Characterization of Extended-Spectrum Beta-Lactamase–Producing Salmonella enterica Serotype Brunei and Heidelberg at the Hussein Dey Hospital in Algiers (Algeria)

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Nosocomial outbreaks of extended-spectrum beta-lactamase-producing
Salmonella enterica serotype Brunei and Heidelberg at the Hussein Dey
hospital in Algiers (Algeria)

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Abstract

Objectives: The purpose of this work was to study the genetic determinants responsible for extended-spectrum β-lactamase (ESBL) resistance of *Salmonella* isolated from children during the period 1995 to 2008 at the Hussein Dey hospital in Algiers (Algeria).

Methods: Fourteen ESBL-resistant *Salmonella* isolates were tested towards 22 antimicrobial agents. PCR and sequencing were used to determine the underlying genetic determinants responsible for the ESBL phenotypes. The transferability of the ESBL phenotypes was tested by conjugation, and ERIC-PCR was employed to typing the isolates.

Results: All isolates were resistant to ticarcillin, ticarcillin-clavulanate, piperacillin, amoxicillin-clavulanate, cefuroxime, aztreonam, ceftazidime, cefotaxime (except 2 isolates), cefepime and cefpirome. PCR and DNA sequencing identified these extended-spectrum β-lactamasces as TEM-48 (n=6), TEM-4 (n=3), CTX-M-15 (n=4), and one new TEM, designated TEM-188.

Conclusion: this study demonstrates the emergence of a public health risk related to ESBL in *Salmonella* in Algeria.

Keywords:

Outbreaks, Children, *Salmonella* Brunei, Heidelberg, Extended-spectrum β-lactamase, TEM-188
INTRODUCTION

Non-typhoidal salmonellae are one of the principal pathogens implicated in food-borne gastroenteritis worldwide. Animals and their products, particularly meat, chicken eggs, and milk, are major sources of human infection. The incidence of non-typhoidal *Salmonella* infections has increased considerably in many countries, but with marked differences among countries (Makanera et al., 2003). Although antibiotics are not usually recommended in cases of *Salmonella* Enterocolitis, they are crucial in systemic infections. Extra-intestinal infectious complications, including meningitis, sepsis and bacteremia are more common in infants and the elderly, and in immunocompromised patients. In these potentially life-threatening cases, the antibiotics of choice are fluoroquinolones and extended-spectrum cephalosporins. *Salmonella* spp. resistant to extended-spectrum cephalosporins have been recognized since 1988, and are increasing in prevalence worldwide. This is of particular concern for the treatment of salmonellosis in children, because fluoroquinolones should not be used in this age group (Kruger et al., 2004; Yates and Amyes, 2005).

To date, more than 340 β-lactamases have been described. *Salmonella* have been found to express a wide variety of ESBL types, including TEM (Ait Mhand et al., 2002), SHV (Hammami et al., 1991), PER (Casin et al., 2003), OXA (Hanson et al., 2002), and CTX-M (Tamang et al., 2011), and, more recently, plasmid-mediated AmpC type enzymes, such as DHA-1 (Barnaud et al., 1998), CMY-2 (Koeck et al., 1997), and ACC-1 (Rhimi-Mahjoubi et al., 2002).
In Algeria, ESBLs have been identified in nosocomial isolates of various Enterobacteriaceae, such as *E. coli*, *K. pneumoniae* and *E. cloacae* (Touati *et al.*, 2006; Iabadene *et al.*, 2008; Messai *et al.*, 2008, Ramdani-Bouguessa *et al.*, 2011). However, only few reports for the presence of these enzymes in *Salmonella* have been published (Naas *et al.*, 2005; Touati *et al.*, 2008; Iabadene *et al.*, 2009; Naas *et al.*, 2011; Bouzidi *et al.*, 2011).

In this study, we characterized the ESBLs in a collection of putative ESBL positive *Salmonella* spp. isolated from 1995 to 2008 at the Hussein Dey hospital in Algiers (Algeria).
METHODS

Bacterial isolates
A collection of 14 non-duplicate amoxicillin-resistant *S. enterica* isolates was examined. They were obtained from stool samples of children between 1995 and 2008 at the Hussein Dey hospital in Algiers (Algeria). All isolates were biochemically identified by using the API 20E identification system (BioMérieux, Marcy l’Étoile, France).

All isolates were serotyped at the French National Reference Center for Salmonella, Institut Pasteur, Paris, France on the basis of somatic O, phase 1 flagellar, and phase 2 flagellar antigens by agglutination tests with antisera (BioRad, Marnes-la-coquette, France). The serotypes were designated according to the White-Kauffmann-Le Minor scheme.

