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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Rachida Kermas,1 Abdelaziz Touati,1 Lucien Brasme,2 Elisabeth Le Magréx-Debar,2 Sadjia Mehrane,3 François-Xavier Weill,4 and Christophe De Champs2

Abstract

The purpose of this work was to study the genetic determinants responsible for extended-spectrum cephalosporin (ESC) resistance of *Salmonella* collected during the period of 1995–2008 at the Hussein Dey hospital in Algiers (Algeria). Fourteen ESC-resistant *Salmonella* isolates were tested towards 22 antimicrobial agents. Polymerase chain reaction (PCR) and sequencing were used to determine the underlying genetic determinants responsible for the extended-spectrum beta-lactamase (ESBL) phenotypes. Enterobacterial Repetitive Intergenic Consensus PCR was employed to type the isolates. All tested isolates were resistant to ticarcillin, ticarcillin-clavulanate, piperacillin, cefuroxime, aztreonam, cefotaxime, and cefpirome. PCR and DNA sequencing identified these ESBLs as TEM-48 (n = 6), TEM-4 (n = 3), CTX-M-15 (n = 4), and one new TEM, designated TEM-188. Thus, continued surveillance for the presence of ESBL-producing (non-typhoidal) salmonellae in Algeria is essential.

Introduction

Non-typhoidal salmonellae are one of the principal pathogens implicated in foodborne 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 antimicrobials are not usually recommended in cases of *Salmonella* enterocolitis, they are crucial in systemic infections. Extraintestinal 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 (ESCs). *Salmonella* spp. resistant to ESCs 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).

*Salmonella* has been found to express a wide variety of extended-spectrum beta-lactamase (ESBL) types, including TEM (Ait Mhamed et al., 2002), SHV (Hammami et al., 1991), PER (Casin et al., 2003), OXA (Hanson et al., 2002), and CTX-M (Taman et al., 2011); and plasmid-mediated AmpC type enzymes, including 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 *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (Touati et al., 2006; Iabadene et al., 2008; Messai et al., 2008; Ramdani-Bougouessa et al., 2011). However, only a few reports on the presence of these enzymes in *Salmonella* have been published (Naas et al., 2005, 2011; Touati et al., 2008; Iabadene et al., 2009; Bouzidi et al., 2011).

In this study, we characterized the ESBLs in a collection of ESC-resistant *Salmonella* isolated from 1995 to 2008 at the Hussein Dey hospital in Algiers (Algeria).

Methods

**Bacterial isolates**

A collection of 14 non-duplicate ESC-resistant *Salmonella enterica* isolates were examined. They were obtained from

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4Institut Pasteur, Centre National de Référence des *Salmonella*, Unité des Bactéries Pathogènes Entériques, Paris, France.
stool samples of children between 1995 and 2008 at the Hussein Dey hospital in Algiers (Algeria).

Selenite enrichment broth was inoculated with stool samples and incubated for 24 h at 37°C. The enrichment broth was subcultured onto Hektoen agar and incubated for 24 h at 37°C. The suspicious colonies were biochemically identified by using the API 20E identification system (BioMérieux, Marcy l’Etoile, 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.

The following β-lactamase–producing isolates were used as control isolates: Enterobacter aerogenes CF 2403 for TEM; K. pneumoniae Kps12 for SHV; E. coli MEN, E. coli 27, and K. pneumoniae Bhe CD13 for CTX-M, K. pneumoniae Kps760 for DHA-1, E. coli TN13 for CMY-2, K. pneumoniae 1734 for FOX, K. pneumoniae SLK54 for ACC, and E. coli C600 PMC251 for MIR. These control isolates were kindly provided by Guillaume Arlet (Service de Bactériologie, Hôpital Tenon AP-HP, Paris, France).

