Original Article

Emergence of Extended-Spectrum β-Lactamases in Clinical Isolates of Salmonella enterica in Tehran, Iran

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SUMMARY: The purpose of the current study was to investigate the presence and molecular type(s) of extended-spectrum β-lactamases (ESBLs) in Salmonella spp. isolates obtained from patients with diarrhea in hospitals of Tehran, Iran. Over a period of 17 months, 129 Salmonella spp. were isolated from fecal samples and tested for susceptibility using the Kirby-Bauer disk diffusion method; then, screening for ESBL-producing isolates and determination of their minimum inhibitory concentrations were carried out using the combined disk method and standard agar dilution method, respectively. The presence and type of ESBL-encoding genes were determined by PCR and sequence analysis. The isolates were all identified as Salmonella enterica of different serovars. The highest resistance in the collected Salmonella isolates was to nalidixic acid (45.7%), followed by tetracycline (43.4%), trimethoprim-sulfamethoxazole (36.4%), ampicillin (15.5%), and chloramphenicol (14.7%). All the isolates were susceptible to ciprofloxacin, gentamicin, and cefoxitin. Three S. enterica isolates were resistant to ampicillin, piperacillin, cefazidime, ceftaxime, ceftriaxone, cefpodoxime, cephalothin, and aztreonam. PCR and DNA sequencing revealed that two of the three isolates harbored both a blaCTX-M-15 gene and a blaTEM gene while the third one carried only a blaCTX-M-15 gene. This is the first study providing structural data for a CTX-M-type β-lactamase produced by Salmonella isolates recovered in Iran.

INTRODUCTION

In recent decades, the incidence of human Salmonella infections has been on the rise; such infections have become one of the most frequent causes of food-borne diseases (1, 2). In the early 1980s, the first extended-spectrum β-lactam antibiotics were introduced for clinical use, and the antimicrobials have been successfully used for treatment of Salmonella infections in both animals and humans (3, 4).

Shortly after release of the first extended-spectrum β-lactam antibiotics, bacteria resistant to such antibiotics began to appear (5). Bacterial resistance to extended-spectrum β-lactam antibiotics has been reported with increasing frequency, and these therapeutic failures have turned into a significant worldwide problem (5).

The Salmonella genome is naturally devoid of genes coding for serine active-site β-lactamases (6), a finding that is also supported by surveys of completed genomes or genomes under assembly from several different serovars of Salmonella enterica. Imported genes may code for β-lactamases that inactivate extended-spectrum antibiotics, and such β-lactamases are therefore denoted as extended-spectrum β-lactamases (ESBLs), which may belong to different molecular families, like SHV, TEM, or CTX-M-type β-lactamases (5).

In Iran, ESBLs have been identified in nosocomial and community isolates of various members of Enterobacteriaceae, such as Escherichia coli and Klebsiella spp. (7-9), but no documentation for the presence of ESBLs in Salmonella spp. has been published.

The aim of the present study was, thus, to investigate whether ESBLs were present in Salmonella spp. collected from patients suffering from diarrhea and hospitalized in Tehran, Iran.

MATERIALS AND METHODS

Study design, bacterial strains, and serological typing: During July 2007 to December 2008, 129 fecal samples were collected from 129 patients suffering from diarrhea. The patients were hospitalized in four different hospitals in Tehran, Iran.

The collected samples were cultured in three different media and an enrichment medium for optimal isolation, and subsequently inoculated into xylose-lysine deoxycholate agar. Colonies that morphologically resembled Salmonella spp. were identified by biochemical tests and by the slide agglutination test according to the Kauffmann-White scheme (10) using commercially available antisera (MAST House, Merseyside, UK).

Antimicrobial susceptibility testing: Isolates identified as Salmonella spp. were tested for susceptibility using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Oxoid) using ampicillin (10 μg), piperacillin (100 μg), cefuroxime (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), cefuroxime axetil (30 μg), aztreonam (30 μg), gentamicin (10 μg), tobramycin (10 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), ampicillin (10 μg), cefoxitin (30 μg), and nalidixic acid (30 μg) disks.
Cycling conditions were as follows: (i) an initial denaturation at 94°C, 30 s at 58°C, 57°C, and 59°C for \( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{SHV}} \), \( \text{bla}_{\text{CTX-M/R}} \); 5´-GAGTTTCCCCATTCCGTTTCC-3´ and \( \text{bla}_{\text{CTX-M/R}} \); 5´-CAGAATAAGGAATCCCA TGGTT-3´ (current study) for amplification of a 880-bp fragment covering the entire allele.

