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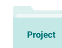
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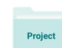
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Original Article

Characterization of the First Extended-Spectrum β-Lactamase–Producing Nontyphoidal *Salmonella* Strains Isolated in Tehran, Iran

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Abstract

The infections caused by *Salmonella* remain a significant public health problem throughout the world. β-Lactams and fluoroquinolones are generally used to treat invasive *Salmonella* infections, but emergence and spread of antibiotic-resistant strains are being increasingly notified in many countries. In particular, detection of extended-spectrum β-lactamases (ESBLs) in *Salmonella* spp. is a newly emerging threat worldwide. This study was carried out to characterize β-lactamase–producing *Salmonella* strains identified in Tehran, Iran. Over the 2-year period from 2007 to 2008, 6 of 136 *Salmonella* isolates recovered from pediatrics patients, including three *Salmonella enterica* serotypes Enteritidis (*S*. Enteritidis) and three *S*. Infantis, showed an ESBL-positive phenotype. Polymerase chain reaction and sequencing were used to identify the genetic determinants responsible for ESBL phenotypes. The *Salmonella* isolates were also compared by pulsed-field gel electrophoresis. All ESBL-producing strains, but one, carried the $bla_{\text{CTX-M-15}}$ gene. Moreover, three of four strains that proved to be positive for a bla_{TEM} gene were producing a TEM-1 β-lactamase. Two strains of *S*. Infantis tested positive for a previously unidentified CTX-M and TEM ESBL, respectively. All ESBL-producing strains carried the insertion sequence IS*Ecp1* 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 *S*. Enteritidis and Infantis strains in Iran.

Introduction

The infections caused by *Salmonella* remain a significant public health problem throughout the world (Su *et al*., 2005). *Salmonella* species causing nontyphoidal disease are among the most common enteric bacterial pathogens isolated from children (Li *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 *Salmonella* (Rotimi *et al*., 2008). Moreover, third-generation cephalosporins are first-choice drugs for treatment of patients with nontyphoidal *Salmonella* infections (Egorova *et al*., 2008). However, their effectiveness is being compromised by the emergence of extended-spectrum β-lactamases (ESBLs) (Rotimi *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 *Salmonella* strains have been recognized since 1988 and are increasing in prevalence worldwide. This resistance in *Salmonella* may be encoded by an increasing number of different ESBLs: most are derivatives of TEM and SHV β-lactamase families, whereas other groups, such as CTX-M, PER, and KPC, have been described in these last years (Tzouvelekis *et al*., 2000; Winokur *et al*., 2000; Mulvey *et al*., 2003). Moreover, genes encoding AmpC β-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 nontyphoidal *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), ceftazidime (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-lactamases

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 bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$, was performed with Go-*Taq* DNA polymerase (Promega, Madison, WI) using primers listed by Kiratisin *et al.* (2008). Detection of IS*Ecp1* was performed for all isolates carrying $bla_{CTX\text{-}M}$ 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). Antimicrobial 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 *Xba*I (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 bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$. $bla_{CTX\text{-}M}$ sequences were detected in all *Salmonella* strains, whereas bla_{TEM} in four isolates, two Enteritidis and two Infantis, respectively (Table 1). None of the *Salmonella* isolates was positive for bla_{SHV}. Amplification with primer IS*Ecp1* yielded a PCR product of 615 bp in all isolates. Amplification

Table 1. Characteristics of the Extended-Spectrum β-Lactamase–Producing *Salmonella enterica* Strains Under Study