Susceptibility testing and ESBL detection

Disk diffusion susceptibility tests for aztreonam, ticarcillin, piperacillin, amoxicillin-clavulanate, ticarcillin-clavulanate, cefoxitin, cefpirome, cefepime, ceftriaxone, imipenem, tobramycin, amikacin, gentamicin, kanamycin, sulfonamide, trimethoprim-sulfamethoxazole, nalidixic acid, ciprofloxacin, tetracycline, and chloramphenicol (BioRad, Hercules, CA) were performed according to the recommendations of the Antibiotic Committee of the French Society for Microbiology (Anonymous, 2010). 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. Escherichia coli ATCC 25922 was used as the control. The results were interpreted according to the recommendations of the Antibiotic Committee of the French Society for Microbiology (Anonymous, 2010). ESBL production was detected by a double-disk synergy test (DDST) and was performed by placing disks of cefazidime, cefotaxime, aztreonam at a distance of 20 mm (center to center) from a disk with amoxicillin/clavulanic acid (20/10 μg). Enhancement of the inhibition zone between the disks containing clavulanic acid and cefotaxime, cefazidime, or aztreonam indicated the ESBL production (Jarlier et al., 1988).

β-Lactamase characterization

Total DNA was extracted by using the QI Amp DNA mini kit (Qiagen, Courtabeuf, France) according to the instructions of the manufacturer.

Detection of β-lactamase genes (including blaTEM, blaSHV, and blaCTX-M) was carried out by polymerase chain reaction (PCR) using specific primers: TEM-A (5′-TAAATTCTTT GAAGAGC-3′) and TEM-B (5′-TTACCAATGCTTACCA-3′) were used to amplify the blaTEM genes (Chanal et al., 2000), and SHV-F (5′-ATGCCATTATCCGCTG-3′) and SHV-R (5′-TTAGGGTTGGAGGCTTAC-3′) were used for the blaSHV genes (Kojima et al., 2005). CTX-M1-A2 (5′-CTTCCGAATAAGGAATC-3′)/628R (5′-CCTTTCATCCATGTCACCA-3′) and 405F (5′-GTGGCGATGAATAAGCTGA-3′)/CTX-M1-B2 (5′-CCGTTCGGCTATTACAA-3′) were used to amplify the blaCTX-M gene (Doutour et al., 2002; Brasme et al., 2007). The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of DNA denaturation at 94°C for 30 s, primer annealing (at 55°C for SHV, 55°C for TEM, and 51°C for CTX-M) for 30 s, and primer extension at 72°C for 1 min, with a final extension step at 72°C for 7 min. PCR products were sequenced by Sanger’s dideoxy chain termination procedure on an Applied Biosystems 3730 XL DNA analyser using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). PCR amplicons of blaTEM genes were cloned by using the pDrive cloning Vector 3.85 kb and the Qiagen PCR cloning kit (Qiagen, Courtabeuf, France) according to the manufacturer’s instructions. The insert sequences were determined by direct sequencing with primers designed with the SP6 promoter and T7 promoter of the pDrive cloning vector surrounding inserts. DNA sequencing was performed as described above. The DNA alignments and deduced amino acid sequences were examined using the BLAST program (Altschul et al., 1990).

Detection of plasmid-mediated bla Amp-C was performed by using multiplex PCR as described previously (Perez-Perez et al., 2002).

PCR fingerprinting

Salmonella isolates with the same antibiotype were typed by Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR using ERIC1 (5′-ATGTAAGCTTCCGAGATTCA-3′) and ERIC2 (5′-AGTAAGACTGAGGTGACG-3′) primers. Whole-cell DNA of isolates was extracted using the QI Amp DNA mini kit (Qiagen). 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 isolates belonging to Salmonella enterica serotypes Brunei (10 isolates) and Heidelberg (4 isolates) were isolated and serotyped in the laboratory and confirmed at the Pasteur Institute in Paris, France.

