Detection of TEM-, SHV- and CTX-M-type \( \beta \)-lactamase production among clinical isolates of *Salmonella* species

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Enteric fever is a major public health problem in developing countries. Due to the problem of resistance to first-line drugs and fluoroquinolone, cephalosporins are currently used for treatment of enteric fever. Cephalosporin resistance in *Salmonella* spp. is mainly due to production of extended-spectrum \( \beta \)-lactamases (ESBLs). The majority of ESBLs in *Salmonella* are derivatives of the TEM and SHV \( \beta \)-lactamase families. The objectives of this study were to detect antibiotic susceptibility patterns, ESBL production and TEM-, SHV- and CTX-M-encoding genes (\( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{SHV}} \) and \( \text{bla}_{\text{CTX-M}} \)) among clinical isolates of *Salmonella* spp. A total of 134 *Salmonella* isolates \([\text{Salmonella} \text{Typhi} (n=101), \text{Salmonella} \text{Paratyphi A} (n=31), \text{Salmonella} \text{Paratyphi B} (n=1)\) and *Salmonella* Typhimurium \((n=1)\)] were included in this study. Multidrug resistance was seen in 5/134 (3.73 %) isolates, all of which belonged to serotype S. Typhi. A better susceptibility profile was observed for first-line drugs (ampicillin, chloramphenicol, co-trimoxazole and tetracycline) and cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefixime and cefepime). However, 131 (97.76 %) of the 134 isolates were resistant to nalidixic acid and one (0.75 %) was resistant to ciprofloxacin. TEM-1-type \( \beta \)-lactamase (\( \text{bla}_{\text{TEM-1}} \)) was detected in six (4.47 %) of the 134 isolates, which belonged to the serotype S. Typhi. All six TEM-positive isolates were negative for the \( \text{bla}_{\text{SHV}} \) gene and none of the isolates was positive for the \( \text{bla}_{\text{CTX-M}} \) gene. The presence of the \( \text{bla}_{\text{TEM}} \) gene encoding TEM-1 \( \beta \)-lactamase is believed to confer resistance only to penicillins and early cephalosporins; however, the resistance spectrum of TEM-1 descendants may extend to second-, third- and fourth-generation cephalosporins. The ESBLs derived from TEM-1 differ from their progenitors by as few as 1 aa, and have the ability to hydrolyse third-generation cephalosporins. Therefore, appropriate selection and rotation of antibiotics as well as continuous monitoring of antibiotic susceptibility profiles could help to control the emergence and spread of resistant strains.

**INTRODUCTION**

Enteric fever includes typhoid and paratyphoid fever. It is an important public health problem caused by *Salmonella enterica* serovar Typhi and *S. enterica* serovars Paratyphi A, B and C respectively. Both serotypes are solely human pathogens. Infection occurs mainly due to ingestion of food or water contaminated with human waste (Scherer & Miller, 2001). Typhoid fever is a major cause of morbidity and mortality, estimated to cause 21.7 million illnesses and 217 000 deaths per year, whereas paratyphoid fever causes illness in an estimated 5.4 million people per year worldwide (Crump et al., 2004). Appropriate treatment reduces the mortality rate as low as 0.5 % (Cooke & Wain, 2004). Multidrug-resistant (MDR) *S. enterica* (resistant to chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole) has emerged worldwide in the last two decades (Madhulika et al., 2004). Invasive *Salmonella* infections can be treated with fluoroquinolones and \( \beta \)-lactam (cephalosporins) antibiotics (Rotimi et al., 2008). However, *S.
enterica isolates have developed resistance to fluoroquinolones in the Indian subcontinent and other regions (Effa & Bukirwa, 2008).

In Salmonella, resistance to cephalosporins is largely due to the production of extended-spectrum β-lactamases (ESBLs) and is often plasmid mediated (Rotimi et al., 2008). The majority of ESBLs in Salmonella are derivatives of the TEM and SHV β-lactamase families. Other β-lactamases like CTX-M have also been described in Salmonella (Bradford, 2001; Bonnet, 2004). CTX-M-type ESBLs or cefotaximases are encoded by bla_CTX-M genes located on a plasmid or on the chromosome (Rodríguez et al., 2004). Studies on ESBL-producing Salmonella strains have revealed the presence of insertion sequenceISEκp1 upstream of thebla_CTX-Mgene, which may contribute to the mobility and dissemination of these genes to other bacteria in the environment (Sjölund et al., 2008; Tamang et al., 2011).

