shigella agar and XLD agar (Merck, Hamburg, Germany)) were incubated at 37°C for 18–24 h. Presumptive Shigella colonies were identified based on the following biochemical tests: triple sugar iron agar, motility-indole-urea agar and IMVIC (indole, methyl red, Voges-Proskauer and citrate). A subset of 37 viable isolates (the other 7 isolates did not survive storage) were included in this study and sent to the National Food Institute, Technical University of Denmark (DTU Food) for further analysis.

Serotyping

Presumptive Shigella isolates were sent to the WHO National Salmonella and Shigella Center, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Bangkok, Thailand, and serotyped based on serological slide agglutination, using the polyvalent antisera A, A1, B, C, C1, C1-3 and D and the following monovalent antisera: S. dysenteriae type 1–12; S. flexneri type I–VI and group factor (3)/, 6 and 7(8); S. boydii type 1–18; and S. sonnei phase I–II (S & A Reagents Ltd, Bangkok, Thailand). The serotypes were interpreted according to the Manual of Clinical Microbiology. 20

Antimicrobial susceptibility

MIC testing was performed at DTU Food using the following antimicrobials: ampicillin, amoxicillin/clavulanic acid, apramycin, cefotaxime, ceftriaxone, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, nalidixic acid, neomycin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim. For ESBL-producing and/ or AmpC β-lactamase-producing isolates, susceptibility to the following β-lactams was also tested: cefazolin, cefepime, cefoxitin, cefpirome, ceftazidime, ceftriaxone, cefalotin, imipenem and meropenem. Epidemiological cut-off values according to current EUCAST recommendations (http://www.eucast.org) were applied, other than for amoxicillin/clavulanic acid, apramycin, ceftriaxone, cefalotin, cefepime, spectinomycin and sulfamethoxazole where CLSI standards and clinical breakpoints were used. 21–23 As the quality control, E. coli ATCC 25922 was used according to the CLSI standards. 21,23

Detection of resistance genes

Of the 37 Shigella isolates, eight strains exhibiting resistance to cefotiofur and cefotaxime were examined for the presence of blaCTX, blaCTX-M, blaCMY, blaCMY-1 and blaCMY-2 as previously described. 24,25 One isolate showing reduced susceptibility to ciprofloxacin and susceptibility to nalidixic acid was tested by PCR for the presence of PMQR genes [qnrA, qnrB, qnrC, qnrD, qnrS, qepA and ace(′)-ib] as previously described. 25–27

PCR products were purified using a GFX™ PCR DNA Kit (GE Healthcare, Chalfont St Giles, UK) and submitted to Maccrogen Inc. (Seoul, Korea) for sequencing. Sequence analysis and alignments were performed using Vector NTI suite 9 (InforMax Inc., Bethesda, MD, USA). The resulting nucleotide sequences were compared with sequences from public databases (www.ncbi.nlm.nih.gov and www.lahey.org/studies/).

PFGE

All 25 S. sonnei isolates were analysed for genetic relatedness by PFGE using XbaI (Fermentas, Glen Burnie, MD, USA) according to the US CDC PulseNet protocol. 28 Electrophoresis was performed with a CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, USA) using 1% Seakem Gold agarose in 0.5× Tris/borate/EDTA at 6 V with an angle of 120° (one phase 2.2–5.4 s, run time 20 h).

Results

Patient data

The age of infected patients ranged from 2 to 56 years (median 7 years). The ratio between genders was 1:0.85 (females/males).

Serotyping

All four species of Shigella were represented among the 37 isolates. Of these, 25 (68%) were S. sonnei phase II, with 5 (14%) S. flexneri, 5 (14%) S. dysenteriae type 2, and 2 (5%) S. boydii type 2. In addition, three different serotypes of S. flexneri were detected: 2 (5%) type 1b (I: 4, 6), 1 (3%) type 2b (II: 7, 8), and 2 (5%) untypeable (Table 1).

Antimicrobial resistance

All Shigella species showed multidrug resistance to at least three classes of antimicrobial agent. However, susceptibility to the different antimicrobials appeared to differ depending on the species and serotype (Table 1). The 25 S. sonnei phase II isolates exhibited resistance to the highest number of antimicrobials, followed by S. flexneri type 1b. In general, these 27 isolates were resistant to 13 of the 17 antimicrobials tested, but differences between the isolates were observed. Resistance to streptomycin, sulfamethoxazole, tetracycline and trimethoprim was common.
Table 1. Shigella serotypes from patients admitted to Milad Hospital, Tehran, Iran during 2008–10