Susceptibility testing and ESBL detection
Disk diffusion susceptibility tests for aztreonam, ticarcillin, piperacillin, amoxicillin-clavulanate, ticarcillin-clavulanate, cefoxitin, cefpirome, cefepime, piperacillin-tazobactam, cefuroxime, imipenem, tobramycin, amikacin, gentamicin, kanamycin, sulfonamide, trimethoprim-sulfamethoxazole, nalidixic acid, ciprofloxacin, tetracycline and chloramphenicol (BioRad) were performed according to the recommendations of the Antiogram Committee of the French Society for Microbiology (http://www.sfm.asso.fr/). Minimum inhibitory concentrations (MICs) for amoxicillin, cefotaxime, ceftazidime and ceftriaxone were determined by Etest (AB BIODISK, Solna, Sweden) performed on Mueller–Hinton agar plates as recommended by the manufacturer.

Extended-Spectrum β-Lactamase (ESBL) production was detected by a double-disk synergy test (DDST) and was performed by placing disks of ceftazidime, cefotaxime...
and aztreonam at a distance of 20mm (centre to centre) from a disk with amoxicillin/clavulanic acid (20/10 µg). Enhancement of the inhibition zone between the disks containing clavulanic acid and cefotaxime, ceftazidime or aztreonam indicated the ESBL production (Jarlier et al., 1988).

**β-Lactamases characterization**

Total DNA was extracted by using the QIAmp DNA mini kit (Qiagen, Courtaboeuf, France) according to the instructions of the manufacturer. The ESBL-encoding genes \( \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}} \) and \( \text{bla}_{\text{CTX-M}} \) were detected by PCR using specific primers (Table 1) and further identified by nucleotide sequence analysis of the PCR products. Sequences were analyzed using the BLAST 2.0 (Basic Local Alignment Search Tool) software available on the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/BLAST.cgi).

**PCR fingerprinting**

For enterobacterial repetitive intergenic consensus (ERIC) PCR, whole-cell DNA of isolates was extracted using the QIAmp DNA mini kit (Qiagen). The primers were ERIC1 (5’-ATGTAAGCTCCTGGGGATTCAC-3’) and ERIC2 (5’-AAGTAAGTACTGGGGTGAGCG-3’). Each 23 µl PCR reaction mixture contained 25 pmol of each primer, 5mmol/L each deoxynucleotide triphosphate (dNTP) and 2.5 U of Taq polymerase (Qiagen, Courtaboeuf, France) in the manufacturer’s provided buffer. Two microliters of total DNA (about 80 ng) was added to each reaction to reach the final 25µl PCR reaction volume. The ERIC-PCR parameters were as follows: initial denaturation at 95°C for 7 min; 30 cycles of denaturation at 92°C for 30 s, annealing at 50°C for 1 min, and extension at 65°C for 8 min; followed by a final extension at 65°C for 16 min (Cao et al., 2008). PCR amplicons were resolved
on 1% agarose gel containing ethidium bromide by horizontal electrophoresis in Tris-borate-EDTA buffer. Gels were visualized under UV light with Bio-Profil (Vilbert Lourmat, Torcy, France).
RESULTS

Fourteen strains belonging to *Salmonella enterica* serotypes Brunei (10 strains) and Heidelberg (4 strains) were isolated and serotyped in the laboratory and confirmed at the Pasteur Institute in Paris, France. The strains were isolated from infants.

All isolates exhibited resistance or decreased susceptibilities to ticarcillin, ticarcillin-clavulanate, piperacillin, amoxicillin-clavulanate, cefuroxime, aztreonam, ceftazidime, cefotaxime, ceftazidime and cefpirome (table 2). They remained susceptible to imipenem, cefoxitin and piperacillin-tazobactam. The DDS test was positive for all of these isolates. MICs determination showed that S. Brunei examined (Table 3) were resistant to amoxicillin (MIC > 256µg/ml), ceftazidime (MIC = 64µg/ml), cefotaxime (MIC = 16µg/ml) and ceftriaxone (MIC = 6µg/ml). For S. Heidelberg strains, resistance were observed for all strains (amoxicillin: MIC > 256µg/ml, ceftazidime: MIC = 48µg/ml, cefotaxime: MIC > 32µg/ml and ceftriaxone: MIC > 32µg/ml).

All isolates were resistant to gentamicin and tobramycin. The isolates of *S. Brunei* were resistant to kanamycin and amikacin (except isolates of S9 and S12). Resistance to nalidixic acid was observed in all isolates of *S. Heidelberg*. All of the strains were susceptible to ciprofloxacin, cotrimoxazole, tetracycline and chloramphenicol.
TEM consensus PCR assays gave the expected PCR fragments for the 10 strains of S. Brunei (Table 2) and CTX-M amplifications were positive for the 4 strains of S. Heidelberg. SHV amplification was negative.

