Introduction

The spread of resistance to β-lactam antibiotics by extended-spectrum β-lactamase (ESBL) production among members of the Enterobacteriaceae is a serious concern. ESBLs are increasingly associated with the genus Salmonella. In parallel with the increasing number of Salmonella serotypes, an increase in the variety of ESBLs has been reported. The ESBLs described in Salmonella typhimurium comprise SHV-2, CTX-M2, PER-1, PER-2, CTX-M4, CTX-M5 and CTX-M6. We report on TEM-3 production by S. typhimurium isolates recovered from children in Casablanca.

Materials and methods

Bacterial isolates

The isolates had low levels of resistance to cefotaxime (MIC 4–8 mg/L) and ceftazidime (4–8 mg/L) and were resistant to gentamicin and co-trimoxazole. They were fully susceptible to imipenem, cefoxitin, cefotetan, kanamycin, tobramycin, amikacin, tetracycline, chloramphenicol and ciprofloxacin. The isolates were closely related and only one representative was used in the following experiments.

Plasmid analysis and transfer of resistance determinants

Plasmid analysis was performed by the alkaline lysis method as described by Sambrook et al. Extracts were run on 0.8% agarose gels at 7 V/cm and stained with ethidium bromide. The sizes of the plasmids were estimated by comparing their migration with molecular weight standards (Supercoiled DNA Ladder; Gibco BRL, Cergy Pontoise, France).

Conjugation experiments were carried out with Escherichia coli K12 J53-2. Individual colonies of the donor and recipient isolates were suspended in 2 mL of Luria–Bertani broth supplemented with 0.5% sucrose and incubated for 18 h. The transconjugants were selected on MacConkey agar supplemented with rifampin (100 mg/L) and ampicillin (100 mg/L).

Each plasmid of the clinical isolates was electroeluted and transformed into E. coli DH5α using the CaCl2 procedure. The transformants were selected on MacConkey agar supplemented with ampicillin (100 mg/L) and cefotaxime (1 mg/L). The transformants and the transconjugants were assessed for their plasmid content, their antibiotic susceptibilities and ESBL production by the double disc synergy test.

Plasmid curing was performed by inoculating 10³–10⁴ cells of S. typhimurium, transformants and transconjugants with chloramphenicol (100 mg/L) for 72 h. The cured transformants and transconjugants were selected on MacConkey agar supplemented with ampicillin (100 mg/L) and cefotaxime (1 mg/L).

Isolates of extended-spectrum β-lactamase (ESBL)-producing Salmonella typhimurium were recovered from children admitted to the IbnRochd University Hospital of Casablanca in 1994. These isolates produced TEM-3 as shown by PCR, isoelectric focusing and sequencing. Production of TEM-3 and resistance to gentamicin were encoded by a 10 kb plasmid that could be transferred by conjugation and transformation. This report extends the list of ESBLs produced by S. typhimurium and stresses the need for continuous surveillance of non-typhoidal Salmonella to adapt antibiotic treatment and preventive measures.
into a series of tubes containing acridine at varying concentrations (10–10,000 mg/L). After incubation the cultures were streaked on to MacConkey agar with ampicillin (100 mg/L) and cefotaxime (1 mg/L).

**Isoelectric focusing**

Analytical isoelectric focusing was carried out with *E. coli* transconjugants using the method described by Matthew et al. Cells were harvested from overnight Luria–Bertani broth culture by centrifugation and resuspended in sodium phosphate buffer 0.05 M pH 7.0. β-Lactamase was released by sonication. Enzymes were identified by isoelectric focusing in polyacrylamide minigels (Phast Gels IEF, pH gradient 3–9; Pharmacia, Uppsala, Sweden) and subsequent staining with nitrocefin (Oxoid, Dardilly, France); ESBLs of known pI were used as markers.

**DNA amplification by PCR**

PCR amplification of the TEM or SHV genes from *S. typhimurium* and from the transconjugants were carried out on a DNA thermal cycler, Progene (Techne, Duxford, UK). The PCR mixture contained in a total volume of 50 μL: 10 pmol of each primer, 0.2 pmol of deoxynucleotide triphosphates, 1 U of Taq polymerase (Promega) and 10 μL of bacterial lysate obtained by heating bacterial colonies to 100°C for 10 min. The following oligonucleotide primers specific for the SHV and TEM genes were obtained from Genosphere Biotechnologie (Paris, France): for SHV genes: 5'-GCCGGGGTTATTCTTATTTGTCGC-3' and 5'-TCTTTCCGATGCGCCGCAGTCA-3'; and for TEM genes 5'-ATAAAAATCTTGAGAC-3' and 5'-TTACCAATGCTTAATCA-3'. The PCR programme consisted of an initial denaturation at 94°C for 12 min, followed by 35 cycles of 30 s at 94°C, 30 s at 45°C (TEM) or 65°C (SHV), 90 s at 72°C. A final extension was performed at 72°C for 10 min. The PCR products were analysed on a 2% agarose gel stained with ethidium bromide and visualized by UV light. The specificities of the TEM and SHV primers for amplification of SHV and TEM genes, respectively, were tested using known controls.