<table>
<thead>
<tr>
<th>Species and serotype</th>
<th>No. (%) of isolates resistant to various antimicrobial agents a at the indicated breakpointsb</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AMC, amoxicillin/clavulanic acid; AMP, ampicillin; XNL, cefoxitin; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; CTX, cefotaxime; NAL, nalidixic acid; NEO, neomycin; SPT, spectinomycin; STR, streptomycin; SMX, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.</td>
</tr>
<tr>
<td></td>
<td>All strains were susceptible to apramycin, carbapenems (imipenem and meropenem), colistin and florfenicol.</td>
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<td>MIC breakpoints in mg/L.</td>
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<td>MIC breakpoints in mg/L.</td>
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Identification of genes conferring resistance to third- and fourth-generation cephalosporins and PMQR determinants

Among the six isolates of S. sonnei phase II resistant to third-generation cephalosporins, five harboured bla_{CTX-M-15} and one harboured bla_{CMY-2}. The two S. flexneri type 1b were also positive for the presence of bla_{CTX-M-15}. Additionally, one of the S. flexneri type 1b isolates was also resistant to cefoxitin. In addition, the two S. flexneri type 1b isolates were resistant to ß-lactam antimicrobials with the exception of cefoxitin. None of the strains was resistant to aminoglycosides, carbapenems (imipenem and meropenem), colistin or florfenicol (Table 1).

PFGE

A subset of 25 S. sonnei phase II isolates were subtyped by PFGE, and seven unique XbaI PFGE patterns were observed. The three XbaI PFGE clusters contained 15, 4 and 2 isolates, with distinct profiles (Figure 1). Within each of the clusters containing four or two isolates, the strains exhibited the same antimicrobial resistance profile (Figure 1). Furthermore, in the main cluster, 10 of the 15 isolates displayed the same antimicrobial resistance profile. No correlation was detected between the PFGE profile and age or gender of the patient or time of infection.

Discussion

Over the past decade, an emergence of ESBL-producing Shigella spp. has been observed in the Middle East, South-East Asia, China and Argentina, where the presence of bla_{CTX-M-15}, bla_{CTX-M-14} and bla_{CTX-M-3} genes has been reported.6–15 However, worldwide only a few reports have described the presence of AmpC ß-lactamases (bla_{CMY-2}) and PMQR (qnrS1, qnrB6 and qnrB19) in Shigella spp.16–18,29,30

In 2010, the first characterization of ESBL-producing Salmonella enterica harbouring bla_{CTX-M-15}, bla_{CTX-M-88} and bla_{TEM-169} from Iran was described.31 This study reports the first known description of ESBL producers (harbouring bla_{CTX-M-15}), AmpC ß-lactamase producers (harbouring bla_{CMY-2}) and PMQR (harbouring qnrS1) in S. sonnei phase II and S. flexneri type 1b isolated from patients in Iran.

This is indeed a worrisome development in antimicrobial resistance, especially as the S. sonnei phase II isolates were highly resistant to many of the antimicrobials tested, including decreased susceptibility to ciprofloxacin, complicating the
treatment of the patients. Interestingly, the patients selected for this study claimed not to have been subjected to any antimicrobial treatment prior to their hospitalization. This underlines the extensive selection pressure present in the Iranian community, where self-medication due to ‘over the counter’ antimicrobial consumption and abuse of prescribed antimicrobials is widespread.32 Our results emphasize the need for prudent and controlled use of antimicrobials, especially third-generation cephalosporins and fluoroquinolones, in the treatment of invasive infections to avoid further acquisition of resistance to these antimicrobials, which have been designated by the WHO as critically important for human health.33 The detection of ESBL producers, AmpC ß-lactamase producers and PMQR in Shigella spp. for the first time in Iran (to our knowledge) was surprising and indicates a lack of data on the prevalence of these types of resistance genotype, and illustrates the need to establish a proper antimicrobial resistance surveillance system34-35 to monitor the emergence and development of antimicrobial resistance. Furthermore, it is important to initiate strategies for prevention and control, such as those mentioned by Niyogi36 provision of safe and abundant water, effective faeces disposal, education on how to avoid faecal contamination of food and water, awareness programmes focusing on hand washing and breast feeding, promotion of oral rehydration therapy to patients suffering from acute diarrhoea and supply of additional nutrients to children recovering from acute diarrhoea or dysentery.7,36

A high genetic homogeneity was observed among S. sonnei phase II isolates, of which many clustered with similar antimicrobial susceptibility patterns. Based on the isolates in this study, it is uncertain whether this is the result of sporadic outbreaks or clonally related endemic isolates present in Iran. Limited PFGE data are available in Iran; however, Ranjbar et al.37 suggested endemic circulation of S. sonnei in Tehran, Iran. Additionally, the PFGE profile of the main cluster seems to correspond to both the antimicrobial resistance pattern and PFGE profile of the second largest PFGE cluster obtained during the 18 years of our study and by Ranjbar et al.37 Further studies aimed at understanding the clonal relatedness of S. sonnei strains need to be initiated in order to investigate the hypothesis raised in this study and by Ranjbar et al.37