Strain no.	*Time of isolation*	*Serotype*	*Antibiotic resistance pattern*	*Type of ESBL*	*PFGE pattern*
60	May 2007	Enteritidis	AMP, AMC, PIP, TIC, CF, AT, CR, CX, CZ, FE, CT	CTX-M-15, TEM-1	E1
109	April 2008	Enteritidis	AMP, AMC, PIP, TIC, CF, AT, CR, CX, CZ, FE, CT, SXT, S, K, TE, NA	CTX-M-15, TEM-1	E2
159	Oct 2008	Enteritidis	AMP, AMC, PIP, TIC, CF, AT, CR, CX, CZ, FE, CT, SXT, S, TE, NA	CTX-M-15	E3
105	Jan 2007	Infantis	AMP, AMC, PIP, TIC, CF, AT, CR, CX, CZ, FE, CT, SXT, S, TE, NA	CTX-M-15, TEM-1	I1
120	May 2008	Infantis	AMP, AMC, PIP, TIC, CF, AT, CR, CX, CZ, FE, CT, SXT, S, TE, NA	CTX-M-88	I2
149	Sept 2008	Infantis	AMP, AMC, PIP, TIC, CF, AT, CR, CX, CZ, FE, CT, SXT, S, TE, NA	CTX-M15, TEM-169	I3

ESBL, extended-spectrum β-lactamase; PFGE, pulsed-field gel electrophoresis; AMP, ampicillin; AMC, amoxicillin–clavulanic acid; AT, aztreonam; CF, cephalothin; CR, ceftriaxone; CX, cefotaxime; CZ, ceftazidime; CT, ceftizoxime; FE, cefepime; K, kanamycin; NAL, nalidixic acid; PIP, piperacillin; SXT, sulfamethoxazole-trimethoprim; TIC, ticarcillin; S, streptomycin; TET, tetracycline.

with primer IS*Ecp1* and an internal $bla_{\text{CTX-M}}$ primer yielded a PCR product of $\approx$1000 bp.

DNA sequencing revealed that $bla_{\text{CTX-M}}$ from five of the six *Salmonella* isolates was identical and encoded for $bla_{\text{CTX-M15}}$. A novel CTX-M nucleotide sequence (GenBank accession no. FJ873739), designated $bla_{\text{CTX-M 88}}$, 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 bla_{TEM} detected $bla_{\text{TEM-1}}$ in three of four strains of *Salmonella*. A novel TEM nucleotide sequence, designated $bla_{\text{TEM-169}}$, 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

	Amino acid substitution[b]				
Amino acid no.[a]	*TEM-1*	*TEM-39 (IRT-10)*	*TEM-78 (IRT-22)*	*TEM-83*	*TEM-169*
16	Phe				
69	Met	Leu	Val	Leu	Leu
165	Trp	Arg	Arg	Cys	Gly
275	Arg			Gen	
276	Asn	Asp	Asp		

[a]Numbering is according to the system of Ambler *et al.* (1991).
[b]The amino acid that will be substituted is indicated when a point mutation generates an amino acid substitution compared with the sequence of TEM-1.
IRT, inhibitor-resistant TEM-type enzymes.

All *Salmonella* strains and the respective transconjugants were tested negative for AmpC β-lactamases by the spot-inoculation method. All the results were replicated in duplicate experiments. No further investigation was conducted for detection of these enzymes.

Based on the conjugation assay, all strains, but the *S*. Infantis strains 120, were able to transfer the *bla* genes to transconjugants, suggesting that they were plasmid mediated. The MIC range of the transconjugants for CX, CR, and CZ was 64–128, 128–256, and 16–32 μg/mL, respectively. Only *S*. Infantis 149 proved to be able to horizontally transfer resistance to AMC; indeed, the transconjugants demonstrated reduced susceptibility (MIC, 16 μg/mL) compared with the recipient strain (MIC, 1 μg/mL).

The PFGE banding patterns of the three ESBL *S*. Enteritidis and *S*. Infantis isolates, respectively, were distinguishable (Fig. 1) and, hence, likely clonally unrelated.