In this background, the present study was carried out to document recent antibiotic susceptibility profiles of ESBL production and to screen for the presence of TEM-, SHV- and CTX-M-encoding genes (bla_TEM, bla_SHV and bla_CTX-M) among clinical isolates of Salmonella spp.

METHODS

**Bacterial isolates and serotyping.** A total of 134 Salmonella isolates (98 isolates were collected in 2007–2009 and the remaining 36 were collected in 2011–2012) from blood samples of patients attending tertiary-care hospitals and other diagnostic laboratories in Chennai, India, were included in this study. Characterization and identification of the isolates were performed using a battery of biochemical tests (Old, 1996). Serotyping of the Salmonella isolates was performed using specific antisera procured from the King Institute of Preventive Medicine and Research, Chennai, India. Institutional ethical clearance was obtained to conduct this study.

**Antibiotic susceptibility testing.** Antibiotic susceptibility testing of the isolates was carried out by a standard Kirby-Bauer disk-diffusion method using Clinical and Laboratory Standards Institute guidelines (CLSI, 2011). The following antibiotics were tested: ampicillin (10 μg), chloramphenicol (30 μg), co-trimoxazole (1.25/23.75 μg), tetracycline (30 μg), cefotaxime (30 μg), cefazidime (30 μg), ceftriaxone (30 μg), cefixime (5 μg), cefepime (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), ofloxacin (5 μg), levofloxacin (5 μg) and imipenem (10 μg). Antibiotic discs were obtained from HiMedia Laboratories.

The MICs of ampicillin, cefotaxime and cefazidime (HiMedia Laboratories) were determined by an agar dilution method (CLSI, 2011). Escherichia coli ATCC 25922 was used as the control strain.

**Detection of β-lactamase production.** Isolates were screened for ESBL production by a combination disc method using cefotaxime (30 μg) and ceftazidime (30 μg) and a disc of cefotaxime + clavulante (30 + 10 μg) and ceftazidime + clavulante (30 + 10 μg) (HiMedia Laboratories). A ≥ 5 mm increase in diameter of the inhibition zone of the cefotaxime + clavulante and ceftazidime + clavulante disc compared with the cefotaxime and ceftazidime disc alone was interpreted as phenotypic evidence of ESBL production (CLSI, 2011).

**PCR for β-lactamase-encoding genes and nucleotide sequencing analysis.** The β-lactamase genes bla_TEM, bla_SHV and bla_CTX-M were detected by PCR using forward and reverse primer pairs with a boiled suspension of bacterial cells as DNA template (Lim et al., 2009). The primers used were bla_TEM: TEM-F, 5'-AGTGGATACACAATTTCG-3', and TEM-R, 5'-CTGACAGTTCACAACTGTA-3'; bla_SHV: SHV-F, 5'-GTTGATGGTATTCAATCGC-3', and SHV-R, 5'-TAAGGTTGCAATGCTG-3'; and bla_CTX-M:CTX-M1, 5'-ATGTGCAGYACCAGTAARGT-3', and CTX-M2, 5'-GGGTR-AARTARGTSCACCAGA-3'. Reaction mixtures of 25 μl containing 1.5 U Tag DNA polymerase (New England BioLabs) in the reaction buffer provided by the manufacturer containing 1.5 mM MgCl₂, 200 μM dNTPs, 0.2 μM primer and 2 μl DNA template were prepared in a thermal cycler (Eppendorf). The cycling parameters employed comprised one cycle of 5 min at 96 °C, followed by 35 cycles of 1 min at 96 °C, 1 min at 58 °C for bla_TEM or at 60 °C forbla_SHV and 1 min at 72 °C, with a final extension of 10 min at 72 °C. The thermal cycling conditions for the CTX-M gene were one cycle of 7 min at 94 °C, followed by 35 cycles of 50 s at 94 °C, 40 s at 50 °C and 1 min at 72 °C, with a final extension of 5 min at 72 °C. The amplicon size for bla_TEM and bla_SHV was 867 bp, and forbla_CTX-M was 593 bp. Aliquots (10 μl) of each PCR product were subjected to electrophoresis on a 1.5% agarose gel. The amplified product was sequenced using ABI 3730XL DNA Analyser (Applied Biosystems). Nucleotide sequences were analysed by searching GenBank using BLAST (http://www.ncbi.nlm.nih.gov/blast/).

**RESULTS**

Of the 134 isolates recovered from blood cultures of suspected cases of enteric fever, Salmonella Typhi was the predominant serotype (n=101, 75.4 %), followed by Salmonella Paratyphi A (n=31, 23.12 %), Salmonella Paratyphi B (n=1, 0.74 %) and Salmonella Typhimurium (n=1, 0.74 %).