**DNA sequencing**

The sequence was determined by direct sequencing of the specific amplified product obtained as described previously with a crude DNA preparation from *S. typhimurium* isolate as template. It was performed by the dideoxy chain termination procedure of Sanger et al. on an ABI 1377 automatic sequencer with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with Ampli-Taq DNA polymerase FS (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA).

**Results and discussion**

Isoelectric focusing showed that the *S. typhimurium* isolates produced a β-lactamase with a pI of 6.3. PCR amplification of the β-lactamase genes revealed that the ESBL belongs to the TEM group. Sequence analysis of the amplified PCR product confirmed the identification of the ESBL as TEM-3. To the best of our knowledge, this is the first report of TEM-3 β-lactamase in *S. typhimurium*. A broad spectrum of ESBLs in non-typhoidal *Salmonella* has been reported, including molecular class A other than SHV and TEM and molecular class C enzymes. TEM-3 was first described in *Klebsiella pneumoniae*, and recent surveys of ESBLs in France revealed that this enzyme was frequently detected. This β-lactamase has already been described in *Salmonella panama*, *Salmonella kedougou* and *Salmonella enteritidis*.

The *S. typhimurium* isolates harboured three plasmids: P1 (10 kb), P2 (8 kb) and P3 (4.5 kb). Only the 10 kb plasmid was transferred by conjugation to *E. coli* K12 as determined by plasmid profile analysis. Transconjugants were ESBL producers, as determined by PCR and double disc synergy test, and were resistant to gentamicin and cotrimoxazole. Electroelution and transformation showed

<table>
<thead>
<tr>
<th>Isolate</th>
<th>AMP (mg/L)</th>
<th>CTX (mg/L)</th>
<th>CAZ (mg/L)</th>
<th>IPM (mg/L)</th>
<th>GEN (mg/L)</th>
<th>SXT (mg/L)</th>
<th>CIP (mg/L)</th>
<th>TET (mg/L)</th>
<th>CHL (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em></td>
<td>&gt;128</td>
<td>8</td>
<td>4</td>
<td>0.5</td>
<td>128</td>
<td>&gt;32</td>
<td>&lt;0.125</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>E. coli</em> K12 J53-2</td>
<td>2</td>
<td>0.03</td>
<td>0.03</td>
<td>0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;1</td>
<td>2</td>
</tr>
<tr>
<td>Transconjugant</td>
<td>&gt;128</td>
<td>1</td>
<td>1</td>
<td>0.125</td>
<td>32</td>
<td>&gt;32</td>
<td>&lt;0.125</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>&gt;128</td>
<td>&lt;0.125</td>
<td>0.25</td>
<td>0.25</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Transformant</td>
<td>&gt;128</td>
<td>16</td>
<td>32</td>
<td>0.5</td>
<td>32</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;1</td>
</tr>
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</table>

AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; GEN, gentamicin; SXT, trimethoprim–sulfamethoxazole; CIP, ciprofloxacin; TET, tetracycline; CHL, chloramphenicol.
that plasmid P1 (10 kb) was responsible for the ESBL production and resistance to gentamicin but not to cotrimoxazole. Transformation with plasmids P2 and P3 did not confer resistance to β-lactams. MICs for *S. typhimurium*, the two *E. coli* and the transconjugants and transformants are shown in the Table. In the curing experiments, no resistance was lost. Reports vary greatly as to resistance phenotypes, the number and sizes of the plasmids carried by ESBL-producing non-typhoidal Salmonella, probably reflecting different genetic events that occurred in distinct geographical areas. ESBL production and resistance to gentamicin in the isolate reported here are encoded by a 10 kb plasmid whereas in *S. enteritidis* TEM-3 production was encoded by a 12 kb plasmid and was associated with resistance to amikacin, netilmicin, tobramycin, tetracycline and sulphonamides. In *S. kedougou* TEM-3 was due to the presence of an 85 kb plasmid and the isolates were resistant to all aminoglycosides except gentamicin and to sulphonamides and tetracycline.

Resistance is easily transferred by the isolates and the list of antibiotics to which co-resistance is transferred is of great importance. Most isolates are co-resistant to at least one aminoglycoside, while some are resistant to chloramphenicol and/or sulphonamide and even to ciprofloxacin. A major risk would be the transmission of the resistance traits to *Salmonella typhi* via an ESBL-producing Enterobacteriaceae. This event could occur, since *S. typhi* is able to acquire antibiotic resistance *in vivo* and since *in vivo* acquisition of ESBL has been shown to occur in *S. kedougou* and *S. enteritidis*.

This report extends the list of ESBLs produced by *S. typhimurium*, one of the two major serotypes of non-typhoidal *Salmonella*. The increasing number of *Salmonella* serotypes involved in ESBL production, the variety of the ESBLs, the high stability of the genetic determinants, and the co-transfer of resistance to antibiotics recommended for the treatment of systemic non-typhoidal *Salmonella* infection and typhoid fever are a serious problem especially in developing countries where these infections are still endemic.

**Acknowledgements**

This work was partially supported by a grant from the Comité de Recherche of the IbnRochd University Hospital of Casablanca and from the Programme Thématique d’Appui à la Recherche Scientifique (PROTARS P1T2/04).

**References**


Received 13 November 2000; returned 11 April 2001; revised 31 July 2001; accepted 31 August 2001