All isolates exhibited resistance or decreased susceptibilities to ticarcillin, ticarcillin-clavulanate, piperacillin, cefuroxime, aztreonam, ceftazidime, cefotaxime, cefepime, and cefpirome (Table 1). They remained susceptible to imipenem, ertapenem, ceftazidime, cefotaxime, cefepime, and aztreonam. PCR fingerprinting with primers designed with the T7 promoter of the pDrive cloning vector surrounding inserts. DNA sequencing was performed as described above. The DNA alignments and deduced amino acid sequences were examined using the BLAST program (Altschul et al., 1990).

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MICs determination showed that Salmonella Brunei examined were resistant to amoxicillin (MIC > 256 μg/mL), ceftazidime (MIC = 64 μg/mL), cefotaxime (MIC = 16 μg/mL), and ceftriaxone (MIC = 6 μg/mL). For Salmonella Heidelberg isolates, resistance was observed for all isolates (amoxicillin, MIC > 256 μg/mL; ceftazidime, MIC = 48 μg/mL; cefotaxime, MIC > 32 μg/mL; ceftriaxone, MIC > 32 μg/mL).

All isolates were resistant to gentamicin and tobramycin. The isolates of Salmonella Brunei were resistant to kanamycin...
### Table 1. Characteristics of Algerian Clinical Isolates of *Salmonella Brunei* and *Salmonella Heidelberg* Resistant to Broad-Spectrum Cephalosporins

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Isolation date</th>
<th>Ward</th>
<th>CXM</th>
<th>TIC</th>
<th>PIP</th>
<th>AMC</th>
<th>FEP</th>
<th>CPO</th>
<th>TCC</th>
<th>ATM</th>
<th>CTX</th>
<th>CAZ</th>
<th>TZP</th>
<th>IMP</th>
<th>FOX</th>
<th>Additional resistance</th>
<th>ESBL type</th>
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<tr>
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<td>6</td>
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<td>20</td>
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<td>6</td>
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<td>22</td>
<td>20</td>
<td>19</td>
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<td>TEM-48</td>
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<td>Neonatology</td>
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<td>6</td>
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<td>TEM-48</td>
</tr>
<tr>
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<td>18</td>
<td>6</td>
<td>6</td>
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<td>21</td>
<td>20</td>
<td>19</td>
<td>12</td>
<td>23</td>
<td>10</td>
<td>23</td>
<td>35</td>
<td>30</td>
<td>GEN, TOB, KAN, AMK, SUL</td>
<td>TEM-48</td>
</tr>
<tr>
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<td>26/07/1998</td>
<td>Neonatology</td>
<td>18</td>
<td>6</td>
<td>6</td>
<td>19</td>
<td>22</td>
<td>21</td>
<td>19</td>
<td>12</td>
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<td>11</td>
<td>23</td>
<td>35</td>
<td>30</td>
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<td>TEM-4</td>
</tr>
<tr>
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<td>TEM-48</td>
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<td>6</td>
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<td>20</td>
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<td>36</td>
<td>31</td>
<td>GEN, TOB, KAN</td>
<td>TEM-4</td>
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<tr>
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<td>24</td>
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<td>GEN, TOB, NAL</td>
<td>CTX-M-15</td>
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<tr>
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<td>6</td>
<td>6</td>
<td>24</td>
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<td>10</td>
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<tr>
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<td>6</td>
<td>24</td>
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<td>GEN, TOB, NAL</td>
<td>CTX-M-15</td>
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</table>

CXM, cefuroxime; TIC, ticarcillin; PIP, piperacillin; AMC, co-amoxiclav; FEP, cefepime; CPO, cefpirome; TCC, ticarcillin-clavulanate; ATM, aztreonam; CTX, cefotaxime; CAZ, ceftazidime; TZP, piperacillin/tazobactam; IMP, imipenem; FOX, cefoxitin; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; AMK, amikacin; SUL, sulfonamides; NAL, nalidixic acid.
and amikacin (except isolates of S9 and S12). Resistance to nalidixic acid was observed in all isolates of Salmonella Heidelberg. All of the isolates were susceptible to ciprofloxacin, cotrimoxazole, tetracycline, and chloramphenicol.

