Characterization of the First Extended-Spectrum \(\beta\)-Lactamase–Producing Nontyphoidal \textit{Salmonella} Strains Isolated in Tehran, Iran

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Abstract

The infections caused by \textit{Salmonella} remain a significant public health problem throughout the world. \(\beta\)-Lactams and fluoroquinolones are generally used to treat invasive \textit{Salmonella} infections, but emergence and spread of antibiotic-resistant strains are being increasingly notified in many countries. In particular, detection of extended-spectrum \(\beta\)-lactamases (ESBLs) in \textit{Salmonella} spp. is a newly emerging threat worldwide. This study was carried out to characterize \(\beta\)-lactamase–producing \textit{Salmonella} strains identified in Tehran, Iran. Over the 2-year period from 2007 to 2008, 6 of 136 \textit{Salmonella} isolates recovered from pediatrics patients, including three \textit{Salmonella enterica} serotypes Enteritidis (\textit{S}. Enteritidis) and three \textit{S}. Infantis, showed an ESBL-positive phenotype. Polymerase chain reaction and sequencing were used to identify the genetic determinants responsible for ESBL phenotypes. The \textit{Salmonella} isolates were also compared by pulsed-field gel electrophoresis. All ESBL-producing strains, but one, carried the \textit{bla}\textsubscript{CTX-M-15} gene. Moreover, three of four strains that proved to be positive for a \textit{bla}\textsubscript{TEM} gene were producing a TEM-1 \(\beta\)-lactamase. Two strains of \textit{S}. Infantis tested positive for a previously unidentified CTX-M and TEM ESBL, respectively. All ESBL-producing strains carried the insertion sequence ISEcp1 gene. Except for one strain of serotype Infantis, all strains were able to transfer the ESBL determinants by conjugation. Distinct, but closely related, pulsed-field gel electrophoresis patterns were observed among the strains belonging to both serotypes. This study reports for the first time the emergence and characterization of ESBL-producing \textit{S}. Enteritidis and Infantis strains in Iran.

Introduction

The infections caused by \textit{Salmonella} remain a significant public health problem throughout the world (Su \textit{et al.}, 2005). \textit{Salmonella} species causing nontyphoidal disease are among the most common enteric bacterial pathogens isolated from children (Li \textit{et al.}, 2005). These infections commonly cause self-limiting gastroenteritis, but severe infections, including bacteremia and meningitis, have also been reported (Parry, 2003).

Increasing occurrence of antimicrobial resistance in both typhoidal and nontyphoidal salmonellae is a serious public health problem. Cephalosporins are major antimicrobials used for treatment of serious infections caused by \textit{Salmonella} (Rotimi \textit{et al.}, 2008). Moreover, third-generation cephalosporins are first-choice drugs for treatment of patients with nontyphoidal \textit{Salmonella} infections (Egorova \textit{et al.}, 2008). However, their effectiveness is being compromised by the emergence of extended-spectrum \(\beta\)-lactamases (ESBLs) (Rotimi \textit{et al.}, 2008). This is of particular concern for the treatment of salmonellosis in children, because fluoroquinolones could not be used in this age group (Yates and Amyes, 2005).

Cephalosporin-resistant \textit{Salmonella} strains have been recognized since 1988 and are increasing in prevalence worldwide. This resistance in \textit{Salmonella} may be encoded by an increasing number of different ESBLs: most are derivatives of TEM and SHV \(\beta\)-lactamase families, whereas other groups, such as CTX-M, PER, and KPC, have been described in these last years (Tzouvelekis \textit{et al.}, 2000; Winokur \textit{et al.}, 2000; Mulvey \textit{et al.}, 2003). Moreover, genes encoding AmpC \(\beta\)-lactamases, that have historically been reported to be chromosomally located in some genera of the family

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Enterobacteriaceae, have been more recently described to disseminate within various species as plasmid-borne and confer high-level resistance to β-lactams (Qin et al., 2008).

Here we report the characterization of the first ESBL-producing non-typhoidal Salmonella strains isolated in Tehran, Iran, in the years 2007 and 2008.

Methods

Bacterial strains

The study included all Salmonella isolates recovered from pediatric patients aged less than 12 years and admitted to a major children hospital in Tehran, Iran, in the years 2007 and 2008. A single specimen was obtained from each patient and collected on the day of admission at the hospital. The isolates that had been identified as Salmonella by the conventional biochemical methods were serotyped by slide agglutination with specific antisera (Staten Serum Institut, Copenhagen, Denmark) at the Centre for Enteric Pathogens of Southern Italy, University of Palermo, Italy (Bopp et al., 1999).