Interpretation of inhibition zones was carried out according to the criteria stipulated by the Clinical Laboratory Standards Institute (CLSI) (11), and quality control was performed using the E. coli ATCC 25922 reference strain.

**Phenotypic detection of ESBL-producing isolates:** Detection of ESBLs in the isolates was ascertained using the combined disk method as recommended by CLSI (11).

After the preliminary identification of isolates as ESBL-producers, their MICs for nalidixic acid and several \( \beta \)-lactam antibiotics were subsequently determined. The following antimicrobial agents were used for MIC determinations, ampicillin, cefazidime \( + \) clavulanic acid, cefotaxime \( + \) clavulanic acid, and nalidixic acid (all from Glaxo-SmithKline, Greenford, UK), cephaparin and pipercillin (both from Sigma, Steinheim, Germany), ceftriaxone (MAST House), and aztreonam (HiMedia, Mumbai, India).

Determination of MICs was performed using the standard agar dilution method and the resistance break points were specified according to CLSI (11).

Quality control was achieved by using E. coli ATCC 25922 and Klebsiella pneumoniae ATCC 700603 (SHV-18 producing strain [12]) as negative and positive controls, respectively.

**Molecular detection of \( \beta \)-lactam resistance genes:** Whole-cell DNA was extracted from ESBL-producing isolates as previously described (13). The presence of the ESBL genes, \( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{SHV}} \), and \( \text{bla}_{\text{CTX-M/R}} \); 5´-GAGTTTCCCCATTCCGTTTCC-3´ and \( \text{bla}_{\text{CTX-M/R}} \); 5´-CAGAATAAGGAATCCCA TGGTT-3´ (current study) for amplification of a 880-bp fragment covering the entire allele. The amplicon sizes of the \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \) fragments were 310 bp and 854 bp, respectively.

All PCR amplifications were performed using 1 U of Super Taq DNA polymerase (SuperTaq Co., London, UK) and 0.5 \( \mu \)M of each primer (Bioneer Co, Ltd, Daejeon, Korea). Cycling conditions were as follows: (i) an initial denaturation step of 5 min at 94°C; (ii) 30 cycles, each consisting of 60 s at 94°C, 30 s at 58°C, 57°C, and 59°C for \( \text{bla}_{\text{CTX-M/R}} \), \( \text{bla}_{\text{TEM}} \), and \( \text{bla}_{\text{SHV}} \), respectively, and 30 s at 72°C; then (iii) a final extension step of 5 min at 72°C.

Molecular weight determination of the PCR products was performed by gel electrophoresis in 1.2% agarose using molecular weight standards (Roche Diagnostics, Mannheim, Germany). The PCR products were purified using High Pure PCR product purification kit (Roche Diagnostics). Direct sequencing of the amplified products was performed for both strands using an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, Calif., USA). Sequence analyses were done using the Lasergene software package (version 6.00; DNASTar, Madison, Wis., USA).

**RESULTS**

Identification of the 129 Salmonella isolates showed that all of them were S. enterica, and the predominant serovar was S. enterica serovar Paratyphi C (33.3%), followed by S. enterica serovar Enteritidis (20.9%). S. enterica serovar Paratyphi B, Typhi, Paratyphi A, Infantis, and Bonn accounted for 17.1, 14.0, 8.5, 5.4, and 0.8% of the isolates.

**Antimicrobial susceptibility of Salmonella spp. isolates:** Susceptibility tests by the Kirby-Bauer disk diffusion method demonstrated that the highest resistance rate among the identified S. enterica isolates was to nalidixic acid (45.7%), followed by tetracycline (43.4%), trimethoprim-sulfamethoxazole (36.4%), ampicillin (15.5%), chloramphenicol (14.7%), pipercillin (12.4%), aztreonam (8.5%), and cephaparin (6.2%). All isolates were susceptible to ciprofloxacin, gentamicin, and cefotaxime. The predominant multidrug resistance pattern among the isolates was resistance to ampicillin, tetracycline, and nalidixic acid; this pattern was found in 27.9% of the isolates. Multidrug resistance to more than 4 antibiotics was observed in 12.4% of the isolates. Three isolates were resistant to cefotaxime, cefazidime, and ceftriaxone, and they were subjected to further analysis.