In conclusion, this study describes the presence of the first ESBL producers (blaCTX-M-15), AmpC ß-lactamase producers

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**Figure 1.** PFGE (XbaI) analysis and antimicrobial resistance patterns of 25 S. sonnei serotypes from patients admitted to Milad Hospital, Tehran, Iran during 2008-10. The scale is a similarity index in percentages, the key represents the isolate numbers, and the three clusters of identical isolates are identified by 100% similarity. Black squares represent resistance. AMC, amoxicillin/clavulanic acid; AMP, ampicillin; APR, apramycin; XNL, ceftiofur; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; FFN, florfenicol; CTX, cefotaxime; GEN, gentamicin; NAL, nalidixic acid; NEO, neomycin; SPT, spectinomycin; STR, streptomycin; SMX, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

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**Table 1.** Patient age, sex, and isolate date for 25 S. sonnei isolates.

<table>
<thead>
<tr>
<th>Key</th>
<th>Patient age</th>
<th>Patient sex</th>
<th>Isolate date</th>
<th>ESBL/AmpC producing</th>
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</thead>
<tbody>
<tr>
<td>1 S</td>
<td>6</td>
<td>F</td>
<td>24-Sep-2008</td>
<td>AmpC positive</td>
</tr>
<tr>
<td>2 S</td>
<td>4</td>
<td>F</td>
<td>8-Oct-2008</td>
<td></td>
</tr>
<tr>
<td>21 S</td>
<td>5</td>
<td>M</td>
<td>18-Jun-2009</td>
<td></td>
</tr>
<tr>
<td>22 S</td>
<td>9</td>
<td>F</td>
<td>11-Jun-2009</td>
<td></td>
</tr>
<tr>
<td>23 S</td>
<td>6</td>
<td>F</td>
<td>6-Aug-2009</td>
<td></td>
</tr>
<tr>
<td>26 S</td>
<td>6</td>
<td>F</td>
<td>20-Sep-2009</td>
<td></td>
</tr>
<tr>
<td>27 S</td>
<td>56</td>
<td>F</td>
<td>15-Sep-2009</td>
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<tr>
<td>3 S</td>
<td>2</td>
<td>M</td>
<td>13-Oct-2008</td>
<td>ESBL positive</td>
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<tr>
<td>34 S</td>
<td>4</td>
<td>M</td>
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<td></td>
</tr>
<tr>
<td>35 S</td>
<td>7</td>
<td>F</td>
<td>5-Dec-2009</td>
<td></td>
</tr>
<tr>
<td>40 S</td>
<td>10</td>
<td>M</td>
<td>11-Feb-2010</td>
<td></td>
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<td>44 S</td>
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<td>M</td>
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<tr>
<td>5 S</td>
<td>4</td>
<td>F</td>
<td>20-Oct-2008</td>
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<tr>
<td>7 S</td>
<td>33</td>
<td>M</td>
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<tr>
<td>8 S</td>
<td>8</td>
<td>F</td>
<td>12-Nov-2008</td>
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<tr>
<td>4 S</td>
<td>10</td>
<td>M</td>
<td>15-Oct-2008</td>
<td></td>
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<tr>
<td>6 S</td>
<td>7</td>
<td>M</td>
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<tr>
<td>30 S</td>
<td>4</td>
<td>M</td>
<td>3-Nov-2009</td>
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<tr>
<td>32 S</td>
<td>3</td>
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<td>36 S</td>
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<tr>
<td>26 S</td>
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<td>F</td>
<td>13-Aug-2009</td>
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<td>37 S</td>
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<td>M</td>
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<td>19 S</td>
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<td>M</td>
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<tr>
<td>20 S</td>
<td>19</td>
<td>F</td>
<td>5-Jun-2009</td>
<td>ESBL positive</td>
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(blaCMV-2) and PMQR (qnrS1) in S. sonnei and S. flexneri isolated from patients in Iran. Antimicrobial susceptibility testing revealed high levels of antimicrobial resistance in all Shigella species detected. Based on PFGE, a high genetic homogeneity was observed among S. sonnei phase II isolates, many of which clustered with similar antimicrobial susceptibility patterns. Additionally, this is the first known description of serotype distribution among Shigella spp. encountered in a hospital setting in Tehran, Iran, and it revealed that S. sonnei was by far the most common Shigella species, followed by S. flexneri and S. dysenteriae. Since there is a lack of data on the epidemiology of Shigella spp. in Iran, we encourage the Iranian authorities to establish a rigorous antimicrobial resistance surveillance system to monitor the emergence and development of antimicrobial resistance and to initiate strategies for prevention and control to reduce the burden of shigellosis in Iran.

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Transparency declarations
None to declare.

References
29 Huang IF, Chiu CH, Wang MH et al. Outbreak of dysentery associated with ceftriaxone-resistant Shigella sonnei: First report of plasmid-mediated