Three isolates of S. Brunei (S9, S12 and S22) harbored the \(\text{bla}_{\text{TEM}-4}\) gene and the \(\text{bla}_{\text{TEM}-48}\) gene was found in six isolates of S. Brunei (S15, S16, S18, S20, S21 and S23). The four strains of S. Heidelberg were found to produce CTX-M-15.

One strain of S. Brunei (S10) was found to produce a new TEM. This protein has been designated TEM-188 (http://www.lahey.org/studies/webt.htm, GenBank Accession Number JN211012). The new TEM β-lactamase differed from TEM-1 by three substitutions: Leu21Phe, Gly238Ser and Glu240Lys. These substitutions are identical to those found in TEM-48. However, TEM-48 has an additional substitution, Thr265Met. Isolates producing TEM-48 and TEM-188 showed identical antibiotypes (table 2 and 3) suggesting that the substitution Thr265Met in TEM-48 has no effect on β-lactams susceptibility.

The ERIC-PCR method was applied to the six TEM-48-producing S. Brunei strains and the four CTX-M-15-producing S. Heidelberg. Two ERIC-PCR patterns were observed, one for the six S. Brunei and a second one for the four S. Heidelberg, suggesting a clonal expansion for each resistant population defined by its serotype.
Discussion

There are a number of commonly identified serotypes of *Salmonella* associated with human infections. In the United States, the most common serovars were Typhimurium, Enteritidis, Newport, Heidelberg, and Javiana. In other parts of the world, there are some differences in the predominant serovars associated with disease. In the European Union, Enteritidis is the predominant serovar. In many parts of Asia, Choleraesuis is one of the top serovars (Foley and Lynne, 2007). *S. Brunei* has been rarely reported from animals, animal food products, and patients with human salmonellosis. There are only 3 articles found on pubmed when we use Salmonella Brunei as keyword.

In our study, 10 strains of *S. Brunei* were recovered from infants during the period 1995 to 2008 whereas the 4 *S. Heidelberg* were recovered only in October 2008. Unfortunately, data of the commonly identified serotypes in Algeria were not available.

The largest subset of the population for which antibiotic susceptibility of Salmonella is a major concern is children. Although, gastroenteritis is the most frequent clinical manifestation, systemic infections are common, and even cases of meningitis have been reported. Such serious infections are most common in children and the elderly. Antibiotic therapy is strongly recommended in such cases (Arlet et al., 2006). Extended-spectrum cephalosporins are commonly used to treat patient with invasive infections or severe diarrhea caused by *Salmonellae*; however, during the past years extended-spectrum cephalosporins-resistant *Salmonellae* have frequently been reported worldwide, including north Africa (Ahmed et al., 2009; Ohmani et al., 2010; Bouzenoune et al., 2011; Naas et al., 2011).
The 1st salmonella strains with ESBLs in Africa were identified in 1988 in Tunisia (Hammami et al., 1991). TEM-4 β-lactamase, which differed from the TEM-1 β-lactamase sequence by 4 substitutions (Leu21Phe, Glu104Lys, Gly238Ser and Thr265Met), was first reported for *E. coli* in France by Paul et al. (Paul et al., 1989). This enzyme was described in an isolate of *Salmonella* collected during a French national survey in 1998 (De Champs et al., 2000) and reported in isolates of *Salmonella* serotype Mbandaka in Tunisia (Makanera et al., 2003). This was the first reported identification of the TEM-4 ESBL in Algerian *Salmonella* Brunei.

The amino acid substitutions of the sequence of TEM-48 compared to the TEM-1 β-lactamase sequence were Leu21Phe, Gly238Ser, Glu240Lys and Thr265Met. TEM-48 was first described in *K. pneumoniae* strains in Poland (Gniadkowski et al., 1998). To our knowledge, no report on TEM-48 isolated from *Salmonella* has been previously published. Moreover, all isolates of *S. Brunei* producing TEM-48 were resistant to aminoglycosides and sulfonamide.

CTX-M-15 was identified in different salmonella serotypes, but to our knowledge, this is the first report of CTX-M-15 in *S. Heidelberg* isolates. CTX-M-15-producing *Salmonella* isolates were reported in different serotypes in Algeria, including *Infantis* (Naas et al., 2011) and *Kedougou* (Touati et al., 2008). The four isolates of *S. Heidelberg* were found resistant to nalidixic acid, but susceptible to fluoroquinolones. PCR for the plasmid-mediated mechanisms were negative for the four isolates, suggesting that the nalidixic resistance was probably mediated by mutations in topoisomerases.