Disk diffusion tests were performed according to the Clinical and Laboratory Standards Institute’s (formerly National Committee for Clinical Laboratory Standards) recommendations (CLSI, 2005) using disks (Oxoid, Hampshire, England) impregnated with ampicillin (10 μg), amoxicillin–clavulanic acid (AMC, 20–10 μg), aztreonam (30 μg), cefepime (30 μg), cephalothin (30 μg), cefotaxime (CX, 30 μg), ceftizoxime (30 μg), ceftriaxone (CR, 30 μg), cefazidime (CZ, 30 μg), cefoxitin (FOX, 30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), streptomycin (10 μg), kanamycin (10 μg), gentamicin (10 μg), imipenem (10 μg), nalidixic acid (30 μg), piperacillin (100 μg), ticarcillin (75 μg), trimethoprim–sulfamethoxazole (1.25/23.75 μg), and tetracycline (30 μg). Minimum inhibitory concentration (MIC) values of AMC, CX, CR, and CZ were determined by the agar dilution method (CLSI, 2006). For quality control of the culture media and antimicrobial drugs under study, Escherichia coli ATCC 25922 and in-house known ESBL-positive E. coli, Klebsiella pneumoniae, AmpC-positive Enterobacter cloacae, and ESBL-negative strains were tested under the same conditions.

Detection and identification of β-lactamas

ESBL screening was performed by the use of a double disk synergy test (Jarlier et al., 1988). The combination disk method based on the inhibitory effect of clavulanic acid was also used according to the Clinical and Laboratory Standards Institute’s criteria (CLSI, 2005).

The presence and characterization of ESBL genes as well as their genetic environment in the six ESBL-producing Salmonella enterica isolates were investigated by polymerase chain reaction (PCR) and sequencing. PCR amplification of bla genes, including blaTEM, blashv, and blactx-M, was performed with Go-Taq DNA polymerase (Promega; Madison, WI) using primers listed by Kiratisin et al. (2008). Detection of ISEcpI was performed for all isolates carrying blactx-M by PCR using primers and conditions described previously (Kiratisin et al., 2008). All PCR amplicons were verified by agarose gel electrophoresis after staining the gels with ethidium bromide.

Purifications of PCR amplicons were carried out by using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). PCR products of bla genes were subjected to bidirectional nucleotide sequencing using PCR primers to determine their molecular types. The nucleotide sequences and the deduced protein sequences were analyzed with the BLAST and Clustal W programs (multiple sequence alignment, pairwise comparisons of sequences, and dendrograms) (Thompson et al., 1994; Altschul et al., 1997).

Phenotypic detection of AmpC enzymes was performed using FOX (30 μg) and E. coli ATCC 25922 according to the method described by Shahid et al. (2004). Bacterial isolates were directly screened by spot inoculation of the test organisms near a FOX disk.

Conjugation assay

To demonstrate that bla genes detected by PCR were located on plasmids, resistance transfer experiments using a broth mating method were performed for all six isolates (Gray et al., 2006). Rifampicin-resistant E. coli K12J5 was used as the recipient. After 24 h, the mating mixture was centrifuged and the supernatant removed to eliminate cell-free β-lactamases. The mixture was then resuspended in saline and plated onto MacConkey agar (Oxoid, Basingstoke, UK) supplemented with CX (2 μg/mL) and rifampicin (150 μg/mL). Anti-microbial susceptibility, a confirmatory test for ESBL and AmpC phenotypes, and PCR detection for ESBL genes were performed on transconjugants using the aforementioned procedures.

Pulsed-field gel electrophoresis

For molecular typing, chromosomal DNAs of ESBL-producing Salmonella strains were subjected to pulsed-field gel electrophoresis (PFGE) analysis using a CHEF Mapper XA apparatus (Bio-Rad Laboratories, Hercules, CA). Agarose plugs containing bacterial DNA were prepared and processed for PFGE as described elsewhere (Ranjbar et al., 2007, 2008). Restriction analysis of chromosomal DNA with XbaI (New England Biolabs, Beverly, MA) was performed, and separation of DNA was carried out by using 1% pulsed-field gel agarose (SeaKem Gold agarose; Cambrex Bio Science, Rockland, ME). DNA banding patterns were visually compared and interpreted according to the criteria suggested by Tenover et al. (1995).

Results

Six of the 136 pediatric S. enterica isolates identified in Tehran, Iran, during the years 2007 and 2008 showed an ESBL-positive phenotype. Three strains were attributed with serotype Enteritidis and three with serotype Infantis.

The resistance patterns of the six strains are illustrated in Table 1. All isolates were resistant to AMC with a MIC range of 128–256 μg/mL, CX with a MIC range of 128–256 μg/mL, CR with a MIC range of 128–256 μg/mL, and CZ with a MIC range of 64–128 μg/mL. All were susceptible to imipenem and ciprofloxacin.

The six isolates were subjected to PCR experiments to detect ESBL genes, including blatem, blashv, and blactx-M. blactx-M sequences were detected in all Salmonella strains, whereas blatem in four isolates, two Enteritidis and two Infantis, respectively (Table 1). None of the Salmonella isolates was positive for blashv. Amplification with primer ISEcpI yielded a PCR product of 615 bp in all isolates. Amplification
with primer Ecp1 and an internal \( \text{bla}_{\text{CTX-M}} \) primer yielded a PCR product of \( \approx 1000 \) bp.