Tables 1 and 2 show the antibiotic susceptibility pattern and resistance pattern for all the Salmonella isolates. Table 3 depicts the MICs of various antimicrobials for S. Typhi and S. Paratyphi A.

**Table 1. Antibiotic susceptibility pattern of Salmonella isolates (n=134) to various antibiotics**

Results are shown as number of isolates (%).

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Sensitive (%)</th>
<th>Intermediate (%)</th>
<th>Resistant (%)</th>
</tr>
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<tbody>
<tr>
<td>Ampicillin</td>
<td>127 (94.78)</td>
<td>1 (0.75)</td>
<td>6 (4.47)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>128 (95.52)</td>
<td>1 (0.75)</td>
<td>5 (3.73)</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>130 (97.10)</td>
<td>0 (0)</td>
<td>4 (2.9)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>127 (94.78)</td>
<td>2 (1.49)</td>
<td>5 (3.73)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>123 (91.8)</td>
<td>11 (8.2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>134 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>132 (98.5)</td>
<td>0 (0)</td>
<td>2 (1.5)</td>
</tr>
<tr>
<td>Cefixime</td>
<td>133 (99.25)</td>
<td>1 (0.75)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>134 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>2 (1.49)</td>
<td>1 (0.75)</td>
<td>131 (97.76)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>126 (94.03)</td>
<td>7 (5.22)</td>
<td>1 (0.75)</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>134 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>134 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>134 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
Of the 134 Salmonella isolates studied, 127 (94.78 %) were sensitive to ampicillin and tetracycline, 128 (95.52 %) were sensitive to chloramphenicol, 130 (97.10 %) were sensitive to co-trimoxazole, 123 (91.8 %) were sensitive to cefotaxime, 132 (98.5 %) were sensitive to ceftriaxone, 133 (99.25 %) were sensitive to cefixime and 126 (94.03 %) were sensitive to ciprofloxacin. All 134 isolates were susceptible to ceftazidime, cefepime, ofloxacin, levofloxacin and imipenem. Multidrug resistance was seen in 5/134 (3.73 %) isolates, all of which belong to serotype S. Typhi. A better pattern of sensitivity was observed for first-line drugs (ampicillin, chloramphenicol, co-trimoxazole and tetracycline) and cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefixime and cefepime). However, 97.76 % (131/134) of isolates were resistant to nalidixic acid and 0.75 % (1/134) were resistant to ciprofloxacin. All the isolates were negative for ESBL production by the combination disc method.

TEM-type β-lactamase (blaTEM) was detected in six (4.47 %) of the 134 isolates, which belonged to the serotype S. Typhi (Fig. 1), and nucleotide sequencing analysis revealed that the six isolates carried the blaTEM gene (TEM-1-type β-lactamase). All the six TEM-positive isolates were negative for the blaSHV gene and showed resistance to ampicillin (MIC >32 μg ml\(^{-1}\)) but were sensitive to third- and fourth-generation cephalosporins. None of the 134 isolates was positive for the blaCTX-M gene.

### DISCUSSION

Enteric fever is a major systemic health problem in developing countries, including India. Various studies document S. Typhi as the predominant serotype and S. Paratyphi A as the second most common serotype (Hirose et al., 2001; Manchanda et al., 2006; Choudhary et al., 2013), which correlates well with our study result.

The Salmonella Paratyphi A isolates showed a better susceptibility to routinely used antibiotics than S. Typhi. In comparison with previous studies, which reported 11–13 % multidrug-resistant Salmonella (Dutta et al., 2005;...
Verma et al., 2010; Bhattarcharya et al., 2011), we observed a significant decrease in MDR (3.73%) among our study isolates. The majority of the isolates were resistant to nalidixic acid, whereas only one isolate showed resistance to ciprofloxacin. Several studies have shown that *Salmonella* spp. resistant to nalidixic acid may show reduced fluoroquinolone susceptibility *in vivo*, resulting in treatment failure (Crump et al., 2003; Kownhar et al., 2007; Choudhary et al., 2013).

In our study, none of the isolates was found to be positive for ESBLs by a combination disc method. A possible reason for this result is that TEM-1 β-lactamase has the ability to hydrolyse only penicillins/early cephalosporins but not third- or fourth-generation cephalosporins, which could not be detected by the ESBL combination disc method.