Discussion

CTX-M enzymes emerged $\sim$20 years ago, shortly following the introduction in the clinical practice of CX. 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 β-lactamase inhibitor, such as clavulanic acid or sulbactam, has been also compromised by the emergence of the mutant TEM-type β-lactamases, collectively designed as IRT β-lactamases (Chaïbi *et al.*, 1999). Moreover, incidence of plasmid-mediated AmpC-producing strains, driven by the emergence of the plasmid-borne $bla_{\text{CMY-2}}$ 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

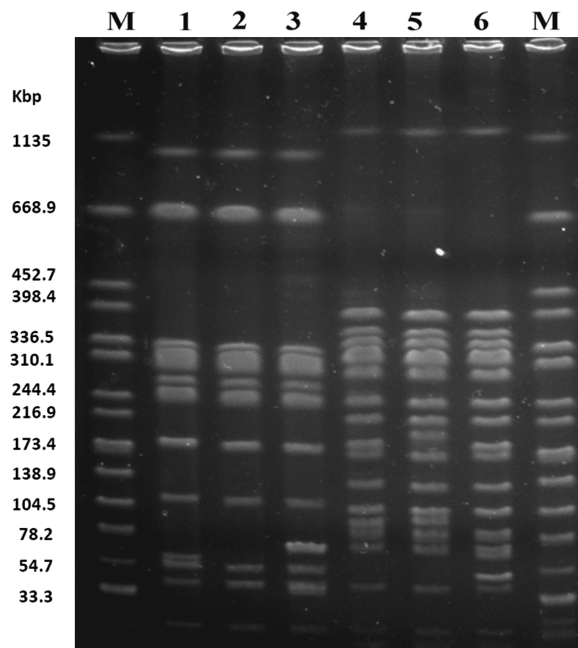

FIG. 1. Pulsed-field gel electrophoresis of *Xba*I-digested genomic DNAs from extended-spectrum β-lactamase–producing *Salmonella enterica* isolates. Lanes M are *Xba*I-digested genomic DNA from *Salmonella* serotype Braenderup strain H9812 which served as a molecular size marker. Lanes 1–3 and 4–6 belong to serotypes Enteritidis and Infantis, respectively.

prevalent serotypes from pediatric cases of salmonellosis in Tehran, Iran (data not published). Prevalence of ESBL-producing *Salmonella* was 4.2% with a lower proportion than that of about 17% attributable to isolates from Kuwait and United Arab Emirates (Rotimi *et al.*, 2008). However, our prevalence was higher than the prevalence of 1.5% in Taiwan, 1.9% in the United States, 2.4% in Latin America, and 0.8% in Europe (Dunne *et al.*, 2000; Winokur *et al.*, 2000; Li *et al.*, 2005).

The pattern of ESBL production was also consistent with data of Rotimi *et al.* (2008) from Kuwait and United Arab Emirates. However, it is interesting to outline the presence within the ESBLs identified in this study of two novel ESBLs, CTX-M-88 and TEM-169, both confirming the anticipated continuous emergence of mutants under the selective pressure of the use of oxymino cephalosporins and β-lactamase inhibitors. The IS*Ecp1* insertion sequence that has been implicated in the expression and mobilization of the $bla_{\text{CTX-M}}$ genes has been also detected in the six isolates under study (Eckert *et al.*, 2004). The finding of the $bla_{\text{CTX-M}}$ genes in *Salmonella* in association with IS*Ecp1* is of serious concern, because of the propensity of the latter to facilitate the spread of resistance. Our finding is particularly worrying as the treatment of *Salmonella* systemic infections in infants and children closely depends on the use of third-generation cephalosporins.

The *S.* Enteritidis and Infantis strains under study proved to be negative to a phenotypic test for detection of AmpC β-lactamases. However, with the exception of the Infantis strain bearing the plasmid-borne IRT $bla_{\text{TEM-169}}$, the apparently chromosome-located resistance to AMC in the remaining five isolates could be supposedly associated to an AmpC-like genetic determinant. Epidemiological data suggest the opportunity for considering AmpC enzymes when screening for β-lactamases by phenotypic and PCR-based techniques in isolates belonging to the family of Enterobacteriaceae.

Molecular typing of isolates demonstrated some degree of genetic diversity that has to be considered in the light of the worldwide clonal dissemination of some serotypes such as Enteritidis. The relative genomic heterogeneity within the isolates along with the absence of time-space clustering of their isolation and allocation of their drug resistance genes on plasmid DNA suggests horizontal gene transfer among strains rather than spread of specific clones.

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 β-lactam antibiotics and other previously life-saving antibacterial 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 β-lactamases and corresponding treatment 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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