Two of the three isolates were identified as S. enterica serovar Enteritidis whereas the third was S. enterica serovar Bonn. All three isolates were recovered from different hospitals in Tehran.

Two of the three isolates had resistance to 11 of the 12 antibiotics while the third was resistant to 9 antibiotics. The various resistance patterns to three or more antibiotics are shown in Table 1.

**Phenotypic and genotypic ESBL investigation:** The three S. enterica isolates that were subjected to further analysis showed a high level of resistance (MIC \( \geq 128 \mu g/ml \)) to most

### Table 1. Distribution of resistance patterns to more than three antimicrobials among Salmonella spp. isolated from patients with acute diarrhea in Tehran, Iran

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHL, TET, AMP</td>
<td>7 (5.4)</td>
</tr>
<tr>
<td>NAL, SXT, TET</td>
<td>36 (27.9)</td>
</tr>
<tr>
<td>SXT, TET, AMP</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>SXT, TET, AMP, CHL</td>
<td>3 (2.3)</td>
</tr>
<tr>
<td>NAL, SXT, TAM, AMP</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>NAL, SXT, TET, AMP</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>NAL, CHL, CPD, TET</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>NAL, SXT, TET, AMP</td>
<td>6 (4.7)</td>
</tr>
<tr>
<td>CRO, CTX, CPD, CAZ, TET, AMP, CEF, PIP, ATM</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>NAL, SXT, CRO, CTX, CPD, CAZ, TET, AMP, CEF, PIP, ATM</td>
<td>2 (1.6)</td>
</tr>
</tbody>
</table>
of the tested β-lactam antibiotics, including the extended-spectrum cephalosporins, cefotaxime, ceftazidime, and ceftriaxone. The MICs to cefotaxime and ceftazidime were severely reduced in the presence of clavulanic acid (Table 2), which is indicative of expression of ESBLs most likely belonging to class A of the β-lactamases.

The sizes of the amplicons of the blaCTX-M and blaTEM genes from the ESBL-producing isolates were determined by agarose gel electrophoresis to be 0.9 kb and 0.3 kb, respectively (data not shown). The blaTEM gene was not amplified from any of the ESBL-producing isolates. Two of the ESBL-producing isolates (S. enterica serovar Enteritidis and S. enterica serovar Bonn) were shown to harbor the blaTEM gene (GenBank accession nos. FJ527491 and FJ527492) together with blaCTX-M-15 (GenBank accession nos. FJ654734 and FJ654733) whereas the third isolate (S. enterica serovar Enteritidis) carried only the blaCTX-M-15 gene (GenBank accession no. FJ774649).

The results of the MICs together with the serotypes of the ESBL-producing isolates are shown in Table 2.

### DISCUSSION

The S. enterica isolates included in this study showed resistance rates of 36.4, 15.5, and 14.7% to trimethoprim-sulfamethoxazole, ampicillin, and chloramphenicol, respectively, whereas Salmonella isolates in previous studies from Iran exhibited higher resistance rates (16,17). The frequent use of extended-spectrum cephalosporins at the expense of ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol probably influenced the gradual reversal of resistance to these drugs and emergence of resistance to extended-spectrum cephalosporins.

As seen in Table 2 the three isolates have similar MICs to the β-lactam antibiotics apart from the reproducibly low MIC to piperacillin of isolate HT-417-60. The susceptibility of all isolates to cefoxitin indicated that plasmid-borne AmpC β-lactamases most likely were not produced by the isolates.

The present study shows the presence of blaCTX-M-15 genes in three isolates, and it is the first identification of these ESBL-encoding genes in Salmonella spp. in Iran.

In the distribution of the bla genes are shown in Table 2.

### ACKNOWLEDGMENTS

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### REFERENCES