DNA sequencing revealed that \( \text{bla}_{\text{CTX-M}} \) from five of the six Salmonella isolates was identical and encoded for \( \text{bla}_{\text{CTX-M15}} \). A novel CTX-M nucleotide sequence (GenBank accession no. FJ873739), designated \( \text{bla}_{\text{CTX-M88}} \), was detected in the S. Infantis 120 strain. The deduced amino acid sequence (291 residues) of the novel CTX-M compared with other related enzymes revealed that it was similar to CTX-M-15, except for the single amino acid substitution of histidine in place of arginine at position 277. Sequence analysis of \( \text{bla}_{\text{TEM1}} \) detected \( \text{bla}_{\text{TEM169}} \) in three of four strains of Salmonella. A novel TEM nucleotide sequence, designated \( \text{bla}_{\text{TEM169}} \), was found in the S. Infantis 149 strain (GenBank accession no. FJ873740). The amino acid sequence (289 residues) of the novel TEM compared with other related enzymes revealed that it differed from the parental enzyme TEM-1 for the amino acid substitutions of leucine in place of methionine at position 69 and glycine in place of tryptophan at position 165 (Table 2). These amino acid substitutions are consistent with the inclusion of the novel TEM in the groups of the TEM-type enzymes resistant to inhibitors (IRT) (4).

**Table 2. Amino Acid Substitutions in TEM-169 Compared with TEM-1, TEM-39, TEM-78, and TEM-83 Sequences**

<table>
<thead>
<tr>
<th>Amino acid no.</th>
<th>TEM-1 (IRT-10)</th>
<th>TEM-78 (IRT-22)</th>
<th>TEM-83</th>
<th>TEM-169</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Phe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>Met</td>
<td>Leu</td>
<td>Val</td>
<td>Leu</td>
</tr>
<tr>
<td>165</td>
<td>Trp</td>
<td>Arg</td>
<td>Cys</td>
<td>Gly</td>
</tr>
<tr>
<td>275</td>
<td>Arg</td>
<td>Asn</td>
<td></td>
<td>Gen</td>
</tr>
<tr>
<td>276</td>
<td>Asn</td>
<td>Asp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Numbers are according to the system of Ambler et al. (1991).

**Discussion**

CTX-M enzymes emerged \( \approx 20 \) years ago, shortly following the introduction in the clinical practice of CTX. Since then, the dissemination of these enzymes is steadily increasing worldwide and today it involves both hospital-acquired and community infections (Kiratisin et al., 2007). In the past, CTX-M ESBL enzymes have been found almost exclusively in E. coli and Klebsiella spp., in association with multiple drug resistance. However, more recently, ESBL-producing Salmonella isolates from human source have been noticed through an increasing number of countries worldwide (Su et al., 2005; Egorova et al., 2008; Rotimi et al., 2008; Riaño et al., 2009). Since 1990, the effectiveness of combinations of hydrolyzable penicillins with a \( \beta \)-lactamase inhibitor, such as clavulanic acid or sulbactam, has been also compromised by the emergence of the mutant TEM-type \( \beta \)-lactamases, collectively named as IRT \( \beta \)-lactamases (Chabi et al., 1999). Moreover, incidence of plasmid-mediated AmpC-producing strains, driven by the emergence of the plasmid-borne \( \text{bla}_{\text{CMY-2}} \) gene, has sharply increased in recent years in both hospital and community settings (Qin et al., 2008).

In this study, CTX-M and TEM ESBLs have been found in six isolates of serotypes Enteritidis and Infantis, the two most...
isolates could be supposedly associated to an AmpC-like ge-

chromosome-located resistance to AMC in the remaining five
digested genomic DNA from

spectively. Lanes 1–3 and 4–6 belong to serotypes Enteritidis and Infantis, re-

strain H9812 which served as a molecular size marker. Lanes

resistance. Our finding is particularly worrying as the treat-

cause of the propensity of the latter to facilitate the spread of

bla

bearing the plasmid-borne IRT
to be negative to a phenotypic test for detection of AmpC

closely depends on the use of third-generation cephalosporins.

The results of our surveillance study would describe the

involvement of Iran in an epidemic, the antimicrobial drug

resistance that is increasingly compromising the usefulness of

the \( \beta \)-lactam antibiotics and other previously life-saving an-
tibacterial drugs. Indeed, because ESBL-producing strain

prevalence greatly varies between different sites and in the

same site over time, regional and local estimates are most

useful to epidemiological assessment of such a public health

threat, but to clinical decision-making also (Winokur et al.,
2001; Pfaller and Segreti, 2006).

Proper detection of \( \beta \)-lactamases and corresponding treat-

ment strategies are of paramount importance in curtailing this

growing epidemic. Prevention and control strategies should

be urgently implemented to stop further spreading of these

strains.

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Disclosure Statement

No competing financial interests exist.

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