PCR-mediated genome fingerprinting based on ERIC or REP has been found useful for the typing of outbreak and sporadic *Salmonella* isolates (Merino et al., 2003).
Nosocomial outbreaks due to ESBLs-producing *Salmonella* have been described in many countries, such as the outbreak in Tunisia due to TEM-4-producing S. Mbandaka (Makanera et al., 2003). The great majority of them have involved pediatric wards and especially neonatology units. In the community, many outbreaks have been reported and were largely foodborne outbreaks (Arl et al., 2006). The two clonal strains observed in our study, were recovered throughout the 13-year study period. The S. Brunei-producing TEM-48 strains were recovered from neonatology ward, except one strain recovered from cradle ward, whereas the S. Heidelberg-producing CTX-M-15 strains were isolated in cradle ward in which the age of children is about 3 months. These observations indicated that gastrointestinal infections were caused mainly by clonally related *Salmonella* serotype isolates and clonal spread was responsible for their dissemination.

Salmonellosis is most often attributed to the consumption of contaminated foods such as poultry, beef, pork, eggs, milk, seafood, nut products, and fresh produce (Foley and Lynne, 2007). In this outbreak, food as a source was excluded because milk was commercially prepared and other infants, hospitalized in the same ward at the same period, were fed with the same preparations but did not become infected with these strains. Therefore, a horizontal transmission of the outbreak strain had probably occurred.

In conclusion, this study demonstrates the emergence of a public health risk related to β-lactams resistance in *Salmonella* in Algeria. The implementation of effective screening methods for the detection of beta-lactamases and ESBLs as well as the establishment of surveillance programs became key factors in the control of hospital outbreaks.
Acknowledgments

230 We thank Janick Madoux for her technical assistance.$^3$
References


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<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Reference</th>
</tr>
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<td>CTX-M1-A2</td>
<td>5’-CTTCCAGAATAAGGAATC-3’</td>
<td>Dutour et al., 2002</td>
</tr>
<tr>
<td></td>
<td>628R</td>
<td>5’-CCTTTCCATCCATGTCACCA-3’</td>
<td>Brasme et al., 2007</td>
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<tr>
<td></td>
<td>405F</td>
<td>5’-GTGGCGATGAATAAGCTGA-3’</td>
<td>Brasme et al., 2007</td>
</tr>
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<td></td>
<td>CTX-M1-B2</td>
<td>5’-CCGTTTCCGCTATTACAA-3’</td>
<td>Dutour et al., 2002</td>
</tr>
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<td>Chanal et al., 2000</td>
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<td></td>
<td>TEM-B</td>
<td>5’-TTACCAATCGTTTAATCA-3’</td>
<td></td>
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<tr>
<td>$b_{a_{SHV}}$</td>
<td>SHV-F</td>
<td>5’-ATGCGTTATATCGCTGTG-3’</td>
<td>Kojima et al., 2005</td>
</tr>
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<td></td>
<td>SHV-R</td>
<td>5’-TTAGCGTTGCGATGCTCGA-3’</td>
<td></td>
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<td>Isolation date</td>
<td>Ward</td>
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<tr>
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</table>
Legend:

**CXM**: cefuroxime, **TIC**: ticarcillin, **PIP**: piperacillin, **AMC**: amoxicillin-clavulanate, **FEP**: cefepime, **CPO**: cefpriom, **CTX**: cefotaxim, **CAZ**: ceftazidime, **TZP**: piperacillin-tazobactam, **IMP**: imipenem, **FOX**: cefoxitin, **GEN**: gentamicin, **TOB**: tobramycin, **Kan**: kanamycine, **AMK**: amikacin, **SUL**: sulfonamide, [**R**]: Resistant, [**S**]: Susceptible, [**I**]: Intermediary.
Table 3: MICs of β-lactams for S. Brunei and S. Heidelberg producing ESBL

<table>
<thead>
<tr>
<th>Code</th>
<th>ESBL</th>
<th>CTX</th>
<th>AMX</th>
<th>CFT</th>
<th>CAZ</th>
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<td>&gt;32</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>48</td>
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<td>CTX-M-15</td>
<td>&gt;32</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>48</td>
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<td>CTX-M-15</td>
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<td>&gt;256</td>
<td>&gt;32</td>
<td>48</td>
</tr>
<tr>
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<td>&gt;32</td>
<td>&gt;256</td>
<td>&gt;32</td>
<td>48</td>
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<tr>
<td>S. Brunei S10</td>
<td>TEM-188</td>
<td>16</td>
<td>&gt;256</td>
<td>12</td>
<td>32</td>
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<tr>
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<td>TEM-4</td>
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<td>64</td>
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<td>6</td>
<td>64</td>
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<td>&gt;256</td>
<td>6</td>
<td>64</td>
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<td>TEM-48</td>
<td>16</td>
<td>&gt;256</td>
<td>6</td>
<td>64</td>
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<tr>
<td>S. Brunei S23</td>
<td>TEM-48</td>
<td>16</td>
<td>&gt;256</td>
<td>6</td>
<td>64</td>
</tr>
</tbody>
</table>

**Legend:** AMX: amoxicillin, CTX: cefotaxime, CAZ: ceftazidime, CFT: ceftriaxone