In Gram-negative pathogens, β-lactamase production remains the most important contributing factor to β-lactam resistance (Medeiros, 1997). The persistent exposure of bacterial strains to a multitude of β-lactams has led to overproduction and mutation of β-lactamases leading to their ability to hydrolyse penicillins and broad-spectrum cephalosporins (Bush, 2001).

In addition to bacteria that have long been known to produce β-lactamase (such as *Staphylococcus aureus* and *Enterobacteriaceae*), these enzymes have spread to pathogens such as *Haemophilus influenzae* and *Neisseria gonorrhoeae* that previously lacked β-lactamase (Jacoby & Munoz-Price, 2005). Additionally, there are a few reports of ESBL production in pathogens such as *Shigella flexneri*, *Shigella dysenteriae*, *Shigella sonnei* and *Vibrio cholerae* (Ahamed & Kundu, 1999; Fortineau et al., 2001; Pai et al., 2001; Petroni et al., 2002).

TEM-type β-lactamases are found mainly in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. The first TEM variant with increased activity against extended-spectrum cephalosporins was TEM-3. In 1983, SHV-2 isolated from *K. pneumoniae* showed transferable resistance to cefotaxime as well as to other cephalosporins (Gupta, 2007). The ESBLs derived from TEM-1, TEM-2 and SHV-1 differ from their progenitors by as few as 1 aa, which results in the ability to hydrolyse third-generation cephalosporins (Paterson & Bonomo, 2005). The CTX-M-type β-lactamases, which differ from TEM- and SHV-type ESBLs, were initially reported in 1980s and are closely related to the β-lactamases of *Klebsiella* spp. (Bonnet 2004). *Salmonella* strains may acquire the gene encoding CTX-M-type ESBLs from *E. coli* and *Klebsiella* spp. in the community and then spread it among the various serotypes of *Salmonella* (Rotimi et al., 2008).

Although we found the *bla*TEM gene encoding TEM-1 β-lactamase in our isolates, which was shown to confer resistance only to penicillins and early cephalosporins, the resistance spectrum of its descendants may extend to second-, third- and fourth-generation cephalosporins (Salverda et al., 2010). This was supported by an outbreak of TEM-1-producing *Klebsiella oxytoca* in a neonatal unit, which was reported in Liverpool, UK, in 1982. Initially, the isolate was sensitive to ceftazidime, and infected patients were treated with this drug, but subsequent isolation of *K. oxytoca* from the same unit found that they harboured the TEM-type ESBL (TEM-12) and showed resistance to ceftazidime (Du Bois et al., 1995). It was demonstrated that the ceftazidime resistance gene was transferrable and carried on a plasmid (Payne et al., 1990).

ESBL production in *Salmonella* spp. was first identified in 1988 and is increasing in prevalence worldwide; it has been detected in *Salmonella enterica* strains of different serovars in a number of countries including Italy, France, Ireland and the Philippines (Morris et al., 2006; Al Naiemi et al., 2008). S. Paratyphi A and S. Typhi with high rates of MDR and ESBL production (SHV-12 and TEM-1) were reported in a study conducted in Nepal (Pokharel et al., 2006). *Salmonella* spp. co-producing CTX-M- and TEM-type β-lactamases have been documented in a few case reports from Bangladesh (Ahmed et al., 2012) and India (Karthikeyan et al., 2011). Plasmid analysis of the resistant isolate revealed that the *bla*CTX-M and *bla*TEM genes were...

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**Fig. 1.** PCR detection of *bla*TEM gene. Lanes: M, 100 bp ladder; 1–6, 876 bp PCR product from the six *S. Typhi* isolates carrying the *bla*TEM gene.
located in the same plasmid, which carried the IS\textit{Ecpl} element upstream of the \textit{bla}\textsubscript{CTX-M} gene to facilitate mobilization and expression (Karthikeyan et al., 2011). The present study is important in understanding the mechanism of resistance operating in these common pathogens, which are also endemic in India.

Extended-spectrum cephalosporins are the drugs of choice for the treatment of infections due to fluoroquinolone-resistant \textit{Salmonella} spp. The emergence of ESBLs in \textit{Salmonella} constitutes a new challenge. ESBLs are the most evolving mechanism of antibiotic resistance among the family of Enterobacteriaceae due to the selective pressure imposed by inappropriate use of third-generation cephalosporins. This emergence may be due to the exchange of mobile genetic elements, such as plasmids and transposons, between enteric bacteria. Therefore, appropriate selection and rotation of antibiotics, as well as continuous monitoring of antibiotic susceptibility profiles, could help to control the emergence and spread of resistance strains.

REFERENCES