TEM consensus PCR assays gave the expected PCR fragments for the 10 isolates of Salmonella Brunei (Table 1), and CTX-M amplifications were positive for the four isolates of Salmonella Heidelberg. SHV and plasmid-mediated \( \text{bla}_{\text{AmpC}} \) amplification was negative.

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

One isolate of Salmonella Brunei (S10) was found to produce a new TEM. This protein has been designated TEM-188 (www.lahey.org/studies/webt.htm; GenBank accession number JN211012). The new TEM \( \beta \)-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 antibotypes (Table 1), suggesting that the substitution Thr265Met in TEM-48 has no effect on \( \beta \)-lactam susceptibility.

The ERIC-PCR method was applied to the six TEM-48–producing Salmonella Brunei isolates and the four CTX-M-15–producing Salmonella Heidelberg. Two ERIC-PCR patterns were observed: one for the six Salmonella Brunei and a second one for the four Salmonella Heidelberg.

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. Salmonella Brunei has been rarely reported from animals, animal food products, and patients with human salmonellosis (Foley and Lynne, 2007).

In our study, 10 isolates of Salmonella Brunei were recovered from infants during the period of 1995 to 2008 whereas the four Salmonella 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). ESCs are commonly used to treat patient with invasive infections or severe diarrhea caused by salmonellae; however, during the past years ESC-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 first Salmonella isolates with ESBLs in Africa were identified in 1988 in Tunisia (Hammami et al., 1991). TEM-4 \( \beta \)-lactamase, which differed from the TEM-1 \( \beta \)-lactamase sequence by four substitutions (Leu21Phe, Glu104Lys, Gly238Ser, and Thr265Met), was first reported for E. coli in France by 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 Mbendaka 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 \( \beta \)-lactamase sequence were Leu21-Phe, Gly238Ser, Glu240Lys, and Thr265Met. TEM-48 was first described in K. pneumoniae isolates in Poland (Gniadkowski et al., 1998).

CTX-M-15 was identified in different Salmonella serotypes, but to our knowledge, this is the first report of CTX-M-15 in Salmonella 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 Salmonella Heidelberg were found resistant to nalidixic acid, but susceptible to fluoroquinolones. PCR for the plasmid-mediated mechanisms was negative for the four isolates, suggesting that the nalidixic acid resistance was probably mediated by mutations in topoisomerases.

All isolates were resistant to gentamycin and tobramycin, and eight isolates of Salmonella Brunei were resistant to amikacin. According to resistance levels conferred by the methylases (amikacin MICs > 256 \( \mu \)g/mL) (Naas et al., 2009, 2011), the presence of methylases in our isolates was unlikely since diameter obtained with the amikacin disc was 12–14 mm. Aminoglycosides resistance was likely due to aminoglycoside-modifying enzyme.

PCR-mediated genome fingerprinting based on ERIC or Repetitive Element (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 Salmonella Mbendaka (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 (Arlet et al., 2006). The presence of different enzymes in the same species could be due to the transfer of different plasmids to a unique clonal isolate. Even an insufficient discrimination between the isolates by ERIC-PCR cannot be ruled out; the weak prevalence of these serovars was an argument for a common source of the isolates. The two clonal isolates observed in our study were recovered throughout the 13-year study period. The Salmonella Brunei–producing TEM-48 isolates were recovered from neonatology ward, except one isolate recovered from the cradle ward, whereas the Salmonella Heidelberg–producing CTX-M-15 isolates were isolated in the 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 contaminated with...
Salmonella (Foley and Lynne, 2007). In this study, food as a source was excluded because milk was commercially prepared. Therefore, a horizontal transmission of the isolates had probably occurred, and we cannot exclude child contamination by their mothers or nurses.

In summary, this study demonstrates the emergence of a public health risk related to \( \beta \)-lactam resistance in Salmonella Heidelberg and Brunei 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.

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References


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